Examining the influence of the menstrual cycle, hormonal contraceptives, biological sex, and gender on cardiovascular and metabolic outcomes in healthy adults.

By Jennifer Williams, BAH, MSc

A Thesis Submitted to the School of Graduate Studies in Partial Fulfilment of the Requirements for the Degree Doctor of Philosophy

DOCTOR OF PHILOSOPY (2023)   McMaster University
(Kinesiology)   Hamilton, Ontario

TITLE: Examining the influence of the menstrual cycle, hormonal
                contraceptives, biological sex, and gender on cardiovascular and
                metabolic outcomes in healthy adults.

AUTHOR: Jennifer Williams
                BAH (Queen's University)
                M.Sc (Queen's University)

SUPERVISOR: Dr. Maureen J. MacDonald

PAGES: xviii, 241

LAY ABSTRACT

Females have been historically understudied in basic science and clinical research. This dissertation set out to explore sex-specific prevalence of research participants in human vascular exercise physiology studies and examined how sex hormones (through the menstrual cycle and oral contraceptive pill use) impact the cardiovascular, respiratory, and skeletal muscle metabolism systems. We found that there is an evident male-bias in vascular exercise physiology research, due in part to the perceived complexity of how sex hormones may impact the cardiovascular system. We also found that the menstrual cycle and oral contraceptive pill cycle have minimal influence on the biological systems examined. While there are evident sex-differences in cardiovascular outcomes, gender expression does not appear to have an impact in young adults. This research is foundational to further the inclusion of female participants in human physiology research and encourage future considerations of how sex/gender may influence physiological outcomes.

ABSTRACT

Sex-differences in cardiometabolic physiology are evident; however, the inclusion of female participants in research studies for the purposes of exploration of sex-specific physiological responses is limited by the perceived complexity due to hormonal cycles. This dissertation examined the prevalence of sex-specific inclusion in human vascular exercise physiology research, investigate the influence of endogenous and exogenous sex hormones on cardiovascular, respiratory, and skeletal muscle metabolism, and consider sex- and gender-differences in peripheral vascular outcomes. The first study confirmed a sex-specific bias towards male inclusion in vascular exercise physiology research, with perceived hormonal complexity noted as one rationale for sex-specific exclusion. To address this perception, we reviewed the literature and identified a small effect of the menstrual cycle, and a more robust influence of oral contraceptive pills, on macrovascular endothelial function, with no influence on smooth muscle function or arterial stiffness. Our next set of studies objectively evaluated the influence of the natural menstrual and two generations of oral contraceptive pills on a comprehensive suite of cardiovascular, respiratory, and metabolic outcomes, and found largely no influence on these outcomes or the underlying vascular cellular regulation, apart from a small effect elevated endogenous and exogenous sex hormones on brachial artery endothelial function. Another area identified in our initial sex-inclusion review was the absence of gender-based research in vascular exercise physiology. Our final study found that biological sex aligned with gender identity, but not gender expression and influenced cardiovascular markers by including elevating systolic blood pressure, central arterial stiffness and endothelial function in males and men compared to females and women. Altogether, this dissertation provides substantial evidence for the lack of hormonal cycle influence of endogenous and exogenous sex hormones on three organ systems, which will open further incorporating females into research study design.

ACKNOWLEDGEMENTS

Five years, six main studies, and over a dozen side project collaborations with EMERG friends and peers and international colleagues. Not to mention the challenges the last five years have brought: pursuing research during a worldwide pandemic and 16-month lab closure, technology issues, freezer meltdown, cell contamination... The following group of advisors, colleagues, friends, and family have provided endless support and encouragement during both the times of adversity, along with the joyful moments in research.

Supervisor: To Maureen, thank you for being an incredible role model and support over the last five years (and in the years prior). I have always looked up to you as a brilliant scientist, fervent support of women in STEM, and strong leader. Thank you for providing endless support and guidance during these years with the Vascular Dynamics Lab, and in particular providing opportunities to collaborate and explore new fields of research.

Supervisory Committee: Thank you to my supervisory committee: Dr. Stuart Phillips and Dr. Stacey Ritz for your guidance in developing projects, providing additional perspective, and all-round support and encouragement. Walking out of every supervisory committee meeting gave me the energy and enthusiasm to get ready for the next step in this dissertation. A special thank you also to Dr. Ritz for including me in many teaching and research opportunities related to sex/gender considerations in biomedical and clinical research. Your guidance has been a turning point for me in my studies and hopeful future academic interests in sex/gender research. Thank you also to my external committee member, Dr. Sofia Ahmed from the University of Calgary. I have enjoyed learning over the years about projects from your lab from Cindy and Chantal. I look forward to our discussions at the upcoming defense and appreciate your support in reviewing my dissertation.

Research Team: To the Vascular Dynamics Lab, thank you for all the support, guidance, and fun lab times we've had over the years (Dr. Jem Cheng, Jenna Stone, Joshua Cherubini, Sydney Valentino, Matthew Badour, Khandra Barrett, Connor Droog, Vanessa Rizzuto, Dr. Emily Dunford). Special thank you to Jem for your early training and getting me up to speed with everything VDL, your stellar organizational skills, and your guidance over the years in pursuing projects. Thank you also to Josh for your stats skills and R support, being there to bounce ideas over the years. Thank you also to the close VDL collaborators, Maddy Byra and Ada Tang's students Elise Wiley, Kenneth Noguchi, Kevin Moncion for your collaborations and support in projects.

An extra special shout out to students I have had an opportunity to mentor of the years (in order of years from 2019-2023): Zaryan Masood, Danielle Joshi, Christina Pizzola, Alicia Au, Rachael Moorhouse, Emily van Berkel, Shantal Stade, Fajjar Aqeel, Claudia Yong, and, of course, Jenna Stone. You have been the reason for all the fun we've had in data collection sessions, endless analysis (especially in the summer), and chatting through research ideas!

TABLE OF CONTENTS

LIST OF FIGURES

CHAPTER 8: GENERAL DISCUSSION

LIST OF TABLES

LIST OF ABBREVIATIONS

ACh	Acetylcholine
AbsFMD	Absolute flow-mediated dilation
AbsNMD	Absolute nitroglycerine-mediated dilation
ANOVA	Analysis of variance
AMS	Artery Measurement System
BA	Brachial artery
BCA	Bicinchoninic acid
BMI	Body mass index
BSRI	Bem Sex Role Inventory
BSRI-30	Bem Sex Role Inventory 30-item questionnaire
BSRI-Feminine	Bem Sex Role Inventory, Feminine subscale
BSRI-Masculine	Bem Sex Role Inventory, Masculine subscale
CCA	Common carotid artery
CI	Confidence intervals
CIHR	Canadian Institutes of Health Research
CVD	Cardiovascular disease
DBP	Diastolic blood pressure
DICOM	Digital Imaging and Communications in Medicine format
DMPA	Depot medroxyprogesterone acetate
DSG	Desogestrel
DXA	Dual-energy X-ray absorptiometry
E_2	Estradiol
ECG	Electrocardiogram
ECGM-2	Endothelial cell growth medium (2)
EE	Ethinyl estradiol
EF	Early follicular
EL	Early luteal
ELISA	Enzyme-linked immunosorbent assay
eNOS	Endothelial nitric oxide synthase
ER	Estrogen receptor
ERα	Estrogen receptor alpha
ERβ	Estrogen receptor beta
FFM	Fat free mass
FMD	Flow-mediated dilation
%FMD	Percent flow-mediated dilation
%FMD$_{scaled}$	Percent flow-mediated dilation allometrically scaled for baseline diameter
GnRHa	Gonadotropin-releasing hormone antagonist
GRADE	Grading of Recommendations Assessment, Development and Evaluation
HH	High hormone
HR	Heart rate
HUVEC	Human umbilical vein endothelial cells

IMT	Intima-media thickness
IQR	Interquartile range
IUD	Intrauterine device
LBF	Leg blood flow
LD	Lumen diameter
LF	Late follicular
LH	Low hormone
LL	Late luteal
LNG	Levonorgestrel
L-NMMA	NG-monomethyl-L-arginine (NO synthase inhibitor)
MAP	Mean arterial pressure
MBV	Mean blood velocity
ML	Mid-luteal
MPA	Medroxyprogesterone acetate
MSNA	Muscle sympathetic nerve activity
NAT	Natural menstrual cycle
NIH	National Institutes of Health
NMD	Nitroglycerine-mediated dilation
%NMD	Percent nitroglycerine-mediated dilation
NO	Nitric oxide
NSERC	Natural Sciences and Engineering Research Council of Canada
OCP	Oral contraceptive pill
OCP2	2nd generation OCP
OCP3	3rd generation OCP
PORH	Postocclusive reactive hyperemia
PP	Pulse pressure
PRISMA	Preferred Reporting Items for Systematic Review and Meta-Analysis
PROSPERO	International Prospective Register of Systematic Reviews
PWV	Pulse wave velocity
Ra	Rate of appearance
Rd	Rate of disappearance
RER	Respiratory exchange ratio
Rf	Breathing frequency
RIPA	Radioimmunoprecipitation Assay Buffer
RPE	Rating of perceived exertion
SAGER	Sex and Gender Equity in Research
SAQOR	Systematic appraisal for observational research
SBP	Systolic blood pressure
SD	Standard deviation
SE	Standard error
SFA	Superficial femoral artery
SMD	Standardized mean difference
SMF	Smooth muscle function

SNP	Sodium nitroprusside
SR	Shear rate
SR AUC	Shear rate area under the curve (to time of peak)
TBST	Tris-Buffered Saline with Tween
TCPS-2	Tri-Council Policy Statement: Ethical Conduct for Research Involving Humans
$\dot{V}CO_2$	Volume of carbon dioxide expired per minute
$\dot{V}e$	Minute ventilation of gas exhaled
$\dot{V}O_2$	Volume of oxygen inspired per minute
$\dot{V}O_2peak$	Peak oxygen uptake
VSM	Vascular smooth muscle
Vt	Tidal volume

DECLARATION OF ACADEMIC ACHIEVEMENT

This dissertation is presented in a "sandwich" format, which contains a general introduction, six independent manuscripts (three reviews, three original studies) to which the candidate is the first author (or a shared first author), and a general discussion. The contributions and status of publishing each manuscript is detailed below:

CHAPTER 2: "Examination of sex-specific participant inclusion in vascular exercise physiology research: a systematic review."

Authors: Lindsay A. Lew*, **Jennifer S. Williams***, Jenna C. Stone, Alicia K.W. Au, Kyra E. Pyke, Maureen J. MacDonald
*Shared first-author contributions

Contributions:
Conceived and designed the research: JSW, LAL, KEP, MJM
Acquired data: JSW, LAL, JCS, AKWA
Analyzed and interpreted data: JSW, LAL
Drafted the manuscript: JW, LAL
Provided critical review and feedback of manuscript: JSW, LAL, JCS, AKWA, KEP, MJM

Status of Manuscript Publishing: **Published** in *Frontiers in Sports and Active Living*

CHAPTER 3: "Impact of the menstrual cycle on peripheral vascular function in premenopausal women: systematic review & meta-analysis."

Authors: **Jennifer S. Williams**, Emily C. Dunford, Maureen J. MacDonald

Contributions:
Conceived and designed the research: JSW, ECD, MJM
Acquired data: JSW, ECD
Analyzed and interpreted data: JSW, ECD
Drafted the manuscript: JSW, ECD
Provided critical review and feedback of manuscript: JSW, ECD, MJM

Status of Manuscript Publishing: **Published** in the *American Journal of Physiology – Heart and Circulatory Physiology*

CHAPTER 4: "Influence of hormonal contraceptives on peripheral vascular function and structure in premenopausal females: a review."

Authors: **Jennifer S. Williams**, Maureen J. MacDonald

Contributions:
Conceived and designed the research: JSW, MJM
Drafted the manuscript: JSW
Provided critical review and feedback of manuscript: JSW, MJM

Status of Manuscript Publishing: **Published** in the *American Journal of Physiology – Heart and Circulatory Physiology*

CHAPTER 5: "Menstrual cycle and oral contraceptive pill phase largely do not influence vascular function, arterial stiffness, and associated cellular regulation in young, healthy premenopausal females."

Authors: **Jennifer S. Williams**, Jem L. Cheng, Jenna C. Stone, Michael J. Kamal, Joshua M. Cherubini, Gianni Parise, Maureen J. MacDonald

Contributions:
Conceived and designed the research: JSW, MJM with support from JCS, MJK, JMC, GP
Acquired data (vascular): JSW, JLC, JCS
Acquired data (cell experiments): JSW, MJK, JMC
Analyzed and interpreted data: JSW, JCS, MJK
Drafted the manuscript: JSW
Provided critical review and feedback of manuscript: JSW, JLC, JCS, MJK, JMC, GP, MJM

Status of Manuscript Publishing: **Submitted** to *Arteriosclerosis, Thrombosis, and Vascular Biology*

CHAPTER 6: "The impact of natural menstrual cycle and oral contraceptive pill phase on substrate oxidation during rest and acute submaximal aerobic exercise."

Authors: **Jennifer S. Williams**, Jenna C. Stone, Zaryan Masood, William Bostad, Martin J. Gibala, Maureen J. MacDonald

Contributions:
Conceived and designed the research: JSW, ZM, WB, MJG, MJM
Acquired data: JSW, ZM, WB
Analyzed and interpreted data: JSW, JCS
Drafted the manuscript: JSW
Provided critical review and feedback of manuscript: JSW, JCS, ZM, WB, MJB, MJM

Status of Manuscript Publishing: **Published** in the *Journal of Applied Physiology*

CHAPTER 7: "Differences in cardiovascular risk factors associated with biological sex, but not gender expression, in young, healthy adults."

Authors: **Jennifer S. Williams**, Elise Wiley, Jem L. Cheng, Jenna C. Stone, William Bostad, Joshua M. Cherubini, Martin J. Gibala, Ada Tang, Maureen J. MacDonald

Contributions:
Conceived and designed the research: JSW, EW, MJM
Acquired data: JSW, JLC, JCS, WB
Analyzed and interpreted data: JSW, EW
Drafted the manuscript: JSW, EW
Provided critical review and feedback of manuscript: JSW, EW, JLC, JCS, WB, JMC, MJG, AT, MJM

Status of Manuscript Publishing: **Submitted** to the *Biology of Sex Differences*

CHAPTER 1: GENERAL INTRODUCTION

1. Sex-Differences in Cardiovascular Disease

Cardiovascular diseases (CVD), including coronary heart disease and myocardial infarction, heart failure, hypertension, and peripheral arterial diseases, exhibit age-related increases in incidence, morbidity and mortality (1-3). Concerningly, despite observing declines in rates of CVD-associated deaths and overall life expectancy in previous years, there is a growing trend to seeing increasing rates in high-income, developed countries (3-5). There also appears to be sex-differences in some CVDs, where the sex-specific rate of most CVDs is lower in women compared to men until menopause where the prevalence rates increase at a faster rate in women (1, 6). There are also some cardiovascular diseases that have a greater age-related prevalence in women, such as cerebrovascular disease, coronary artery dissection, and heart failure with preserved ejection fraction (7, 8).

Despite the increased prevalence of CVDs in older women, early symptoms of a myocardial infarction are missed in 78% of women (9). One reason could be linguistic differences in the ways that men and women describe their symptoms. Men will often describe typical symptomology of squeezing chest pain, back/shoulder and jaw pain, shortness of breath, and nausea. However, women will often describe their symptoms as less specific and use more words to describe their experience, including chest pressure (not gripping pain), fainting and fatigue, indigestion, and mental confusion (10). One rationale for differences in symptomology could be in the way in which CVD, particularly myocardial infarctions, are diagnosed. One study observed that women referred for diagnostic angiography had a higher prevalence of exhibiting normal arteries and less prevalence of severe coronary artery occlusion compared to men, despite experiencing persistent angina (11). The rationale provided by the authors is that women present more often with dysfunction of the microvasculature in the heart, rather than large coronary artery ischemia (6).

Adding to this narrative, women's heart health has been long under-researched (9, 12). A 2018 report by the Heart and Stroke Foundation of Canada called "Ms. Understood: Women's hearts are victims of a system that is ill-equipped to diagnose, treat and

support them" reported that 2/3 of heart disease research focuses on men (9). Further evidence from this report highlighted specific ethnic groups at highest risk of CVD: South Asian and Indigenous populations. While there are many reasons for the exclusion of women from basic science and clinical health research, one reason has been the perceived added methodological complexity of periods of hormonal change or reproductive influence specific to women – such as menstrual cycles, hormonal contraceptive use, pregnancy, and menopause (13). In stark contrast, while variability exists in physiological outcomes, evidence suggests that inclusion of females in animal and human research does not increase variability (13). Recently, there are growing calls from researchers, government agencies, advocacy bodies, and journals to include both men and women in research (9, 14-21), with guidance for methodologies to consider to overcome perceived hormonal complexity (22-24).

Building on these calls, the *Lancet* commissioned a call-to-action review of current disparities in CVD research and healthcare in women, with a focus on better understanding the sex-specific pathophysiology and risk factors underlying CVD development (25). Given the high prevalence of women experiencing CVD, and the underrepresentation of women in clinical research studies, investigations into risk factors associated with CVD in women is necessary for early prevention (26). Some population health studies have been conducted to explore sex-differences in traditional and novel risk factors for CVD. For example, the INTERHEART study examined sex-differences in modifiable risk factors associated with myocardial infarction from 52 countries worldwide. This study found that there were nine modifiable risk factors associated with 90% of the risk associated with a myocardial infarction, five of which had a greater association based on sex (27). In women, having hypertension or diabetes, low physical activity, and low alcohol consumption were more highly associated with having a myocardial infarction compared to men (27). In men, former smoking had a greater association with having a myocardial infarction compared to women (27). This study also reported a 9-year difference between men's earlier incidence of their first myocardial infarction compared to women; however, 80% of this 9-year gap was explained by earlier prevalence of risk factors in men compared to

women (27). Beyond traditional risk factors, there has been a growing interest in the identification and assessment of early risk factors for CVD that can help with predicting future risk such as early dysfunction of arterial function and structure, as detailed in the following section.

2. Assessments of Early Risk Factors for Cardiovascular Disease

The cardiovascular system is a loop of blood vessels connected to a single, valved heart, which pumps oxygen- and nutrient-rich blood unidirectionally to the body and collects oxygen-deprived blood and waste to be pumped to the lungs and other organs (28). Blood moves away from the heart in major conduit arteries that connect to smaller microvascular arterioles before branching at target organs, like skeletal muscle, through single-celled capillaries (28). Macrovascular arteries and microvascular arterioles both contain three primary layers of cells: the tunica intima, tunica media, and adventitia (Figure 1A) (28). The tunica intima contains a single layer of endothelial cells which are responsible for sensing changes in blood flow and associated shear stress and producing vasodilators and vasoconstrictors to regulate vascular tone (28). The tunica media contains layers of smooth muscle cells, which respond to vasoactive substance release from endothelial cells to vasodilate or vasoconstrict (28). Finally, the adventitia layer contains collagen and elastin which provide necessary structure to the inner two layers (28). Macrovascular arteries are more elastic and respond to higher blood pressures coming from left ventricular ejection (systole), while microvascular arteries are more muscular and assist in distributing blood flow to tissues (28). While macro- and microvascular arteries are interrelated in their function and structure, they also demonstrate heterogeneous responses to stimuli related to the relative contribution of vasodilator pathways (29-31). Damage or dysfunction in arterial layers is an early step in the development of atherosclerosis, which is a precursor to the development of CVD (32-34). As a result, investigation of the function and structure of these arterial layers, and mechanisms to improve function, may ward off CVD development. The following section will detail three relevant functional assessments and their association with clinical outcomes: endothelial function, smooth muscle function, and arterial stiffness and structure. While there are other functional and structural measures, including

cerebrovascular function tests, and blood biomarker assessments, this section will focus primarily on outcomes discussed in later chapters of this dissertation.

2.1 Endothelial Function

Endothelial function tests examine the function of endothelial cells in the macro- and microvasculature, in response to stimuli that are known for vasodilation or vasoconstriction, often via blood flow associated changes in shear stress (35, 36). Shear stress is the frictional force of blood flow moving past the endothelial cell layer on the inside of the artery, which starts a cascade of cellular responses, including the generation of nitric oxide from endothelial nitric oxide synthase, through calcium-dependent or -independent mechanisms (37, 38). Animal and limited human trials that have observed no dilation or vasoconstriction when endothelial cells are removed from the artery lumen (39-42). These findings indicate that endothelial cells, and the nitric oxide they produce, are obligatory in shear-associated vasodilation and remodeling (43). One common non-invasive macrovascular assessment of endothelial function is the flow-mediated dilation (FMD) test, often performed in the brachial artery or superficial femoral artery (Figure 1B) (44). FMD occurs when a transient ischemia, often in response to blood flow occlusion in the distal forearm (or thigh) for 5 minutes, and subsequent reperfusion (release of occlusion) naturally increases blood flow associated shear stress (44, 45). This response is termed reactive hyperemia and is the primary stimulus for subsequent vasodilation of the artery, in comparison to baseline or pre-ischemia measurement. Guidelines for FMD have been well established and refined over the last three decades, and often use an ultrasound machine to capture continuous artery images and blood flow for post-testing analysis (45, 46); further, adherence to guidelines improves reproducibility of FMD (47).

Population-levels studies have observed that decreases in brachial artery FMD are associated with increases in cardiovascular events where a 1% decrease in relative FMD is associated with a 13% increased risk of experiencing a cardiovascular event (33, 34). Further, a recent analysis of studies using guideline methodology for the assessment of FMD have found sex-differences in both baseline arterial diameter and

%FMD, with higher arterial diameter but lower %FMD in males compared to females (48). In addition to macrovascular measures, there are also non-invasive methods for examination of microvascular endothelial function. Microvascular endothelial function is commonly quantified as a change in skin blood flow in response to a vascular reactivity assessment and either uses ultrasound imaging, laser Doppler flowmetry, or devices that sense distension in arterioles in the arms or hands (49-51). Stimuli for inducing changes in skin blood flow can range from reactive hyperemia (often performed in concert with the FMD test), local heating or cooling, or pharmacological interventions like acetylcholine infusion or topical application (49-51).

2.2 Smooth Muscle Function

Smooth muscle function tests examine the function of the smooth muscle cells that surround endothelial cells in the macro- and microvasculature (43). These tests are often necessary to help contextualize any changes in endothelial function and to dissociate the endothelial cell mechanisms from any dysfunction of the resulting vasodilation/constriction of the surrounding smooth muscle (45). The most common macrovascular smooth muscle function test is a nitroglycerine mediated dilation (NMD) test, which uses a sublingual dose of nitroglycerine (~0.25-0.4 mg) to induce maximal vasodilation of the smooth muscle in the arteries (45, 52, 53). Ultrasound is used to capture arterial diameters, often in the brachial artery (Figure 1B), prior to and for 10 minutes following administration of the sublingual nitroglycerine dose (45, 52). In the microvasculature, a common method used to assess smooth muscle function is using iontophoresis in response to sodium nitroprusside, another potent vasodilator of the arterioles (49, 51, 54).

2.3 Arterial Stiffness & Structure

Broadly, arterial stiffness assessments can be split into the examination of local artery stiffness in the carotid artery (major conduit artery to the brain) or into regional peripheral artery stiffness across the central body or individual limbs (55, 56). Using ultrasound and applanation tonometry to capture simultaneous carotid artery (Figure 1B) diameter and pulse waveforms with consecutive heart beats, carotid artery stiffness

can be quantified as measures of carotid artery compliance, distensibility, and β-stiffness (56, 57). These measures are reflective of a combination of endothelial functional changes in vascular tone to regulate blood pressure in addition to structural components of the adventitia layer (i.e., elastin, collagen fibers). In addition to functional arterial stiffness measures, the thickness of the intima-media layer can be quantified as it is also an indication of early atherosclerosis development and a predictor of future CVD risk (58-61).

Peripheral artery stiffness is commonly assessed by pulse wave velocity (PWV), which uses applanation tonometry to detect the velocity at which arterial pressure waveforms travel from one segment of the cardiovascular tree to another (57, 62). Methodologically, simultaneous applanation tonometers are applied to two arteries in the body, and the distance between the two points is divided by the pulse transit time (i.e., the time it takes for the pulse to travel from one artery to another) (62, 63). Central PWV – also known as aortic PWV – is the gold standard measurement, assessed between the carotid artery and femoral arteries (Figure 1B), and represents the stiffness of the central arterial segments (62). Central PWV has been associated with CVD, such that a 1 m/s increase in central PWV is associated with a 15% increased risk of CVD (32). In addition to measures of central PWV, peripheral arterial segments can also be assessed for region-specific arterial stiffness (63). For example, the leg arterial stiffness can be examined using assessments at the femoral and dorsalis pedis (foot) arteries (Figure 1B). Similarly, arm arterial stiffness can be examined using assessments at the carotid and radial arteries (Figure 1B). While these latter stiffness measures have not bee prognostically linked to CVD, they do provide mechanistic insight into alterations in these regions of the vascular tree linked to disease (64) and adaptations to vasoactive stimuli (i.e., exercise) (65).
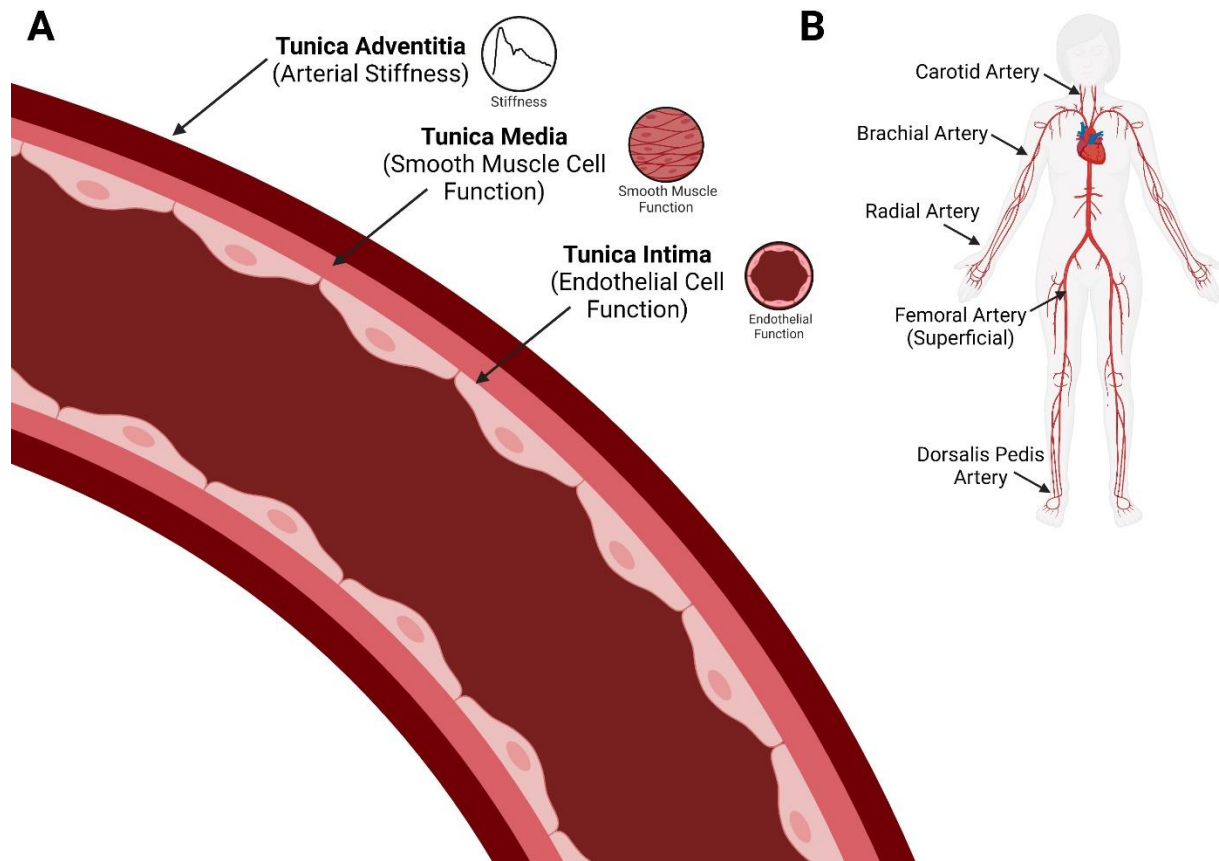
Figure 1. (A) Cross-section of an artery and (B) key arteries of interest in the human body. Figure 1A details three artery layers: tunica adventitia, tunica media, and tunica intima, along with the connection between common vascular assessments of arterial stiffness, smooth muscle cell function, and endothelial cell function and the layers they are associated with in the artery. Figure 1B details the primary arteries examined through the vascular assessments, detailed in-text. Figure created in Biorender.com.

3. Factors Influencing the Cardiovascular System: Biological Sex & Gender

Biological sex and gender have known influences on the cardiovascular system and the progression of CVD (12, 66, 67). Biological sex is most referred to as the sex assigned at birth; however, some scholars argue that sex can be defined using a multi-layer approach from chromosomal sex, gonadal (internal or external) sex, and considering secondary sexual characteristics and development (68). Often in human physiology trials, biological sex is asked in a two-step question with the first question being "What is your sex assigned at birth?" with options of male, female, and intersex (69, 70). With regards to the cardiovascular system, there are several physiological differences

between males and females with the most evident being body composition and stature, as early as adolescence (71, 72). For example, females tend to be shorter and have a greater distribution of body fat associated with secondary sexual characteristics (72), potentially due to the role of sex hormones (73). These differences drive some of the differences in cardiovascular anatomy, including larger artery size and greater blood pressure in males compared to females (71, 74, 75). While these sex-differences are well characterized, often forgotten is that these sex-differences are based on averages and there is substantial variability in physiological factors (76) – for example, a female may be taller than average and cross over into the average heights of males. The same is true for sex-differences in other factors, ranging from sex hormone concentrations to enzyme protein content (76). Some commonly explored sex-related factors include differences in body anthropometrics (height, weight, fat distribution), X-chromosome linked genetic traits, and, commonly, sex hormones; further discussion of the role of sex hormones in females is included in the next section.

Gender is a sociological construct and can be broadly separated into four categories: gender identity, gender expression, gender roles/relations, and institutionalized gender (66, 67, 77):

- Gender identity is how an individual identifies their gender. In a two-step question on biological sex (detailed above) and gender identity, options for gender identity could include man, woman, non-binary (ascribing to no binary gender category), gender-diverse (overarching term to describe individuals whose gender does not align with traditional binary genders), transgender (an individual whose gender identity does not align with their biological sex assigned at birth), among many others.
- Gender expression is the outward expression of an individuals' gender and could include the presentation of gendered traits (detailed in Chapter 7) or gender-specific appearance.
- Gender roles and relations are the socially ascribed roles and relationships that gendered individuals take on, including caregiving, household tasks, and work relationships, among others.

- Individualized gender is policy-driven regulations for how individuals of different genders can interact with social institutions, including spaces like hospitals and healthcare centers, schools and workplaces.

While gender has not been extensively studied in cardiovascular physiology research, there is some evidence that having a feminine gender, or taking on gender roles traditionally ascribed to women, such as caregiving and household tasks, lower educational opportunities, lower socioeconomic status, and higher social stress increases CVD risk (12, 78-80). Further, there is evidence that members of gender diverse communities who do not fall into gender binary categories of men/women (e.g., non-binary, gender diverse, transgender) are at an increased risk of CVD (67, 81, 82). This may be due to gender minority stress, referring to social stress gender diverse individuals face including oppression, discrimination, and stigma (81, 83-85).

4. Factors Influencing the Cardiovascular System: Sex Hormones

As detailed previously, in comparison to age-matched men, premenopausal women appear to be protected against aging-related increases in the development and prevalence of CVD until the menopause transition where there is a steady increase in CVD risk and occurrence in women (12, 86-88). From a sex-based perspective, many researchers point to the dramatic decline in sex hormones – namely 17β-estradiol (primary estrogen) and progesterone – associated with the loss of the menstrual cycle at menopause to explain this increased CVD risk (89, 90). As a result, investigations into how sex hormones influence cardiovascular risk factors (i.e., endothelial function, arterial stiffness) has been a focus of female-specific research (53, 90-94). Mechanistically, 17β-estradiol may have cardioprotective effects, including acting as an oxidative stress scavenger and antioxidant, along with inducing upregulation of vasodilator pathways like the endothelial nitric oxide synthase (eNOS) – nitric oxide (NO) pathway in endothelial cells via estrogen receptors (ERs), such as ER alpha (ERα) and ER beta (ERβ) (88, 93, 95, 96). In contrast, there is some evidence that progesterone, or various synthetic variants of progestins found in oral contraceptive pills (OCPs), may act antagonistically to 17β-estradiol (97-99).

The natural menstrual (NAT) cycle is characterized by the fluctuations in endogenous 17β-estradiol and progesterone, along with other sex hormones, cyclically across a monthly cycle beginning in most females at age of puberty (i.e., ~10-13 years old) and lasting until menopause with the cessation of cycles (22, 28). Characterized by the onset of menses as the first day of the cycle, the cycle is broadly split into two phases: the follicular phase and the luteal phase (100, 101). The early portion of the follicular phase begins with the onset of menses and is characterized by lower levels of endogenous sex hormones (101). The latter half of the follicular phase begins with an increase in 17β-estradiol to its peak, which occurs at the time of ovulation (101). The first half of the luteal phase is characterized by an initial decrease in 17β-estradiol, followed by a rise in both endogenous sex hormones to peak at mid-luteal, followed by a progressive drop to the end of the cycle (101). The cycle then begins anew back in the early follicular phase. These changes in sex hormone levels are represented in Figure 2.

OCPs are used by 150 million women worldwide (102) and are one of the most prescribed drugs to premenopausal women in Canada (103). OCPs contain the synthetic estrogen ethinyl estradiol and one of several progestins, in varying concentrations. Over the course of the last 60 years, four generations of progestins have been developed which differ mainly in the androgenicity of the progestin and its associated side effect profile (104-106). For example, levonorgestrel is one of the most common 2nd generation progestins and is highly androgenic, while 3rd generation progestins like desogestrel are less androgenic. OCPs include one phase called the placebo/non-active pill phase, also known as the hormone withdrawal phase, and the active pill phase (103-105). During this phase, the user either takes placebo/non-active sugar pills or does not take pills to induce a period of exogenous hormone withdrawal. For some users, this will correspond with the onset of menses. This period lasts for 4-7 days, depending on the formulation of OCP (107, 108). During the active pill phase, the user takes 21-24 days of active pills, once per day at the same time of day (107, 108). Depending on the formulation, OCPs can be monophasic, biphasic, or triphasic (106).

Monophasic refers to the same active pill dose taken throughout the entire active pill phase; biphasic includes two levels of increasing progestin dosages in the active pill phase; triphasic refers to three increasing levels (~1 level/week) of progestin dosages through the active pill phase (106). There is some evidence that NAT and OCP cycles may influence early CVD risk outcomes, such as endothelial function, smooth muscle function, and arterial stiffness; this evidence is presented in-depth in later chapters of this dissertation (Chapters 2-4).
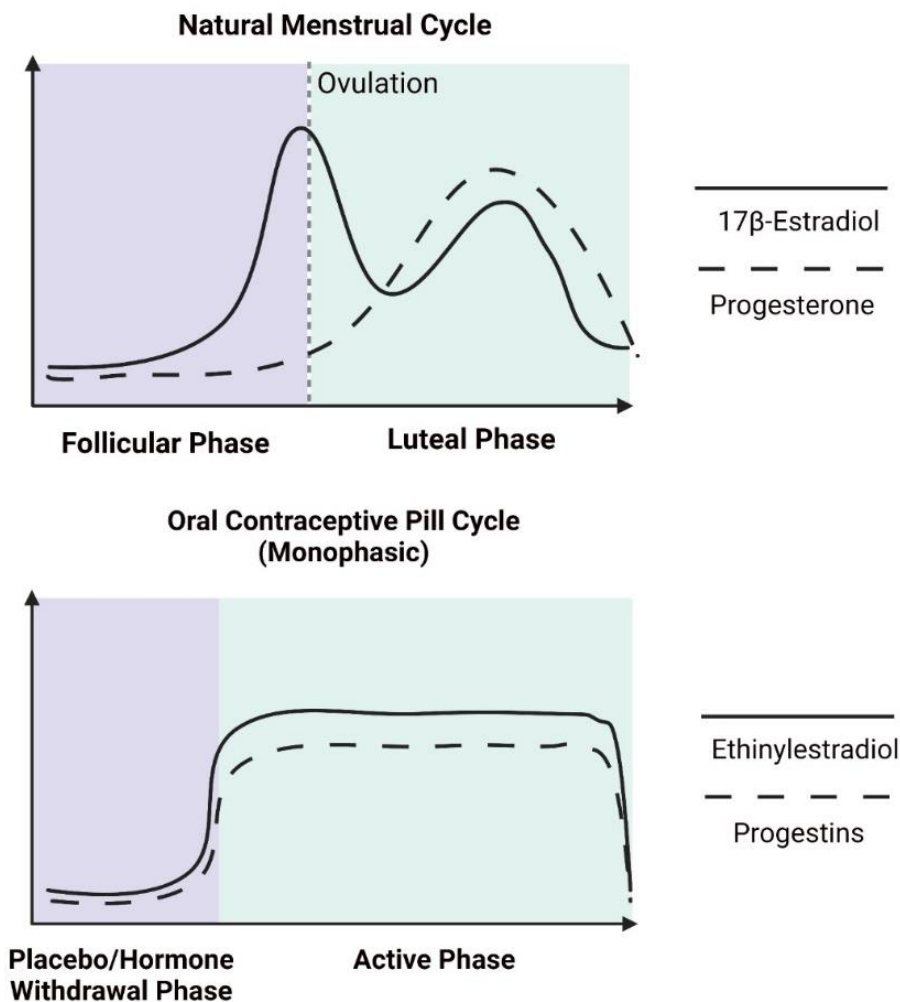
Figure 2. Natural Menstrual and Oral Contraceptive Pill Cycle. The top frame depicts the natural menstrual cycle, separated into the follicular and luteal phases with 17β-estradiol levels represented as a thick line and progesterone levels as a dotted line. The bottom frame depicts a monophasic oral contraceptive pill cycle, with the placebo/hormone withdrawal phase and active phase. Ethinyl estradiol levels are represented by the thick line while progestins (different ones depending on generation of OCP) are represented in the dotted line. Figure created in Biorender.com.

5. Study Research Questions & Hypotheses

The focus of this dissertation was on interrogating questions related to female-specific physiology, using a cell-to-society framework (Figure 3). This framework was inspired by the ecological framework of health (from public health and health promotion literature (109, 110)) and by teachings of Dr. Stacey Ritz (111, 112). While human physiology is commonly examined at an organ/body-level, with questions related to underlying mechanisms at cell and gene levels, the connection from body to surrounding personal environment, community, and society all play a role in shaping human health. This framework permits examinations of the connections between cellular and genetic mechanisms, organ/body-level physiology, personal environment, community, and society/culture (and policy), and their influence on health outcomes.

The overarching purposes of this dissertation were to:

  (a) Examine the sex-specific inclusion of research participants in human vascular exercise physiology literature, including commonly cited barriers to female inclusion;

  (b) Comprehensively summarize prior literature examining menstrual cycle and hormonal contraceptive use, including cycle phase differences, on peripheral vascular function and structure;

  (c) Examine factors that may underscore differences in CVD risk in young adults, including the influence of the natural menstrual and OCP cycles, biological sex, and gender identity and expression; and

  (d) Holistically examine the influence of the natural menstrual and OCP cycles on the cardiovascular, respiratory, and skeletal muscle metabolism systems.

The following section illustrates the brief rationale and significance, research question(s) and hypothesis for each study included in this dissertation.

Chapter 2: "Examination of sex-specific participant inclusion in vascular exercise physiology research: a systematic review." (Published in *Frontiers in Sports and Active living, Physical Activity in the Prevention and Management of Disease*).

This chapter provides a foundation for the dissertation in presenting a systematic review of vascular exercise physiology research. While government bodies, advocacy groups, and groups of researchers have called for increasing female engagement in basic biomedical and clinical research, the quantification of historical sex-specific inclusion has not been documented in the field of vascular exercise physiology. This review examined sex-specific inclusion of participants in vascular endothelial function exercise physiology research, including reporting in published study designs and results. This review also considered the rationales researchers provided for the inclusion/exclusion of participants on the basis of sex. This study provides a foundation in this dissertation for addressing the lack of female inclusion in vascular exercise physiology research and cites several barriers to inclusion including the perceived complexity of hormonal cycles.

Research Question(s) (a) What is the historical sex-specific prevalence of research participants and rationale(s) for sex-specific inclusion/exclusion of participants in human vascular exercise physiology research? (b) Have there been any changes in sex-specific prevalence over the 25 years of endothelial function (measured by flow mediated dilation) research?

Hypotheses: We hypothesized that there would be a sex-bias in exercise physiology research, specifically that males would have a higher proportion of participation in studies and that the rationales for exclusion would be related to the complexity of female hormonal cycles. We also hypothesized that there would be significant improvements in sex-inclusion in more recent years, given changes to policies and pressures from governmental and other advocacy and funding bodies.

Chapter 3: "Impact of the menstrual cycle on peripheral vascular function in premenopausal women: systematic review & meta-analysis." (Published in *American Journal of Physiology – Heart & Circulatory Physiology*).

This chapter provides additional foundation to the dissertation through systematically examining the influence of the menstrual cycle on peripheral vascular function – namely endothelial function and smooth muscle function in the macro- and microvasculature. Since 1995, numerous studies have been published examining the role of the menstrual cycle, and fluctuations in endogenous 17β-estradiol and progesterone on these early CVD risk factors. While some studies have observed improvements in cardiovascular outcomes, many have observed only a subtle (small) effect or no effect of the menstrual cycle. As a result, this study set out to consolidate literature available examining the influence of the menstrual cycle on peripheral vascular outcomes and provide guidance to researchers on "controls" for the menstrual cycle in research study design.

Research Question(s): What is the effect of menstrual cycle phases on peripheral macro- and microvascular endothelial function and smooth muscle function in premenopausal females?

Hypotheses: We hypothesized that the menstrual cycle would have a subtle (small) effect on macro- and microvascular endothelial function, but no influence on smooth muscle function, based previous evidence.

Chapter 4: "Influence of hormonal contraceptives on peripheral vascular function and structure in premenopausal females: a review." (Published in *American Journal of Physiology – Heart & Circulatory Physiology*).

This chapter completes the foundational review chapters, providing context to the state of the literature on hormonal contraceptive use and peripheral vascular function and structure in premenopausal females. While hormonal contraceptives are one of the most widely prescribed drugs used by premenopausal females, their effects on early risk factors for CVD are minimally studied. This review aims to summarize current literature on contraceptive use and vascular function and structure, both across types and generations of contraceptives compared to non-users and examining cycle phase

differences. This review also provided future directions and considerations for researchers investigating contraceptive use and cardiovascular outcomes.

Research Question(s): (a) What is the effect of different generations of OCPs and non-oral contraceptives, compared to non-users (naturally cycling) on peripheral vascular function and structure in females? (b) Are there any OCP phase differences in peripheral vascular outcomes?

Hypotheses: We hypothesized that there would be an effect of both OCP and non-oral contraceptive use, along with phase differences in OCPs, on vascular function and structure, dependent in part on route of administration and progestin type and dosage.

Chapter 5: "Menstrual cycle and oral contraceptive pill phase largely do not influence vascular function, arterial stiffness, and associated cellular regulation in young, healthy premenopausal females." (Submitted to *Arteriosclerosis, Thrombosis, and Vascular Biology*).

As identified in Chapter 2, historical exclusion of female participants in vascular exercise physiology research has been in part based on the perceived complexity of the natural menstrual and OCP cycles. However, findings from Chapter 3 noted only a subtle influence of the natural menstrual cycle on peripheral vascular endothelial function and no influence on smooth muscle function. Further, Chapter 4 identified variable effects of OCPs, dependent on generation type and progestin dose, on peripheral artery endothelial function. Chapter 4 also found no influence on smooth muscle function and arterial stiffness, with mixed effects on carotid artery structure, but an overall paucity of literature on these outcomes. These chapters also highlighted the lack of research on lower limb peripheral vascular function and no investigation into underlying cellular mechanisms governing endothelial function responses. These studies provided the foundation for this study designed to address these gaps in the literature.

Research Question(s): (a) What is the phase influence of 2nd and 3rd generation OCPs and the natural menstrual cycle on endothelial and smooth muscle function in the upper and lower limbs, and arterial stiffness?; (b) What is the chronic impact of 2nd and 3rd generation OCP use compared to naturally cycling participants on peripheral vascular outcomes?; and (3) Are *in vitro* cellular mechanisms linked to *in vivo* endothelial function measures with OCP and natural menstrual cycles?

Hypotheses: Based on previous literature in Chapters 3 and 4, we hypothesized that there would be subtle and acute phasic improvements in endothelial function in the high hormone phases in 3rd generation OCP users and naturally cycling participants, but not in 2nd generation OCP users in both the upper and lower limbs. We also hypothesized that there would be no effect of OCP or natural menstrual cycles on smooth muscle function in the upper and lower limbs or arterial stiffness. We also hypothesized that at a group-level, 3rd generation OCP users would have improved endothelial function compared to 2nd generation OCP users, but with no difference in smooth muscle function in the upper and lower limbs, and no difference in arterial stiffness. Finally, we hypothesized that cellular mechanisms governing alterations in endothelial function would be associated with the ERα-eNOS-NO pathways.

Chapter 6: "The impact of natural menstrual cycle and oral contraceptive pill phase on substrate oxidation during rest and acute submaximal aerobic exercise." (Published in the *Journal of Applied Physiology*).

Based on examination of sex-differences in skeletal muscle and metabolic outcomes, females are reported to have elevated lipid oxidation and lower carbohydrate and protein oxidation compared to males at rest and during submaximal aerobic exercise. One hypothesis provided for known sex-differences in substrate oxidation is the potential influence of sex hormones. However, the acute influence of the natural menstrual and OCP cycles on substrate oxidation and other cardiorespiratory outcomes has been minimally investigated, leaving this hypotheses untested. Note that the participants in this study chapter also completed the study in Chapter 5, so there is

overlap in describing the participant characteristics and ethics associated with the overall study.

Research Question(s): What is the influence of the natural menstrual cycle and 2nd and 3rd generation OCP use on substrate oxidation during rest and submaximal aerobic exercise?

Hypotheses: We hypothesized, based on previous evidence, that at rest and during 40% and 65% $\dot{V}O_2peak$ exercise, there would be an increase in lipid oxidation in the high hormone phase of both natural menstrual and OCP cycles when endogenous/exogenous estrogen and progesterone/progestins are elevated. We also hypothesized that these sex hormones would have similar phase influences on substrate oxidation, and there would be no differences in response between groups.

Chapter 7: "Differences in cardiovascular risk factors associated with biological sex, but not gender expression, in young, healthy adults." (Submitted to *Biology of Sex Differences*).

A central focus of this dissertation has been on female-specific physiology, however, the rationale to pursuing investigations with female-specific research questions is often based on understanding sex-differences in physiological outcomes. As noted in Chapter 2, very few (<1%) of studies examining vascular exercise physiology examined gender. While sex-differences in early risk factors for CVD, including elevated blood pressure and arterial stiffness and lower endothelial function in males compared to females, have been extensively studied, gender has recently emerged as an additional factor to explore. Specifically, feminine gender expression and gender roles traditionally ascribed to women may be associated with increased risk of CVD. However, no study to date had examined the associations of biological sex, gender identity, and gender expression on risk factors for CVD, in a cohort of young, healthy adults.

Research Question(s): (a) What is the impact of biological sex, gender identity, and gender expression on early risk factors for CVD – including blood pressure, arterial stiffness, and endothelial function. (b) Are there associations between gender expression, cardiorespiratory fitness, central arterial stiffness, and endothelial function that help explain the sex- and gender-differences previously observed in CVD?

Hypotheses: We hypothesized that arterial stiffness and blood pressure would be elevated in males and men, but that males and men would also have elevated endothelial function (when differences in baseline diameter are accounted for). We also hypothesized that feminine gender expression would be associated with a blunted cardiovascular profile, namely lower endothelial function and higher arterial stiffness. We hypothesized that increasing cardiorespiratory fitness would be associated with improvements in endothelial function and arterial stiffness, but that increasing feminine gender expression scores would be associated with impaired CV outcomes (decreased endothelial function and increased central arterial stiffness).
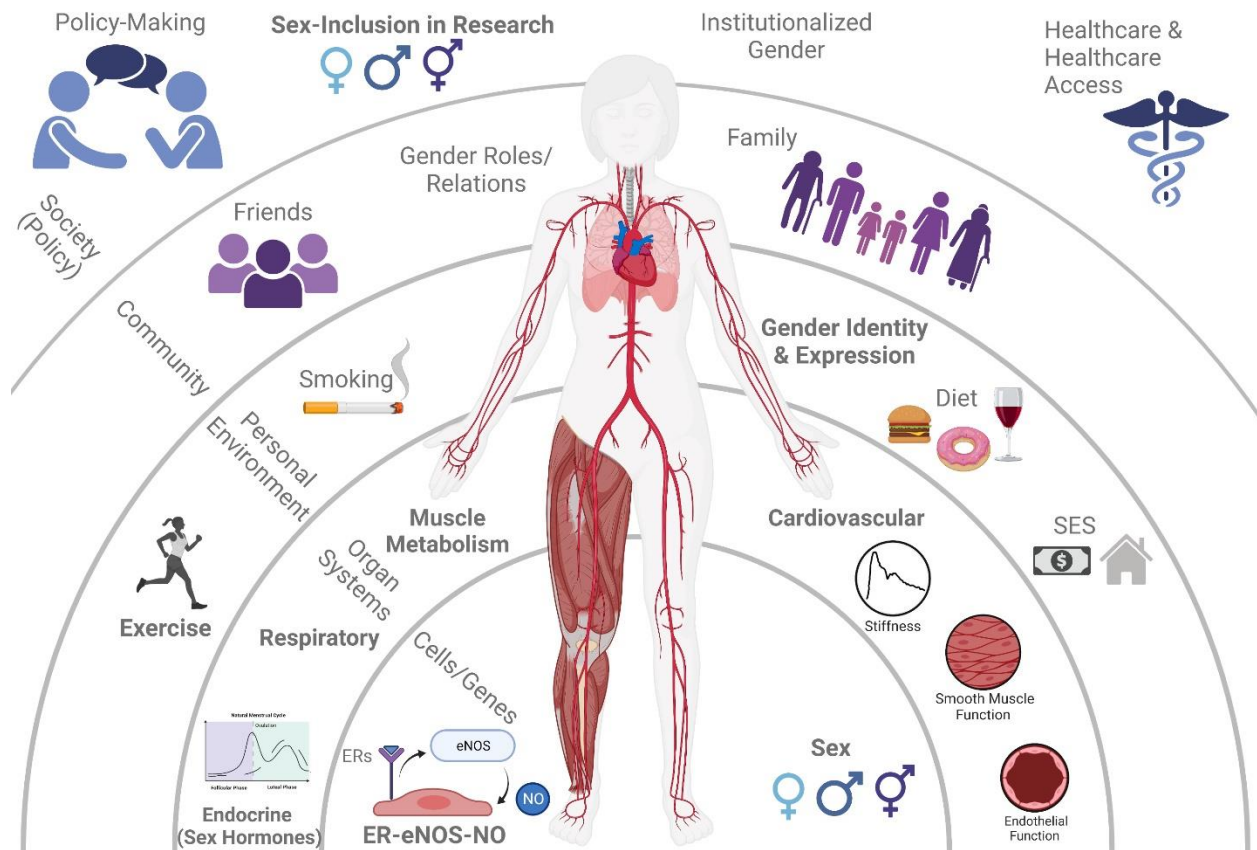
Figure 3. Dissertation Central Figure. Human physiology does not exist in a vacuum; singular organ physiology is connected to cells and gene regulation, other organ-to-organ connections, influences of the personal environment and immediate community (i.e., friends, family, roles within community networks), and societal and policy impacts. While not an extensive listing of all factors influencing human physiology, this dissertation explores factors listed in bold, including sex and the ER-eNOS-NO pathway at the cells/genetic level; endocrine (sex hormones) influence on three interrelated organ systems: cardiovascular, respiratory, muscle metabolism; exercise (fitness) and gender identity and expression, and considerations around policy and societal factors related to sex-inclusion in research study design. Figure created in Biorender.com.

References

1. Stramba-Badiale M, Fox KM, Priori SG, Collins P, Daly C, Graham I, et al. Cardiovascular diseases in women: a statement from the policy conference of the European Society of Cardiology. European Heart Journal. 2006;27(8):994-1005.

2. Wang H, Naghavi M, Allen C, Barber RM, Bhutta ZA, Carter A, et al. Global, regional, and national life expectancy, all-cause mortality, and cause-specific mortality for 249 causes of death, 1980–2015: a systematic analysis for the Global Burden of Disease Study 2015. The Lancet. 2016;388(10053):1459-544.

3. Roth GA, Mensah GA, Johnson CO, Addolorato G, Ammirati E, Baddour LM, et al. Global Burden of Cardiovascular Diseases and Risk Factors, 1990-2019: Update From the GBD 2019 Study. J Am Coll Cardiol. 2020;76(25):2982-3021.

4. Koh HK, Parekh AK, Park JJ. Confronting the Rise and Fall of US Life Expectancy. Jama. 2019;322(20):1963-5.

5. Amini M, Zayeri F, Salehi M. Trend analysis of cardiovascular disease mortality, incidence, and mortality-to-incidence ratio: results from global burden of disease study 2017. BMC Public Health. 2021;21(1):401.

6. Humphries KH, Izadnegahdar M, Sedlak T, Saw J, Johnston N, Schenck-Gustafsson K, et al. Sex differences in cardiovascular disease – Impact on care and outcomes. Frontiers in Neuroendocrinology. 2017;46:46-70.

7. Shufelt CL, Pacheco C, Tweet MS, Miller VM. Sex-Specific Physiology and Cardiovascular Disease. In: Kerkhof PLM, Miller VM, editors. Sex-Specific Analysis of Cardiovascular Function. Cham: Springer International Publishing; 2018. p. 433-54.

8. Sotomi Y, Hikoso S, Nakatani D, Mizuno H, Okada K, Dohi T, et al. Sex Differences in Heart Failure With Preserved Ejection Fraction. Journal of the American Heart Association. 2021;10(5):e018574.

9. Heart and Stroke Foundation of Canada. Ms. Understood: Women's hearts are victims of a system that is ill-equipped to diagnose, treat, and support them. Heart and Stroke Foundation: Annual Heart Report. 2018.

10. Kreatsoulas C, Crea-Arsenio M, Shannon HS, Velianou JL, Giacomini M. Interpreting angina: symptoms along a gender continuum. Open heart. 2016;3(1):e000376.

11. Kreatsoulas C, Natarajan MK, Khatun R, Velianou JL, Anand SS. Identifying women with severe angiographic coronary disease. Journal of Internal Medicine. 2010;268(1):66-74.

12. Norris CM, Yip CYY, Nerenberg KA, Clavel MA, Pacheco C, Foulds HJA, et al. State of the Science in Women's Cardiovascular Disease: A Canadian Perspective on the Influence of Sex and Gender. Journal of the American Heart Association. 2020;9(4):e015634.

13. Beery AK. Inclusion of females does not increase variability in rodent research studies. Current Opinion in Behavioral Sciences. 2018;23:143-9.

14. Meignié A, Toussaint JF, Antero J. Dealing with menstrual cycle in sport: stop finding excuses to exclude women from research. Eur J Appl Physiol. 2022;122(11):2489-90.

15. Lindsey ML, LeBlanc AJ, Ripplinger CM, Carter JR, Kirk JA, Hansell Keehan K, et al. Reinforcing rigor and reproducibility expectations for use of sex and gender in cardiovascular research. American Journal of Physiology-Heart and Circulatory Physiology. 2021;321(5):H819-H24.

16. Lindsey ML, Carter JR, Ripplinger CM, Kassiri Z, Hansell Keehan K, Brunt KR, et al. Sex still matters in cardiovascular research. American Journal of Physiology-Heart and Circulatory Physiology. 2022;324(1):H79-H81.

17. Stranges TN, Namchuk AB, Splinter TFL, Moore KN, Galea LAM. Are we moving the dial? Canadian health research funding trends for women's health, 2S/LGBTQ + health, sex, or gender considerations. Biology of Sex Differences. 2023;14(1):40.

18. Wilson R, Adams ML, Pyke KE. Inclusion of female participants in cardiovascular research: A case study of Ontario NSERC- funded programs. Appl Physiol Nutr Metab. 2020.

19. Beery AK, Zucker I. Sex bias in neuroscience and biomedical research. Neuroscience & Biobehavioral Reviews. 2011;35(3):565-72.

20. Mazure CM, Jones DP. Twenty years and still counting: including women as participants and studying sex and gender in biomedical research. BMC Women's Health. 2015;15(1):94.

21. White J, Tannenbaum C, Klinge I, Schiebinger L, Clayton J. The Integration of Sex and Gender Considerations Into Biomedical Research: Lessons From International Funding Agencies. The Journal of Clinical Endocrinology & Metabolism. 2021;106(10):3034-48.

22. Elliott-Sale KJ, Minahan CL, de Jonge X, Ackerman KE, Sipilä S, Constantini NW, et al. Methodological Considerations for Studies in Sport and Exercise Science with Women as Participants: A Working Guide for Standards of Practice for Research on Women. Sports Med. 2021;51(5):843-61.

23. Sims ST, Heather AK. Myths and Methodologies: Reducing scientific design ambiguity in studies comparing sexes and/or menstrual cycle phases. Exp Physiol. 2018;103(10):1309-17.

24. Chang DH, Dumanski SM, Ahmed SB. Female sex-specific considerations to improve rigor and reproducibility in cardiovascular research. American Journal of Physiology-Heart and Circulatory Physiology. 2023;324(3):H279-H87.

25. Vogel B, Acevedo M, Appelman Y, Bairey Merz CN, Chieffo A, Figtree GA, et al. The Lancet women and cardiovascular disease Commission: reducing the global burden by 2030. Lancet. 2021;397(10292):2385-438.

26. Appelman Y, van Rijn BB, ten Haaf ME, Boersma E, Peters SAE. Sex differences in cardiovascular risk factors and disease prevention. Atherosclerosis. 2015;241(1):211-8.

27. Anand SS, Islam S, Rosengren A, Franzosi MG, Steyn K, Yusufali AH, et al. Risk factors for myocardial infarction in women and men: insights from the INTERHEART study. European Heart Journal. 2008;29(7):932-40.

28. Gerard J. Tortora BHD. Principles of Anatomy and Physiology, 15th Edition. Wiley. 2016.

29. Rizzoni D, Muiesan ML, Porteri E, De Ciuceis C, Boari GEM, Salvetti M, et al. Interrelationships between macro and microvascular structure and function. Artery Research. 2010;4(4):114-7.

30. Hill CE, Phillips JK, Sandow SL. Heterogeneous control of blood flow amongst different vascular beds. Medicinal Research Reviews. 2001;21(1):1-60.

31. Cleuren ACA, van der Ent MA, Jiang H, Hunker KL, Yee A, Siemieniak DR, et al. The in vivo endothelial cell translatome is highly heterogeneous across vascular beds. Proceedings of the National Academy of Sciences. 2019;116(47):23618-24.

32. Vlachopoulos C, Aznaouridis K, Stefanadis C. Prediction of cardiovascular events and all-cause mortality with arterial stiffness: a systematic review and meta-analysis. J Am Coll Cardiol. 2010;55(13):1318-27.

33. Inaba Y, Chen JA, Bergmann SR. Prediction of future cardiovascular outcomes by flow-mediated vasodilatation of brachial artery: a meta-analysis. Int J Cardiovasc Imaging. 2010;26(6):631-40.

34. Yeboah J, Folsom AR, Burke GL, Johnson C, Polak JF, Post W, et al. Predictive value of brachial flow-mediated dilation for incident cardiovascular events in a population-based study: the multi-ethnic study of atherosclerosis. Circulation. 2009;120(6):502-9.

35. Li YS, Haga JH, Chien S. Molecular basis of the effects of shear stress on vascular endothelial cells. J Biomech. 2005;38(10):1949-71.

36. Pyke KE, Tschakovsky ME. The relationship between shear stress and flow-mediated dilatation: implications for the assessment of endothelial function. J Physiol. 2005;568(2):357-69.

37. Green DJ, Jones H, Thijssen D, Cable NT, Atkinson G. Flow-mediated dilation and cardiovascular event prediction: does nitric oxide matter? Hypertension. 2011;57(3):363-9.

38. Tschakovsky ME, Pyke KE, Weisbrod CJ, Maiorana AJ, O'Driscoll G, Green DJ. Relationship between changes in brachial artery flow-mediated dilation and basal release of nitric oxide in subjects with Type 2 diabetes. American Journal of Physiology - Heart and Circulatory Physiology. 2006;291(3):H1193-H9.

39. Pohl U, Holtz J, Busse R, Bassenge E. Crucial role of endothelium in the vasodilator response to increased flow in vivo. Hypertension. 1986;8(1):37-44.

40. Berdeaux A, Ghaleh B, Dubois-Randé JL, Vigué B, Drieu La Rochelle C, Hittinger L, et al. Role of vascular endothelium in exercise-induced dilation of large epicardial coronary arteries in conscious dogs. Circulation. 1994;89(6):2799-808.

41. Langille BL, Reidy MA, Kline RL. Injury and repair of endothelium at sites of flow disturbances near abdominal aortic coarctations in rabbits. Arteriosclerosis (Dallas, Tex). 1986;6(2):146-54.

42. Dawson EA, Rathore S, Cable NT, Wright DJ, Morris JL, Green DJ. Impact of Introducer Sheath Coating on Endothelial Function in Humans After Transradial Coronary Procedures. Circulation: Cardiovascular Interventions. 2010;3(2):148-56.

43. Furchgott RF, Zawadzki JV. The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. Nature. 1980;288(5789):373-6.

44. Celermajer DS, Sorensen KE, Gooch VM, Spiegelhalter DJ, Miller OI, Sullivan ID, et al. Non-invasive detection of endothelial dysfunction in children and adults at risk of atherosclerosis. Lancet. 1992;340(8828):1111-5.

45. Thijssen DHJ, Bruno RM, van Mil A, Holder SM, Faita F, Greyling A, et al. Expert consensus and evidence-based recommendations for the assessment of flow-mediated dilation in humans. Eur Heart J. 2019;40(30):2534-47.

46. Thijssen DH, Black MA, Pyke KE, Padilla J, Atkinson G, Harris RA, et al. Assessment of flow-mediated dilation in humans: a methodological and physiological guideline. Am J Physiol Heart Circ Physiol. 2011;300(1):H2-12.

47. Greyling A, van Mil AC, Zock PL, Green DJ, Ghiadoni L, Thijssen DH. Adherence to guidelines strongly improves reproducibility of brachial artery flow-mediated dilation. Atherosclerosis. 2016;248:196-202.

48. Holder SM, Bruno RM, Shkredova DA, Dawson EA, Jones H, Hopkins ND, et al. Reference Intervals for Brachial Artery Flow-Mediated Dilation and the Relation With Cardiovascular Risk Factors. Hypertension. 2021;77(5):1469-80.

49. Loader J, Roustit M, Taylor F, MacIsaac RJ, Stewart S, Lorenzen C, et al. Assessing cutaneous microvascular function with iontophoresis: Avoiding non-specific vasodilation. Microvasc Res. 2017;113:29-39.

50. Newton DJ, Khan F, Belch JJ. Assessment of microvascular endothelial function in human skin. Clin Sci (Lond). 2001;101(6):567-72.

51. Roustit M, Cracowski JL. Assessment of endothelial and neurovascular function in human skin microcirculation. Trends in pharmacological sciences. 2013;34(7):373-84.

52. Maruhashi T, Soga J, Fujimura N, Idei N, Mikami S, Iwamoto Y, et al. Nitroglycerine-induced vasodilation for assessment of vascular function: a comparison with flow-mediated vasodilation. Arterioscler Thromb Vasc Biol. 2013;33(6):1401-8.

53. Shenouda N, Priest SE, Rizzuto VI, MacDonald MJ. Brachial artery endothelial function is stable across a menstrual and oral contraceptive pill cycle but lower in premenopausal women than in age-matched men. Am J Physiol Heart Circ Physiol. 2018;315(2):H366-H74.

54. Zhou K, Parker JD. The role of vascular endothelium in nitroglycerin-mediated vasodilation. Br J Clin Pharmacol. 2019;85(2):377-84.

55. Pereira T, Correia C, Cardoso J. Novel Methods for Pulse Wave Velocity Measurement. Journal of Medical and Biological Engineering. 2015;35(5):555-65.

56. Gavish B, Izzo JL, Jr. Arterial Stiffness: Going a Step Beyond. Am J Hypertens. 2016;29(11):1223-33.

57. Priest SE, Shenouda N, MacDonald MJ. Effect of sex, menstrual cycle phase, and monophasic oral contraceptive pill use on local and central arterial stiffness in young adults. Am J Physiol Heart Circ Physiol. 2018;315(2):H357-h65.

58. Polak JF, O'Leary DH. Carotid Intima-Media Thickness as Surrogate for and Predictor of CVD. Global Heart. 2016;11(3):295-312.e3.

59. Polak JF, Pencina MJ, Pencina KM, O'Donnell CJ, Wolf PA, D'Agostino RB. Carotid-Wall Intima–Media Thickness and Cardiovascular Events. New England Journal of Medicine. 2011;365(3):213-21.

60. Øygarden H. Carotid Intima-Media Thickness and Prediction of Cardiovascular Disease. Journal of the American Heart Association. 2017;6(1):e005313.

61. Sol AId, Moons KGM, Hollander M, Hofman A, Koudstaal PJ, Grobbee DE, et al. Is Carotid Intima-Media Thickness Useful in Cardiovascular Disease Risk Assessment? Stroke. 2001;32(7):1532-8.

62. Van Bortel LM, Laurent S, Boutouyrie P, Chowienczyk P, Cruickshank JK, De Backer T, et al. Expert consensus document on the measurement of aortic stiffness in daily practice using carotid-femoral pulse wave velocity. J Hypertens. 2012;30(3):445-8.

63. Townsend RR, Wilkinson IB, Schiffrin EL, Avolio AP, Chirinos JA, Cockcroft JR, et al. Recommendations for Improving and Standardizing Vascular Research on Arterial Stiffness: A Scientific Statement From the American Heart Association. Hypertension. 2015;66(3):698-722.

64. Tsuchikura S, Shoji T, Kimoto E, Shinohara K, Hatsuda S, Koyama H, et al. Central versus peripheral arterial stiffness in association with coronary, cerebral and peripheral arterial disease. Atherosclerosis. 2010;211(2):480-5.

65. Sugawara J, Maeda S, Otsuki T, Tanabe T, Ajisaka R, Matsuda M. Effects of nitric oxide synthase inhibitor on decrease in peripheral arterial stiffness with acute low-intensity aerobic exercise. American Journal of Physiology-Heart and Circulatory Physiology. 2004;287(6):H2666-H9.

66. Krieger N. Genders, sexes, and health: what are the connections—and why does it matter? International Journal of Epidemiology. 2003;32(4):652-7.

67. Longpré-Poirier C, Dougoud J, Jacmin-Park S, Moussaoui F, Vilme J, Desjardins G, et al. Sex and Gender and Allostatic Mechanisms of Cardiovascular Risk and Disease. Canadian Journal of Cardiology. 2022;38(12):1812-27.

68. Fausto-Sterling A. Chapter 2 - Of spirals and layers in Sex/Gender: Biology in a Social World. New York: Routledge. 2012:3-11.

69. Lagos D, Compton D. Evaluating the Use of a Two-Step Gender Identity Measure in the 2018 General Social Survey. Demography. 2021;58(2):763-72.

70. Bauer GR, Braimoh J, Scheim AI, Dharma C. Transgender-inclusive measures of sex/gender for population surveys: Mixed-methods evaluation and recommendations. PLOS ONE. 2017;12(5):e0178043.

71. Syme C, Abrahamowicz M, Leonard GT, Perron M, Richer L, Veillette S, et al. Sex Differences in Blood Pressure and Its Relationship to Body Composition and Metabolism in Adolescence. Archives of Pediatrics & Adolescent Medicine. 2009;163(9):818-25.

72. Bredella MA. Sex Differences in Body Composition. In: Mauvais-Jarvis F, editor. Sex and Gender Factors Affecting Metabolic Homeostasis, Diabetes and Obesity. Cham: Springer International Publishing; 2017. p. 9-27.

73. Wells JCK. Sexual dimorphism of body composition. Best Practice & Research Clinical Endocrinology & Metabolism. 2007;21(3):415-30.

74. Joyner MJ, Wallin BG, Charkoudian N. Sex differences and blood pressure regulation in humans. Experimental Physiology. 2016;101(3):349-55.

75. Holder SM, Bruno RM, Shkredova DA, Dawson EA, Jones H, Hopkins ND, et al. Reference Intervals for Brachial Artery Flow-Mediated Dilation and the Relation With Cardiovascular Risk Factors. Hypertension (0194911X). 2021;77(5):1469-80.

76. Junker A, Juster R-P, Picard M. Integrating sex and gender in mitochondrial science. Current Opinion in Physiology. 2022;26:100536.

77. Clayton JA, Tannenbaum C. Reporting Sex, Gender, or Both in Clinical Research? Jama. 2016;316(18):1863-4.

78. Pelletier R, Khan NA, Cox J, Daskalopoulou SS, Eisenberg MJ, Bacon SL, et al. Sex Versus Gender-Related Characteristics: Which Predicts Outcome After Acute Coronary Syndrome in the Young? Journal of the American College of Cardiology. 2016;67(2):127-35.

79. Seeland U, Nemcsik J, Lønnebakken MT, Kublickiene K, Schluchter H, Park C, et al. Sex and Gender Aspects in Vascular Ageing - Focus on Epidemiology, Pathophysiology, and Outcomes. Heart, lung & circulation. 2021;30(11):1637-46.

80. Dev R, Adams AM, Raparelli V, Norris CM, Pilote L. Sex and Gender Determinants of Vascular Disease in the Global Context. Canadian Journal of Cardiology. 2022;38(12):1799-811.

81. Juster RP, Pruessner JC, Desrochers AB, Bourdon O, Durand N, Wan N, et al. Sex and Gender Roles in Relation to Mental Health and Allostatic Load. Psychosom Med. 2016;78(7):788-804.

82. Poteat TC, Divsalar S, Streed CG, Feldman JL, Bockting WO, Meyer IH. Cardiovascular Disease in a Population-Based Sample of Transgender and Cisgender Adults. American Journal of Preventive Medicine. 2021;61(6):804-11.

83. Kerr P, Barbosa Da Torre M, Giguère C, Lupien SJ, Juster RP. Occupational gender roles in relation to workplace stress, allostatic load, and mental health of psychiatric hospital workers. J Psychosom Res. 2021;142:110352.

84. Mereish EH, Goldstein CM. Minority Stress and Cardiovascular Disease Risk Among Sexual Minorities: Mediating Effects of Sense of Mastery. International journal of behavioral medicine. 2020;27(6):726-36.

85. Morgan E, D'Aquila R, Carnethon MR, Mustanski B. Cardiovascular disease risk factors are elevated among a cohort of young sexual and gender minorities in Chicago. Journal of Behavioral Medicine. 2019;42(6):1073-81.

86. Khoudary SRE, Aggarwal B, Beckie TM, Hodis HN, Johnson AE, Langer RD, et al. Menopause Transition and Cardiovascular Disease Risk: Implications for Timing of Early Prevention: A Scientific Statement From the American Heart Association. Circulation. 2020;142(25):e506-e32.

87. Hayward CS, Kelly RP, Collins P. The roles of gender, the menopause and hormone replacement on cardiovascular function. Cardiovascular Research. 2000;46(1):28-49.

88. Davezac M, Buscato M, Zahreddine R, Lacolley P, Henrion D, Lenfant F, et al. Estrogen Receptor and Vascular Aging. Frontiers in Aging. 2021;2.

89. Moreau KL, Hildreth KL, Klawitter J, Blatchford P, Kohrt WM. Decline in endothelial function across the menopause transition in healthy women is related to decreased estradiol and increased oxidative stress. Geroscience. 2020;42(6):1699-714.

90. Moreau KL, Hildreth KL, Meditz AL, Deane KD, Kohrt WM. Endothelial function is impaired across the stages of the menopause transition in healthy women. J Clin Endocrinol Metab. 2012;97(12):4692-700.

91. Moreau KL, Stauffer BL, Kohrt WM, Seals DR. Essential role of estrogen for improvements in vascular endothelial function with endurance exercise in postmenopausal women. J Clin Endocrinol Metab. 2013;98(11):4507-15.

92. Hildreth KL, Ozemek C, Kohrt WM, Moreau KL, Blatchford PJ, Hurt KJ, et al. Effects of resveratrol or estradiol on postexercise endothelial function in estrogen-deficient postmenopausal women. Journal of applied physiology (Bethesda, Md : 1985). 2020;128(4):739-47.

93. Gavin KM, Seals DR, Silver AE, Moreau KL. Vascular endothelial estrogen receptor alpha is modulated by estrogen status and related to endothelial function and endothelial nitric oxide synthase in healthy women. J Clin Endocrinol Metab. 2009;94(9):3513-20.

94. Charney P. Coronary artery disease in young women: the menstrual cycle and other risk factors. Annals of Internal Medicine. 2001;135(11):1002-4.

95. Iorga A, Cunningham CM, Moazeni S, Ruffenach G, Umar S, Eghbali M. The protective role of estrogen and estrogen receptors in cardiovascular disease and the controversial use of estrogen therapy. Biol Sex Differ. 2017;8(1):33.

96. Sudoh N, Toba K, Akishita M, Ako J, Hashimoto M, Iijima K, et al. Estrogen Prevents Oxidative Stress–Induced Endothelial Cell Apoptosis in Rats. Circulation. 2001;103(5):724-9.

97. Wassmann K, Wassmann S, Nickenig G. Progesterone Antagonizes the Vasoprotective Effect of Estrogen on Antioxidant Enzyme Expression and Function. Circulation Research. 2005;97(10):1046-54.

98. Miner JA, Martini ER, Smith MM, Brunt VE, Kaplan PF, Halliwill JR, et al. Short-term oral progesterone administration antagonizes the effect of transdermal estradiol on endothelium-dependent vasodilation in young healthy women. American Journal of Physiology-Heart and Circulatory Physiology. 2011;301(4):H1716-H22.

99. Bentur OS, Schwartz D, Chernichovski T, Ingbir M, Weinstein T, Chernin G, et al. Estradiol augments while progesterone inhibits arginine transport in human endothelial cells through modulation of cationic amino acid transporter-1. American Journal of Physiology-Regulatory, Integrative and Comparative Physiology. 2015;309(4):R421-R7.

100. Reed BG, Carr BR. The Normal Menstrual Cycle and the Control of Ovulation. In: De Groot LJ, Chrousos G, Dungan K, Feingold KR, Grossman A, Hershman JM, et al., editors. Endotext. South Dartmouth (MA)2000.

101. Hackney AC, Smith-Ryan AE, Fink JE. Methodological Considerations in Exercise Endocrinology. In: Hackney AC, Constantini NW, editors. Endocrinology of Physical Activity and Sport. Cham: Springer International Publishing; 2020. p. 1-17.

102. Park H, Kim K. Trends and Factors Associated with Oral Contraceptive Use among Korean Women. Healthcare (Basel, Switzerland). 2021;9(10).

103. Rotermann M, Dunn S, Black A. Oral contraceptive use among women aged 15 to 49: Results from the Canadian Health Measures Survey. Statistics Canada. 2015;26(10).

104. Darney PD. The androgenicity of progestins. The American Journal of Medicine. 1995;98(1, Supplement 1):S104-S10.

105. Shufelt CL, Bairey Merz CN. Contraceptive hormone use and cardiovascular disease. Journal of the American College of Cardiology. 2009;53(3):221-31.

106. Christin-Maitre S. History of oral contraceptive drugs and their use worldwide. Best Practice & Research Clinical Endocrinology & Metabolism. 2013;27(1):3-12.

107. Schlaff WD, Lynch AM, Hughes HD, Cedars MI, Smith DL. Manipulation of the pill-free interval in oral contraceptive pill users: the effect on follicular suppression. American Journal of Obstetrics & Gynecology. 2004;190(4):943-9.

108. Sulak PJ, Kuehl TJ, Ortiz M, Shull BL. Acceptance of altering the standard 21-day/7-day oral contraceptive regimen to delay menses and reduce hormone withdrawal symptoms. Am J Obstet Gynecol. 2002;186(6):1142-9.

109. McLeroy KR, Bibeau D, Steckler A, Glanz K. An ecological perspective on health promotion programs. Health education quarterly. 1988;15(4):351-77.

110. Stokols D. Establishing and maintaining healthy environments. Toward a social ecology of health promotion. The American psychologist. 1992;47(1):6-22.

111. Greaves L, Ritz SA. Sex, Gender and Health: Mapping the Landscape of Research and Policy. Int J Environ Res Public Health. 2022;19(5).

112. Ritz DS. HLTHSCI 2T03: Sex, Gender and Health Course Outline. McMaster University Bachelor of Health Sciences Program. 2021.

CHAPTER 2: Examination of sex-specific participant inclusion in vascular exercise physiology research: a systematic review.

Lindsay A. Lew*, **Jennifer S. Williams***, Jenna C. Stone, Alicia K. W. Au, Kyra E. Pyke, Maureen J. MacDonald

*Shared first author contributions

Supplemental Material from this Chapter can be found here:
https://www.frontiersin.org/articles/10.3389/fspor.2022.860356/full#supplementary-material

frontiers
in Sports and Active Living

Examination of Sex-Specific Participant Inclusion in Exercise Physiology Endothelial Function Research: A Systematic Review

Lindsay A. Lew [1†], Jennifer S. Williams [2†], Jenna C. Stone [2], Alicia K. W. Au [2], Kyra E. Pyke [1] and Maureen J. MacDonald [2*]

[1] Cardiovascular Stress Response Lab, School of Kinesiology and Health Studies, Queen's University, Kingston, ON, Canada, [2] Vascular Dynamics Lab, Department of Kinesiology, McMaster University, Hamilton, ON, Canada

Background: To combat historical underrepresentation of female participants in research, guidelines have been established to motivate equal participation by both sexes. However, the pervasiveness of female exclusion has not been examined in vascular exercise physiology research. The purpose of this study was to systematically quantify the sex-specific prevalence of human participants and identify the rationales for sex-specific inclusion/exclusion in research examining the impact of exercise on vascular endothelial function.

Methods: A systematic search was conducted examining exercise/physical activity and vascular endothelial function, assessed via flow mediated dilation. Studies were categorized by sex: male-only, female-only, or mixed sex, including examination of the sample size of males and females. Analysis was performed examining sex-inclusion criteria in study design and reporting and rationale for inclusion/exclusion of participants on the basis of sex. Changes in proportion of female participants included in studies were examined over time in 5 year cohorts.

Results: A total of 514 studies were identified, spanning 26 years (1996–2021). Of the total participants, 64% were male and 36% were female, and a male bias was identified (32% male-only vs. 12% female-only studies). Proportions of female participants in studies remained relatively constant in the last 20 years. Male-only studies were less likely to report sex in the title compared to female-only studies (27 vs. 78%, $p < 0.001$), report sex in the abstract (72 vs. 98%, $p < 0.001$) and justify exclusion on the basis of sex (15 vs. 55%, $p < 0.001$). Further, male-only studies were more likely to be conducted in healthy populations compared to female-only studies ($p = 0.002$). Qualitative analysis of justifications identified four themes: sex-specific rationale or gap in the literature, exclusion of females based on the hormonal cycle or sex-differences, maintaining congruence with the male norm, and challenges with recruitment, retention and resources.

Conclusions: This systematic review provides the first analysis of sex-based inclusion/exclusion and rationale for sex-based decisions in human vascular exercise

physiology research. These findings contribute to identifying the impact of research guidelines regarding inclusion of males and females and the perceived barriers to designing studies with equal sex participation, in an effort to increase female representation in vascular exercise physiology research.

INTRODUCTION

Sex-specific inclusion/exclusion in physiology research has been a long-standing issue, with male human, animal, and cell models often preferentially selected, as observed in basic science (Coiro and Pollak, 2019; Kim et al., 2021), pre-clinical human (Feldman et al., 2019), and clinical research trials (Heart and Stroke, 2018; Feldman et al., 2019). For example, a recent study by Cowley et al. examined sex-bias in sport and exercise science research, finding that during 2014–2020 in 6 major sports science journals, two-thirds of participants overall across studies were male and 31% of studies exclusively assessed males (compared to only 6% of studies exclusively assessing females) (Cowley et al., 2021). Further, these authors consistently observed sex-bias in the number of participants and number of sex-specific studies over the years of study (Cowley et al., 2021), which agreed with earlier findings by Costello et al., of studies published between the years of 2011 and 2013 (Costello et al., 2014). However, information is lacking on the rationale(s) for inclusion/exclusion on the basis of sex, or additional elements of sex-bias in the presentation of the articles examined, such as how information on sex is reported in the abstract and methodology of the manuscripts (Wilson et al., 2020).

"Sex" refers to the biological attributes, such as chromosomes, anatomy, and hormones, which determine male and female sex, while "gender" refers to socially constructed identity, roles, and behaviors that govern men and women (Tannenbaum et al., 2016); however, a nuanced approach to sex/gender identifies these constructs as more complex than a binary categorization (Fausto-Sterling, 2012; Bhargava et al., 2021). Responding to the concerns regarding sex/gender representation in human research, expert guidelines, government policies, grant guidelines, and recent journal publication requirements have been established. For example, the Sex and Gender Equity in Research (SAGER) guidelines, established in 2012, detail how to consider sex/gender in research design and reporting (Heidari et al., 2016). Expanding to examine government policies, in Canada, three federally-funded research councils, established the "Tri-Council Policy Statement: Ethical Conduct for Research Involving Humans" (TCPS 2) first established in 2010, and updated in 2018 (Tri-Council Policy Statement, 2018). Article 4.2 of this statement identifies that "women shall not be inappropriately excluded from research solely on the basis of gender or sex," recognizing the historical and discriminatory exclusion of women in human research (Tri-Council Policy Statement, 2018). Similarly, in the United States, originally dating back to 1994 but recently updated in 2017, all NIH-funded clinical research must consider sex/gender, alongside

other participant characteristics such as race and/or ethnicity in study design "…to ensure that research findings can be generalizable to the entire population" (National Institute for Health Research, 2017). Likewise, in the United Kingdom in 2017, the NIHR-INCLUDE Framework and Guidance was established to provide a "roadmap" for improving inclusion and representation in health and care research, including examining under-served groups including groups based on sex (National Institute for Health Research, 2020; Witham et al., 2020). These recent guidelines from health research bodies have identified clear direction for sex-specific inclusive practices in research.

Further, recent changes in grant guidelines require researchers to consider sex and/or gender in establishing research studies for federal funding. These changes were recently quantified in a 10-year longitudinal study, evaluating integration of sex and/or gender in grant submissions to the Canadian Institutes of Health Research (CIHR) (Haverfield and Tannenbaum, 2021). This study found that integration of sex in grant submissions rose from 22% in 2011 to 83% in 2021; while integration of gender increased from 12 to 33% (Haverfield and Tannenbaum, 2021). Moreover, applications with high scores in the integration of sex/gender have a higher likelihood of being funded (sex: 92% higher, gender: 153% higher) (Haverfield and Tannenbaum, 2021). Finally, some journals have endeavored to create guidelines or requirements for justification of sex/gender inclusion in study designs. For example, the *American Journal of Physiology – Heart and Circulatory Physiology* recently released new requirements that as of January 2023, all studies must include both sexes/genders, unless there is "strong scientific justification" for studying a single sex (e.g., studying hormonal contraceptive use in females, studying prostate cancer in males) (Lindsey et al., 2021).

Despite the burgeoning body of literature and policy changes aimed at integrating sex/gender considerations in human research, females continue to be excluded. For example, a recent case study of Ontario's NSERC-funded programs on the inclusion of female participants in cardiovascular physiology research found that females were underrepresented in or excluded from 63% of studies, with no temporal changes since the establishment of the TCPS 2 policy in 2010 (Wilson et al., 2020). Further, the study interviewed a limited number of Principal Investigators with NSERC Discovery Grant funding and identified notions of a "male norm" contributing to the preferential selection of male research participants as males are seen as the "standard" research subject and the female body is seen as more complex with considerations regarding the menstrual cycle, technical difficulties in acquiring measures, and/or disease prevalence (Wilson et al., 2020). An example of this can be seen in a recent

paper by Naylor et al., which examined comparisons in brachial and femoral artery function in male athletes and excluded females due to the potential influence of sex hormones on flow-mediated dilation (FMD) and the need for male-specific data (Naylor et al., 2021).

Macrovascular endothelial function is commonly assessed using a standard FMD test via vascular ultrasound technology, which examines the artery response to occlusion-induced hyperemia (Thijssen et al., 2019). The FMD response of the brachial artery is directly correlated with endothelial function of the coronary arteries (Raitakari and Celermajer, 2000), and endothelial function is of clinical relevance as its dysfunction is a precursor in the development of atherosclerosis, stroke, and hypertension (Yeboah et al., 2009). Current guidelines (updated in 2019) for the assessment of vascular endothelial function, and specifically FMD, detail that "premenopausal women should be examined in a standardized phase of the menstrual cycle, since hormonal changes can affect FMD" (Thijssen et al., 2019). However, recent studies from our lab groups have repeatedly identified lack of changes in FMD across the menstrual and oral contraceptive cycle (D'Urzo et al., 2018; Shenouda et al., 2018; Williams et al., 2019; Liu et al., 2021). In agreement, a recent meta-analysis found that the menstrual cycle has only a small effect on FMD, which was largely accounted for by methodological differences in FMD acquisition (Williams et al., 2020). Therefore, the topic of how to consider "controlling" for the hormonal cycle has been long debated, with a Point-Counterpoint discussion published in 2020 (Stanhewicz and Wong, 2020; Wenner and Stachenfeld, 2020a) and recent methodological guidance papers (Sims and Heather, 2018; Elliott-Sale et al., 2021). Ongoing discourse on the topic of hormonal cycling controls indicates that testing females during a standardized phase of the hormonal cycle (e.g., early follicular phase or placebo phase) is recommended (Thijssen et al., 2019); however, the need for control may depend on the study design and population of interest (Stanhewicz and Wong, 2020).

Despite the ongoing discourse surrounding the need to have more inclusion of female participants in exercise physiology research, quantification of the historical sex-specific inclusion in vascular exercise physiology studies and identification of rationale(s) for inclusion/exclusion has yet to be published. Therefore, the purpose of this study was to systematically quantify the sex-specific prevalence of participants and identify the rationale(s) for sex-specific inclusion/exclusion of participants in human research examining the impact of exercise on vascular endothelial function. FMD was selected as the primary outcome of interest for its clinical relevance and prevalence as a macrovascular assessment method. Aligned with previous studies identifying sex-bias in exercise physiology research (Costello et al., 2014; Cowley et al., 2021), we hypothesized observing a male sex-bias in vascular exercise research, with rationales for exclusion related to the perceived complexity of female bodies. However, we also anticipated that there would be significant improvements in sex parity in vascular exercise physiology research in recent years, in concert with the implementation of guidelines and policy addressing the issue.

METHODS

This systematic review was conducted following the Preferred Reporting Items for Systematic Review and Meta-Analysis (PRISMA) statement. This review was also registered with the International Prospective Register of Systematic Reviews (PROSPERO).

Search Strategy

A systematic search was conducted to investigate the current and past prevalence of sex-specific participant inclusion in vascular exercise physiology research. Studies were selected for inclusion through a systematic search of three online databases EMBASE, MEDLINE, and SPORTDiscus, from inception to October 2021. The search strategy (**Appendix A**) was aimed to select articles evaluating macrovascular endothelial function in response to acute or chronic exercise or physical activity interventions, or cross-sectional studies examining athlete/active vs. non-athlete/sedentary populations. The search consisted of the following combination of keywords: "exercise" OR "training" OR "physical activity" or "athlete" OR "cycling" OR "running" AND "vascular function" OR "endothelial function" OR "endothelium-dependent dilation" OR "flow-mediated dilation" OR "flow mediated dilation."

Eligibility Criteria

Only peer-reviewed, original studies, written in English were eligible for inclusion in this review. Studies were excluded if they were not available in English, or were reviews (e.g., narrative, literature, systematic, meta-analyses), case studies, commentaries, letters to the editor, conference abstracts, or non-peer reviewed (e.g., thesis manuscripts). Studies must have included human participants (cell and animal models were excluded) of any age and clinical status (*Population*). Studies must have incorporated any type of exercise, training or physical activity intervention or a cross-sectional comparison (*Intervention*). Finally, studies were required to include flow-mediated dilation (FMD) methodology assessed via ultrasound technology as an outcome variable (*Outcome*).

Study Selection

Eligibility of studies was assessed by two reviewers. Initial title and abstract screening for all studies was conducted independently by two reviewers (LAL and JSW). Any discrepancies about eligibility were settled through consensus following a discussion with the two reviewers (LAL and JSW). Next, a full-text screening was conducted independently by two reviewers (LAL and JSW). Similarly, any discrepancies about eligibility or the reason for exclusion were settled by consensus following a discussion with the two reviewers (LAL and JSW).

Data Extraction

Data was extracted from each study by one of four reviewers (LAL, JSW, JCS or ACWA), following the piloting of the data extraction sheet (**Appendix B**). Information regarding participant and study characteristics, sex of participants, results, and discussion of sex/gender throughout the article were extracted from all included studies. Data extracted about

PhD Thesis – J. Williams; McMaster University - Kinesiology

participant and study characteristics included: age, hormonal status, clinical status, type of study, and exercise/physical activity intervention length and type. Data extracted about sex of participants included: sex of participants, total sample size, sample size of males and females, and questions regarding the reporting of sex throughout the manuscript (**Appendix B**). Data on studies confusing or conflating terminology for sex compared to gender (e.g., study examining biological males and females, but using the term gender, or interchanging with men/women), and whether studies examined gender were extracted. Finally, justification/rationale of inclusion/exclusion of sex throughout the manuscript was recorded, where applicable and available.

Data Synthesis and Analysis
Quantitative Analysis
Quantitative data was aggregated and reported across all years as Chi squared analysis (Microsoft Excel 2016). Proportion of female participants included in studies was compared across cohorts of years (i.e., every 5 years) to examine changes over time in sex-specific inclusion in research trials, using one-way ANOVA with the factor being the year cohort. Games-Howell corrected *post-hoc* tests were conducted as the homogeneity of variance was violated (Levene's test, $p < 0.001$; SPSS, Version 22.0). Significance was set at $p \leq 0.05$. Five-year cohorts were selected, as the range of studies included in the review spanned 25 and 5-year cohorts provides a reasonable number of groups for comparative analysis. The proportion of male-only, female-only and mixed-sex studies were examined. Mixed-sex studies were also assessed for proportion of females (40–60% proportion of females/total sample size = equal; < 40% females = unequal favoring males, > 60% females = unequal favoring females), as previously published (Wilson et al., 2020). Where a study did not specify sex ($n = 11$), an assumption was made that the study was in only males, as per Wilson et al. (2020). Number of studies including various participant characteristics, study designs and types of exercise interventions in each sex-specific inclusion grouping was examined and reported. Sex-based analysis performed in studies and discussion of sex/gender throughout the paper were examined and reported.

Qualitative Analysis
Qualitative data was thematically coded using reflexive thematic analysis to identify patterns and themes of rationales provided for sex-specific inclusion/exclusion (Braun and Clarke, 2021). Two authors (LAL and JSW) analyzed extracted quotes from the articles that provided justification or rationales for the inclusion/exclusion of either sex, and sorted related quotes and defined and named common themes for inclusion in the results.

RESULTS
Study Selection and Characteristics
The systematic search revealed 5,052 articles after duplicates were removed, that underwent title and abstract screening to result in 694 articles for full-text review. Following full-text review, 514 articles remained for inclusion in the systematic review, with exclusions identified in the flow diagram figure

(**Figure 1**). Examining the year-ranges of studies, 37% (192) studies were published in 2021–2017, 30% (156) from 2016 to 2012, 21% (107) from 2007 to 2011, 10% (49) from 2002 to 2006, and 2% (10) from 1996 to 2001. Examining the types of participants included in the studies, there was an even split between healthy populations (49%) and clinical populations (51%). Further details regarding the types of studies included in this review can be found in **Figure 2**. The majority of trials were randomized controlled trials (**Figure 2A**), chronic exercise training interventions (**Figure 2B**), and specifically involved aerobic exercise interventions (**Figure 2C**). Similarly, the participants represented in the included trials varied by age (**Figure 2D**) and menopausal status (**Figure 2E**), with a large proportion of studies not reporting menopausal status (42%). Similarly, the majority of studies including female participants did not specify the phase of the hormonal cycle tested or did not control for the hormonal cycle (72%; **Figure 2F**); only 28% tested in a consistent hormonal phase (e.g., early follicular phase/placebo phase or another consistent phase) as per the FMD guidelines (Thijssen et al., 2019).

Sex-Inclusion in Study Design
The total number of participants in the review was 25,364, with 16,140 males (64%) and 9,247 females (36%). The proportion of female participants in studies was different across time cohorts (main effect of time cohorts: $p = 0.004$); however, *post-hoc* testing revealed that the only difference was a lower %females in the 1996–2001 cohort compared to all others ($p < 0.001$). However, there were only 10 studies in this time cohort (2% of all studies). While the total number of studies increased, there was no difference in the proportion of female participants across time cohorts in the last 20 years (average: 35%; **Figure 3A**).

While the majority of trials reported sex of participants (97%), in 3% of studies sex was not disclosed; these studies were assumed to be "male-only" as described by Wilson et al. (2020). Examining further the number of female-only, male-only and mixed sex studies, the number of mixed-sex studies (56%) was greater than that of male-only studies (32%), which was greater than that of female-only studies (12%; **Figure 3B**). Of the studies that were mixed-sex, the number of studies that favored the inclusion of females (20%) was lower than the number of studies that included equal male and female participants (37%) and studies that favored inclusion of males (43%; **Figure 3C**).

Sex-Inclusion in Study Reporting
Of studies in single-sex populations (i.e., male-only or female-only studies), ~40% of studies included sex in the title, while nearly 80% of studies included sex in the abstract. Similarly, of studies that were single-sex in nature or mixed-sex with an underrepresentation of one sex, ~17% of studies justified the exclusion or underrepresentation of a sex. Finally, 32% of studies in single-sex populations recognized the lack of generalizability of their study.

When comparing female-only and male-only studies, it was determined that male-only studies were less likely to report sex in the title compared to female-only studies [27% male-only vs. 78% female-only, $\chi^2 (1,223) = 45.86, p < 0.001$; **Figure 3D**]. The same

52

FIGURE 1 | Systematic review flow-diagram.
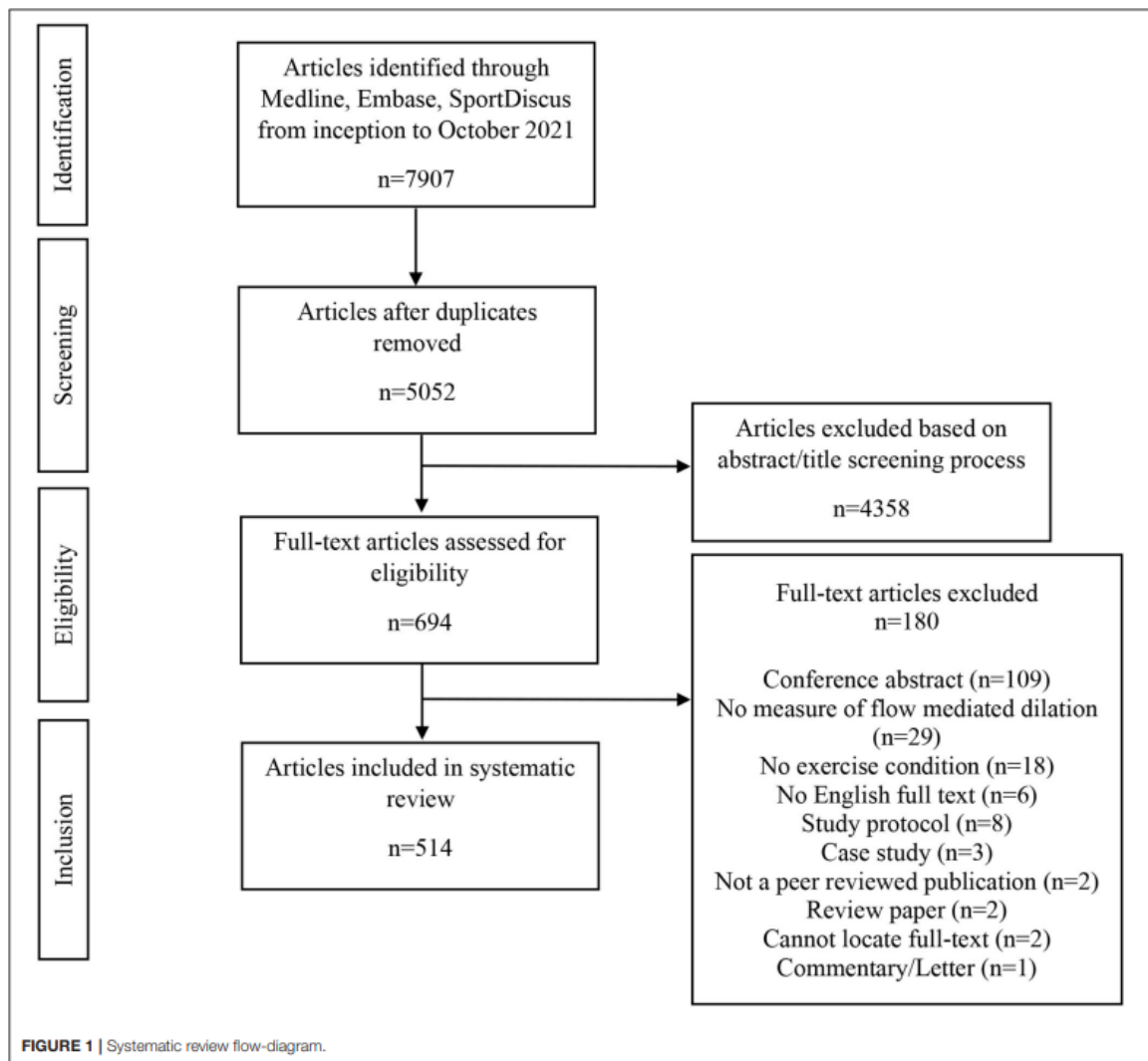
was true for reporting sex in the abstract [72 vs. 98%, χ^2 (1,222) = 17.71, $p < 0.001$; **Figure 3E**], and providing a justification for the exclusion on the basis of sex [15 vs. 55%, χ^2 (1,206) = 29.20, $p < 0.001$]. However, there was no difference in the proportion of studies which identified sex-exclusion as a limitation in their ability to generalize from the study population [30% male-only vs. 39% female-only, χ^2 (1,193) = 1.12, $p = 0.291$].

Further, when comparing mixed-sex and single-sex (i.e., male-only or female-only) studies, mixed-sex studies were more likely to be conducted in clinical populations (65% of mixed-sex studies versus 33% of single-sex studies), while single-sex studies were more likely to be conducted in healthy populations [35% of mixed-sex studies vs. 67% of single-sex studies; χ^2 (1,514) = 50.90, $p < 0.001$; **Figure 3F**]. Examining whether

male-only or female-only studies were driving this difference, it was determined that male-only studies were more likely to be conducted in healthy populations (72% of male-only studies vs. 50% of female-only studies), while female-only studies were more likely to be conducted in clinical populations [28% of male-only studies vs. 50% of female-only studies; χ^2 (1,227) = 9.99, $p = 0.002$].

Finally, when examining the two most common exercise interventions in studies (i.e., resistance vs. aerobic), it was found that male-only studies were more likely to include resistance exercise interventions (26% of male-only studies vs. 15% of mixed-sex/female-only studies), while mixed-sex/female-only studies were more likely to involve aerobic exercise interventions [74% of male-only studies vs. 85% of mixed-sex/female-only

FIGURE 2 | Study characteristics of studies included in review. **(A)** Type of studies; **(B)** Types of interventions; **(C)** Types of exercise; **(D)** Age of participants; **(E)** Menopausal status of female participants; **(F)** Hormonal cycling control in female participants.
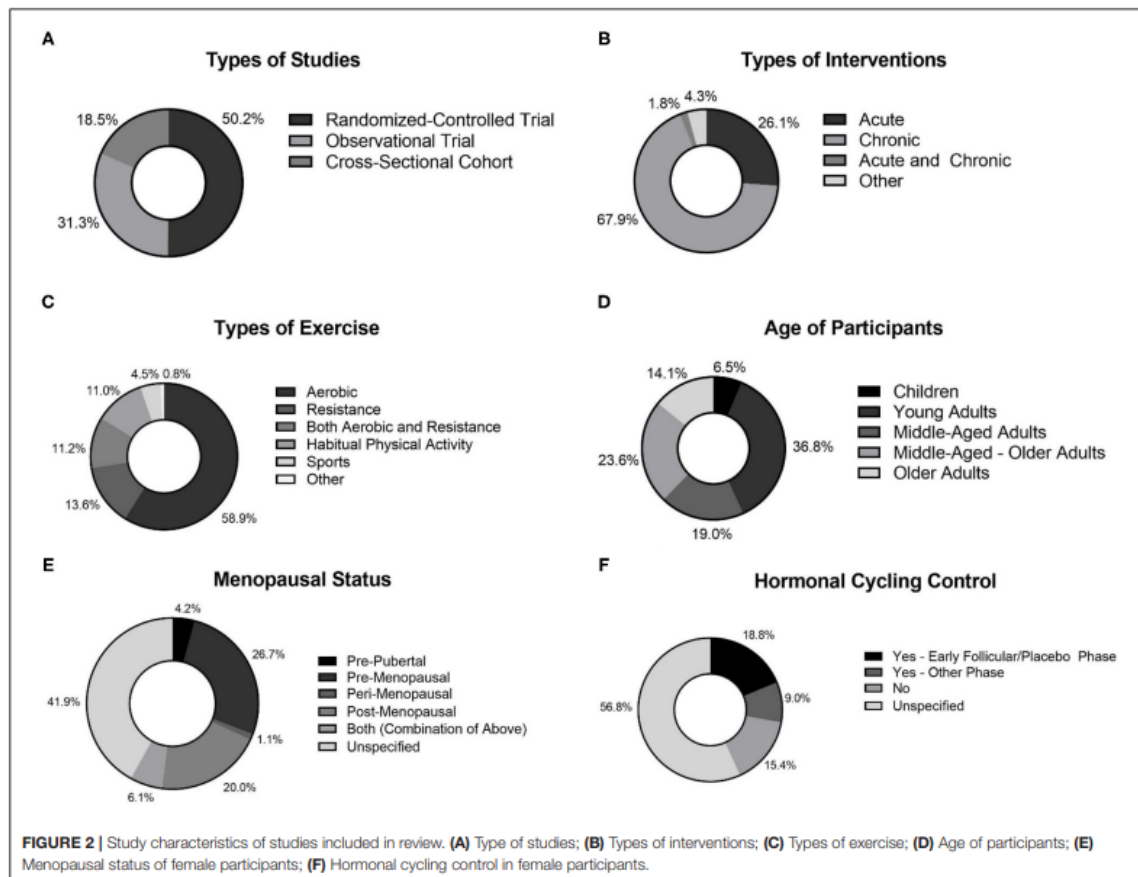
studies; χ^2 (1,373) = 7.50, p = 0.006]. In addition, male-only studies were more likely to include acute exercise interventions (44% of male-only studies vs. 20% of mixed-sex/female-only studies), while mixed-sex or female-only studies were more likely to be chronic exercise training studies [56% of male-ony studies vs. 80% of mixed-sex/female-only studies, χ^2 (1,483) = 29.62, p < 0.001].

Qualitative Analysis of Study Reporting

Examining the justifications provided for the exclusion or inclusion of certain sexes, there were four main themes: need to study a specific sex for a sex-specific rationale or a gap in the literature, the need to exclude females on the basis of the hormonal cycle, maintaining the male norm, and challenges with recruitment, retention and resources. One of the first justifications identified was the need to study a specific sex given the sex-specific nature of a condition or a clear gap in the literature. For example, studies highlighted common sex-specific conditions in females, such as menopause or the influence of hormone therapy, polycystic ovarian syndrome, pregnancy and

amenorrhea, or in males, such as prostate cancer and testosterone therapy. Similarly, the decision to only examine one sex was reported in some studies to be based on a paucity of literature in that sex.

Another common theme in the studies was exclusion of female participants on the basis of the hormonal cycle and/or attempting to remove the influence of sex that may confound the study findings. Alongside this theme was the notion of pursuing research that aligns with past populations as a "proof of concept," aligning with past identification of a male norm. An additional recurring theme in the studies was the notion that researchers may face recruitment, retention and resource barriers when attempting to recruit both sexes. For example, financial barriers were identified, stating that it is more costly to examine sex equally. Several other studies identified challenges with recruitment and retention, citing low numbers of females as part of exercise programs or where clinical conditions are more common in males compared to females. Examples of these themes are illustrated in quotes in **Figure 4** (Casey et al., 2007; Currie et al., 2012; Atkinson et al., 2015; Restaino et al.,

54

FIGURE 3 | Sex-specific inclusion. **(A)** Average percentage of female and male participants from studies in cohorts of 5 years with the dotted line as the average over all studies; **(B)** Number of male-only, female-only and mixed-sex studies in cohorts of 5 years; **(C)** Number of mixed-sex studies with equal male-female participants, unequal participants favoring females and unequal participants favoring males, over cohorts of 5 years; **(D)** Number of male-only and female-only studies including sex in the article title; **(E)** Number of male-only and female-only studies including sex in the article abstract; **(F)** Number of studies in mixed-sex and sex-specific groups for healthy and clinical populations.

2015; Paditsaeree and Mitranun, 2018; Santos-Parker et al., 2018; Claes et al., 2020; Papadakis et al., 2020; Boidin et al., 2021; Waclawovsky et al., 2021). In examining the identification of where generalizability is limited, studies primarily highlighted that because of the sex-specific nature of the study or the lack of equal participants across sex, study findings could not be generalized to other populations.

Sex-Differences

Within studies examining mixed-sex populations, only ∼10% of studies intended to test for sex-differences in their methods with an *a priori* design, with approximately one-third of these studies disaggregating based on sex to perform the analysis, and two-thirds of these studies incorporating sex-based comparison into

their statistical analysis. Similarly, when considering all mixed-sex studies, only 17% reported analyzing data based on sex and reporting differences or lack thereof. Of studies reporting on sex-differences, 83% reported no sex-differences, while 17% reported that there were sex-differences in response to an exercise-based intervention. Finally, examining all studies in the review, only 20% included a discussion on how sex/gender may or may not influence the study's results; in mixed-sex studies, only 22% included a discussion on sex/gender.

Examining Gender

Examining the inclusion of gender in the studies, approximately 40% of studies conflated or confused the terms sex and gender in reporting on participants (e.g., using terms "male" and "female"

55

FIGURE 4 | Summary of qualitative themes for sex-inclusion/exclusion and representative quotes.
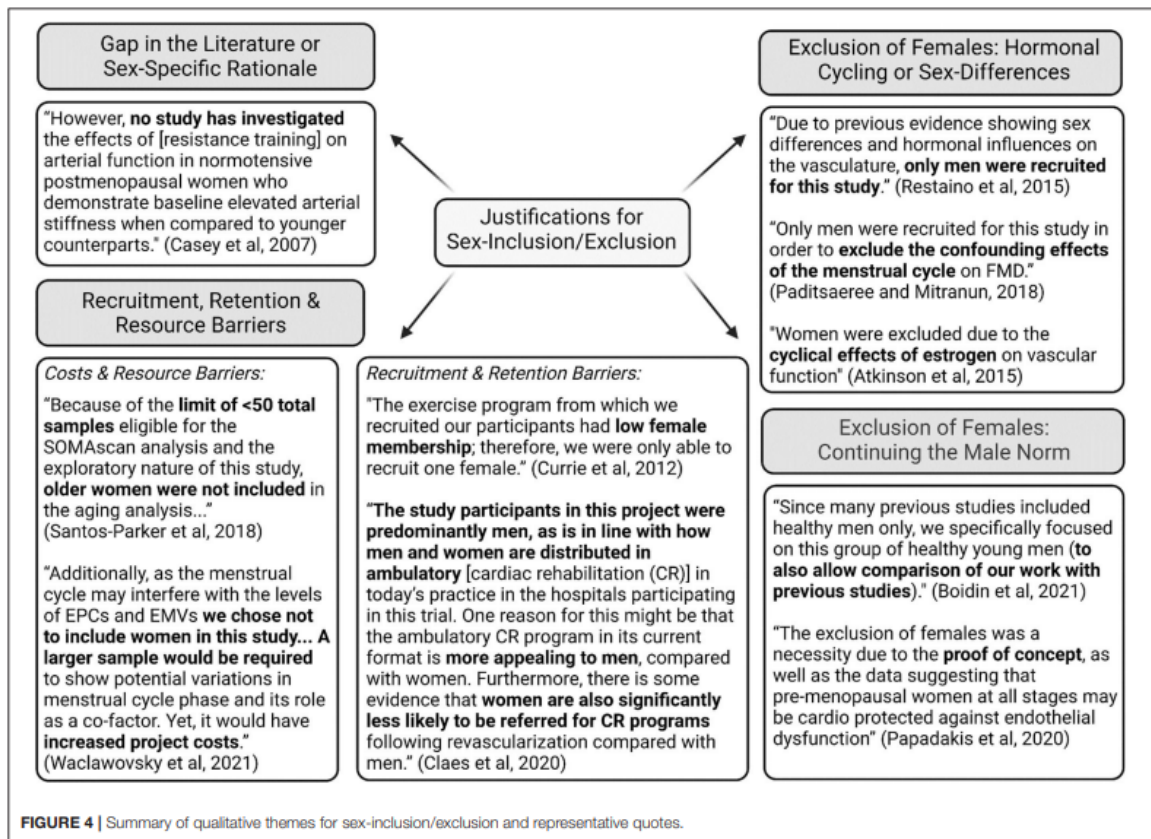
as gender instead of sex or using terms "woman" and "man" as sex instead of gender, or using both the terms "sex" and "gender" interchangeably throughout the paper). In addition, the vast majority of studies did not explicitly examine gender (e.g., identity, roles, behaviors) and its impact on the outcome (i.e., FMD) (99%).

DISCUSSION

Summary of Study Findings

The purpose of this study was to quantify and characterize sex-specific prevalence of human participants in research examining the impact of exercise on vascular endothelial function and to identify the rationales justifying sex-specific inclusion/exclusion of participants. Overall, it is evident that females remain underrepresented in vascular exercise physiology studies, as indicated by a lower total prevalence of both female participants and female-only studies. Summarizing over 500 studies with ~25,000 participants, this study found evidence of a male-bias, with male participants included more than female participants (64 vs. 36%), and 32% of studies conducted with male-only populations (compared to 12% in female-only populations).

In mixed-sex studies, favoring of female participants was less common (20%) than favoring of male participants (43%) or equal male and female representation (37%). Furthermore, underrepresentation of female participants was largely unaltered across time despite the advances in policy and recommendations related to sex and gender considerations. In addition, this study found that male-only studies were less likely to report sex in the title and abstract, and justify exclusion on the basis of sex, compared to female-only studies. Further, our analysis found that male-only studies tended to be conducted in healthy populations and involve acute interventions and resistance exercise interventions. Qualitative analysis found common rationales regarding unequal sex inclusion to be based on sex-specific conditions or paucity of research in a given sex, female exclusion on the basis of the hormonal cycle or sex-differences, perpetuation of the male norm, and concerns regarding the recruitment, retention and resources needed to pursue sex-parity. Finally, only 17% of mixed-sex studies performed sex-based analysis, demonstrating the paucity of sex difference research in the field of vascular exercise physiology, even in those studies that included mixed-sex in their participant pools.

Sex-Specific Inclusion

Approximately one-third (36%) of participants included in all studies were female, highlighting the imbalance between male and female participant inclusion in vascular endothelial exercise physiology research. Although this present study was narrow in scope including only studies assessing FMD in response to an exercise intervention, the results align with similar research of broader scope (Costello et al., 2014; Wilson et al., 2020; Cowley et al., 2021). A case study of research by five cardiovascular physiology investigators in Ontario reported a slightly lower average female enrollment of 24% (Wilson et al., 2020). Moreover, two studies investigating the sex of participants in original articles of three (Costello et al., 2014) and six (Cowley et al., 2021) high-impact sport and exercise medicine journals similarly found that females participants encompassed 39 and 34% of all included participants, respectively. The proportion of single-sex studies further exemplifies the existence of a sex bias skewed toward male inclusion with 32% of the studies being male-only, and 12% female-only. This higher prevalence of male-only studies was also noted previously (Costello et al., 2014; Wilson et al., 2020; Woitowich et al., 2020; Cowley et al., 2021). For example, previous work in exercise physiology by Costello et al. identified 4–13% female-only proportion, Cowley et al. identified 6% female-only proportion, and work by Wilson et al. identified 5% proportion of female-only studies in vascular research (Costello et al., 2014; Wilson et al., 2020; Cowley et al., 2021). The lack of female-inclusion in research studies directly contributes to expanding gaps in basic biomedical and clinical understanding of how exercise influences vascular function in female cardiovascular systems. For example, with evidence of known sex-differences in vascular endothelial responses to exercise training (Seals et al., 2019), establishing sex-specific exercise training interventions is integral to improving cardiovascular health of both males and females and understanding underlying mechanisms responsible for sex-specific responses.

Interestingly, male-only studies are more likely to be conducted in healthy populations and utilize acute exercise interventions in comparison to female-only/mixed-sex studies. Alternatively, mixed-sex studies were more likely to be conducted in clinical populations compared to sex-specific studies. Discrepancies in prevalence of mixed-sex studies in clinical vs. healthy populations may stem from the specific policies and protocols mandated in clinical trials. For example, the National Institutes of Health (NIH) established the policy in 1994 for Inclusion of Women and Minorities to improve sex-based equality of participants in NIH-funded clinical research (NIH: Grants Funding, 2017). According to this policy, in addition to research proposals outlining female inclusion *a priori* and plans for appropriate outreach programs and activities to increase recruitment/retention of this population, investigators must also provide annual progress reports detailing sex/gender of participants. These mandatory checkpoints included in the rigorous clinical trial protocols increase the accountability of researchers to conduct mixed-sex studies. Whereas, equal inclusion of sex in participants is often recommended rather than mandated in research predominantly conducted in healthy populations and is not guided throughout the research process apart from investigator-driven design and funding body decisions. In addition, female-only inclusion may have been more prevalent in clinical studies as females are often studied for the complexity of sex-specific conditions and hormonal experiences (e.g., pregnancy, hormone use, menopause, amenorrhea). Further, based on the observations in the qualitative findings of this review further detailed below in the *Reported Rationales for Exclusion* section, it may be speculated that females were disproportionately excluded from studies involving healthy populations and acute-based interventions, due to the perceived influence of hormonal cycle/sex hormones on basic mechanistic research outcomes.

Reporting Sex in Studies

The SAGER guidelines were created in 2021 to promote a systematic reporting of sex and gender in research and provide greater transparency of scientific data (Heidari et al., 2016). Key components of these guidelines include reporting sex when detailing participant characteristics, as well as reporting the sex of participants in the title and abstract if only one sex is included. This present study provides evidence that many single-sex vascular exercise physiology studies fail to adhere to these guidelines as 3% did not detail sex, 60% did not report sex in the title, and 20% did not report sex in the abstract. Additionally, the SAGER guidelines emphasize the importance of reporting results disaggregated by sex and performing sex-based analysis when possible (Heidari et al., 2016). In this review, sex-based analysis was very limited, with only 10% of mixed-sex studies indicating an *a priori* decision to conduct sex-based analysis and 17% conducting a sex-based analysis. Alongside the apparent need for more sex-difference research, there have been calls in the literature emphasizing the need for appropriately powered sex-based analysis (Aulakh and Anand, 2007).

Females undergo acute and chronic variations in sex hormones throughout their lives, including but not limited to cyclic fluctuations in endogenous sex hormones across the menstrual cycle or synthetic hormones across a contraceptive cycle, substantial reductions in sex hormones during the menopause transition, or temporary increases in synthetic hormones with the use of hormone therapy. As some evidence has suggested an impact of sex hormones on endothelial function (Hashimoto et al., 1995; Moreau et al., 2012) or modulating the endothelial response to exercise (Moreau et al., 2013), it remains imperative to report participant hormonal status. Specifically, identifying whether female participants are pre-menopausal, peri-menopausal, or post-menopausal, alongside details regarding hormonal cycle phase and contraceptive/hormone therapy use, where applicable, provides additional context for researchers to understand and interpret research findings.

Interestingly, reporting of hormonal status of female participants was limited in this review, with 42% of studies including females failing to specify hormonal status of participants. Further, there is little control for menstrual phase in studies including premenopausal women demonstrated

by approximately three quarters of studies that did not control for menstrual phase or did not report phase. While controlling for hormonal cycle phase is debated (Stanhewicz and Wong, 2020), recent FMD guidelines suggest collecting data on premenopausal women in a standard phase of the menstrual cycle, alongside other standardized controls such as diet, exercise, alcohol/caffeine consumption (Thijssen et al., 2019). Considerations of the hormonal cycle should be evaluated similar to other necessary study design controls, considering the research question and study design, and establishing controls wherever possible. While there remains a need to *consider* the hormonal cycle in study design and reporting, female participants should not be excluded on the basis of hormonal variation, as female-inclusive research provides meaningful contributions to vascular exercise physiology. Altogether, these results suggest a need for not only improved quantity, but also quality, of reporting in vascular exercise physiology research conducted in females.

Reported Rationales for Exclusion

In contrast to previous reviews (Costello et al., 2014; Cowley et al., 2021), this was the first study to examine justifications for exclusion on the basis of sex, and recognition of limits to generalizability with exclusion. This study found that male-only studies were less likely to provide justification for exclusion on the basis of sex, compared to female-only studies (15 vs. 55%), while both male-only and female-only studies equally recognized the limitations in generalizability of the study findings to broader population groups (30 vs. 39%). In examining the qualitative rationales for justification, four central themes emerged. First, sex-specific nature or a clear gap in the literature was a justified rationale for sex-exclusion, such as researchers exploring the influence of hormonal therapies like hormone replacement therapy or testosterone therapy, or sex-specific conditions like pregnancy and prostate cancer. The SAGER guidelines detail that sex-exclusion on the basis of a sex-specific research question is justified (Heidari et al., 2016). Similarly, some studies identified clear literature gaps in the introduction of the study, such as the recognition of the paucity of research in one sex for a given exercise intervention.

Another theme emerging from the qualitative analysis was the exclusion of females based on the more variable hormonal cycle influence, and the perceived need to perpetuate a "male norm" in aligning with prior research to ensure validity and comparison of study findings. This observation has been a consistent theme in the exclusion or underrepresentation of female participants for maintaining a status quo of studying males (Beery and Zucker, 2011; Yoon et al., 2014; Woitowich et al., 2020). This male bias has been identified in many fields, including both basic cell and animal research (Yoon et al., 2014) and human research, including physiology (Beery and Zucker, 2011; Will et al., 2017) and more recently in exercise physiology (Cowley et al., 2021). Further, qualitative examination of interviews by Wilson et al. identified that primary investigators believe this may be due, in part, to females being perceived as more complex, with considerations to the hormonal cycle (Wilson et al., 2020). Early work examining the influence of the menstrual cycle on vascular

endothelial function established large fluctuations in FMD, and was used as a justification for excluding females on the basis of this complexity (Hashimoto et al., 1995). However, a recent meta-analysis has identified that the menstrual cycle may have only a small effect on vascular endothelial function, and variability may instead be explained by other methodological factors (Williams et al., 2020). Nonetheless, controlling for hormonal phase is still recognized as best practice, but should no longer be a justification for exclusion as several recent articles offer excellent guidance on how to account for hormonal status while investigating females (Sims and Heather, 2018; Wenner and Stachenfeld, 2020b; Elliott-Sale et al., 2021).

Finally, another theme that emerged from the qualitative analysis was various recruitment, retention and resource barriers that exist when attempting to recruit both sexes. For example, specifically in clinical studies, exclusion or underrepresentation of female participants was noted due to poor recruitment or higher prevalence of drop-outs. This is in line with earlier findings by Wilson et al., noting relative disease prevalence may limit recruitment efforts (Wilson et al., 2020). However, according to the SAGER guidelines, an effort to recruit equally across sex is necessary (Heidari et al., 2016), and concerted effort is required to target recruitment to participants who may be underrepresented due to inherent barriers in research participation. For example, women have been historically underrepresented in cardiac rehabilitation programs due to in part to systematic under referrals, and poorer retention due in part to other gender-based commitments such as family care and a lack of social support (Jackson et al., 2005; Supervía et al., 2017; Colbert et al., 2020). Finally, researchers noted as part of justification the concern that integrating male and female participants would result in increased costs for an experiment with the need to double sample size. While the increased costs associated with sample size and potential hormonal testing cannot be overlooked, it has been argued that some trials may not require an increased sample size when integrating both sexes (Beery, 2018), examining sex-specific responses in pre-clinical research may provide long-term savings at the stage of clinical trials, and further examining sex-differences may lead to future untapped areas of research development (Klein et al., 2015).

Examining Gender

When reviewing study treatment of gender, approximately 40% of studies conflated or confused the terms sex and gender, often interchanging terminology throughout the article (e.g., using the term gender and then referring to "male" or "female" participants). In addition, almost no studies explicitly examined gender, specifically the socially constructed identity, behaviors, roles, and institutional interactions that humans experience, and that have been known to influence health (Tannenbaum et al., 2016). Within vascular exercise physiology research, exploring gender offers an untapped area of future research to offer a more nuanced approach to examining apparent sex-differences that cannot only be explained by biological sex variables, like chromosomes, anatomy, and hormones.

Limitations

The scope of the project was narrowed to include only exercise physiology studies that utilized FMD to assess conduit artery endothelial function. Therefore, these results cannot be extrapolated further, although the findings of this project appear to be in line with studies of broader scope and in different disciplines (Costello et al., 2014; Wilson et al., 2020; Woitowich et al., 2020; Cowley et al., 2021). More research is needed to extend these findings to other assessments of cardiovascular function (e.g., arterial stiffness, carotid artery compliance, microvascular function) and other physiological interventions. Further, only English, peer-reviewed studies were included in this review. Thus, these findings do not encompass all vascular exercise physiology research due in part to the potential publishing bias against null or statistically insignificant results (Hopewell et al., 2009). Additionally, studies included in this review were from various geographic locations, however data was not extracted regarding the country in which the research originated. As such, differing local policies surrounding sex/gender inclusion in research and reporting, which may influence the integration of sex/gender (Merriman et al., 2021), could not be accounted for in the temporal analysis of sex-specific inclusion/exclusion. This remains an important future direction to understand the effectiveness of sex/gender guidelines and mandates from governing bodies. Similarly, journal-specific requirements for sex/gender reporting were not accounted for in the analysis, and this is therefore a potential future direction for research. Lastly, despite an attempt to mitigate investigator bias by creating a structured extraction template prior to analysis, the team of investigators are all female-identifying and share a common interest in integrating females into exercise physiology research which may have influenced the analysis and results.

Guidance for Future Researchers

Based on the findings from this review, the first guidance for researchers is to consider improved data collection and reporting practices when considering sex/gender in future research studies. For example, sex identification in the title and abstract where studies are sex-specific. This recommendation can be reinforced by journals including reporting requirements around sex/gender during the manuscript submission and peer review process. For example, research by the *American Journal of Physiology – Heart and Circulatory Physiology* has found an increase in mixed-sex studies and reporting on sex/gender in articles since integrating strategies in manuscript submission and peer review (Lindsey et al., 2021). Similarly, work by Clayton and Tannenbaum have identified a simple structure of reporting of sex/gender in clinical research, noting that the *Journal of the American Medical Association* has integrated requirements for sex-specific reporting and justification for exclusion on the basis of sex (Clayton and Tannenbaum, 2016). However, journal instructions may not always result in improved sex/gender inclusion, and researchers should independently consider these factors in study design (Merriman et al., 2021). Further, reporting hormonal status of participants (e.g., menopausal status, hormonal cycling controls) is necessary to provide additional context to research findings. As detailed previously, researchers should consider how hormonal status, including hormonal cycling and hormonal therapy (including contraceptive use), may influence research outcomes and incorporate appropriate controls into research study design where appropriate; however, this should not come at the cost of arbitrary exclusion of female participants. Hormonal considerations should be balanced alongside other study design controls, with the central principle of inclusion of both males and females.

While it is recommended to aim for mixed-sex studies with equal male/female participation (Heidari et al., 2016), there are rationales for sex-specific research. For example, some thoughtfully designed studies included in this review identified the notion of "intentional design" for sex-specific research, where researchers noted a paucity of research or a direct rationale for sex-specificity (e.g., disease more prevalent in one sex, sex-specific condition), detailed in the introduction of a study. In contrast to the omission of rationale for sex-exclusion, or justification after design (e.g., in the discussion), it is recommended for researchers to consider sex-inclusion in the design of studies. However, as detailed in the review, some researchers have noted the limitations of sex-inclusion, specifically in clinical populations. For example, in some clinical populations recruitment and retention may be limited; however, researchers are still urged to work toward parity in study design and mitigate barriers for recruitment and retention on the basis of sex. Similarly, few studies examined sex-based analysis in study design; where appropriate, *a priori* analysis is encouraged to examine sex-differences in response to exercise interventions. Finally, some studies conflated sex/gender and nearly no studies examined more complex constructs within gender. Researchers are encouraged to consider including structured questions around sex/gender, such as the two-step sex and gender question (Bauer et al., 2017), or more in-depth gender questionnaires (Schmitt and Millard, 1988; Pelletier et al., 2015), alongside other sex/gender tools summarized in a recent review (McGregor et al., 2016).

CONCLUSION AND FUTURE DIRECTIONS

This is the first study to quantitatively assess sex-inclusion in vascular exercise physiology research studies over 25 years, building on prior research to qualitatively identify rationales for inclusion/exclusion on the basis of sex. There was clear evidence of the underrepresentation of female participants in vascular exercise physiology research, and this trend appears to be unaltered over time despite recent attention to this topic. In particular, healthy populations involving acute interventions appear to be an area for attention, recognizing increased rates of female exclusion in these studies. Researchers are urged to consider sex/gender inclusion in research study design and reporting, with the aim for improved female inclusion. With recent attention to the considerations of sex/gender in research study design, it is anticipated that future analyses in vascular exercise physiology research will identify improved sex-specific inclusion in the next 25 years.

PhD Thesis – J. Williams; McMaster University - Kinesiology

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

JW and LL conceived and designed the study, with support from KP and MM. JW and LL performed searches, title and abstract screening, full-text screening, wrote the manuscript, and prepared figures. JW, LL, JS, and AA performed data extraction. All authors edited and revised the manuscript prior to final submission. All authors contributed to the article and approved the submitted version.

FUNDING

ACKNOWLEDGMENTS

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fspor.2022.860356/full#supplementary-material

REFERENCES

Atkinson, C., Carter, H., Dawson, E., Naylor, L., Thijssen, D., and Green, D. (2015). Impact of handgrip exercise intensity on brachial artery flow-mediated dilation. *Eur. J. Appl. Physiol.* 115, 1705–1713. doi: 10.1007/s00421-015-3157-1

Aulakh, A. K., and Anand, S. S. (2007). Sex and gender subgroup analyses of randomized trials. *Women's Health Issues* 17, 342–350. doi: 10.1016/j.whi.2007.04.002

Bauer, G. R., Braimoh, J., Scheim, A. I., and Dharma, C. (2017). Transgender-inclusive measures of sex/gender for population surveys: Mixed-methods evaluation and recommendations. *PLoS ONE* 12, e0178043. doi: 10.1371/journal.pone.0178043

Beery, A. K. (2018). Inclusion of females does not increase variability in rodent research studies. *Curr. Opin. Behav. Sci.* 23, 143–149. doi: 10.1016/j.cobeha.2018.06.016

Beery, A. K., and Zucker, I. (2011). Sex bias in neuroscience and biomedical research. *Neurosci. Biobehav. Rev.* 35, 565–572. doi: 10.1016/j.neubiorev.2010.07.002

Bhargava, A., Arnold, A. P., Bangasser, D. A., Denton, K. M., Gupta, A., Hilliard Krause, L. M., et al. (2021). Considering sex as a biological variable in basic and clinical studies: an endocrine society scientific statement. *Endocr. Rev.* 42, 219–258. doi: 10.1210/endrev/bnaa034

Boidin, M., Erskine, R. M., Thijssen, D. H. J., and Dawson, E. A. (2021). Exercise modality, but not exercise training, alters the acute effect of exercise on endothelial function in healthy men. *J. Appl. Physiol.* 130, 1716–1723. doi: 10.1152/japplphysiol.00004.2021

Braun, V., and Clarke, V. (2021). Can I use TA? Should I use TA? Should I not use TA? Comparing reflexive thematic analysis and other pattern-based qualitative analytic approaches. *Counsel. Psychother. Res.* 21, 37–47. doi: 10.1002/capr.12360

Casey, D. P., Pierce, G. L., Howe, K. S., Mering, M. C., and Braith, R.W. (2007). Effect of resistance training on arterial wave reflection and brachial artery reactivity in normotensive postmenopausal women. *Eur. J. Appl. Physiol.* 100, 403–408. doi: 10.1007/s00421-007-0447-2

Claes, J., Gillain, A., Cornelissen, V., Pattyn, N., Cornelis, N., Buys, R., et al. (2020). Feasibility, acceptability, and clinical effectiveness of a technology-enabled cardiac rehabilitation platform (physical activity toward health-I): Randomized controlled trial. *J. Med. Internet Res.* 22, e14221. doi: 10.2196/14221

Clayton, J. A., and Tannenbaum, C. (2016). Reporting sex, gender, or both in clinical research? *JAMA* 316, 1863–1864. doi: 10.1001/jama.2016.16405

Coiro, P., and Pollak, D. D. (2019). Sex and gender bias in the experimental neurosciences: the case of the maternal immune activation model. *Transl. Psychiatry.* 9, 90. doi: 10.1038/s41398-019-0423-8

Colbert, J. D., Martin, B.-J., Haykowsky, M. J., Hauer, T. L., Austford, L. D., Arena, R. A., et al. (2020). Cardiac rehabilitation referral, attendance and mortality in women. *Eur. J. Prev. Cardiol.* 22, 979–986. doi: 10.1177/2047487314545279

Costello, J. T., Bieuzen, F., and Bleakley, C. M. (2014). Where are all the female participants in Sports and Exercise Medicine research? *Eur. J. Sport Sci.* 14, 847–851. doi: 10.1080/17461391.2014.911354

Cowley, E. S., Olenick, A. A., McNulty, K. L., and Ross, E. Z. (2021). "Invisible Sportswomen": The sex data gap in sport and exercise science research. *Women Sport Phys. Activ. J.* 29, 146–151. doi: 10.1123/wspaj.2021-0028

Currie, K. D., McKelvie, R. S., and MacDonald, M. J. (2012). Flow-mediated dilation is acutely improved after high-intensity interval exercise. *Med. Sci. Sports Exerc.* 44, 2057–2064. doi: 10.1249/MSS.0b013e318260ff92

D'Urzo, K. A., King, T. J., Williams, J. S., Silvester, M. D., and Pyke, K. E. (2018). The impact of menstrual phase on brachial artery flow-mediated dilatation during handgrip exercise in healthy premenopausal women. *Exp. Physiol.* 103, 291–302. doi: 10.1113/EP086311

Elliott-Sale, K. J., Minahan, C. L., de Jonge, X., Ackerman, K. E., Sipilä, S., Constantini, N. W., et al. (2021). Methodological considerations for studies in sport and exercise science with women as participants: a working guide for standards of practice for research on women. *Sports Med.* 51, 843–861. doi: 10.1007/s40279-021-01435-8

Fausto-Sterling, A. (2012). *Chapter 2 - Of Spirals and Layers in Sex/Gender: Biology in a Social World.* New York, NY: Routledge. doi: 10.4324/9780203127971

Feldman, S., Ammar, W., Lo, K., Trepman, E., van Zuylen, M., and Etzioni, O. (2019). Quantifying sex bias in clinical studies at scale with automated data extraction. *JAMA Netw. Open.* 2, e196700–e. doi: 10.1001/jamanetworkopen.2019.6700

Hashimoto, M., Akishita, M., Eto, M., Ishikawa, M., Kozaki, K., Toba, K., et al. (1995). Modulation of endothelium-dependent flow-mediated dilatation of the brachial artery by sex and menstrual cycle. *Circulation* 92, 3431–3435. doi: 10.1161/01.CIR.92.12.3431

Haverfield, J., and Tannenbaum, C. (2021). A 10-year longitudinal evaluation of science policy interventions to promote sex and gender in health research. *Health Res. Policy Syst.* 19, 94. doi: 10.1186/s12961-021-00741-x

Heart and Stroke, F. (2018). *Understood: Women's Hearts Are Victims of a System That Is Ill-Equipped to Diagnose, Treat, and Support Them.* Heart and Stroke Foundation: Annual Heart Report.

60

PhD Thesis – J. Williams; McMaster University - Kinesiology

Heidari, S., Babor, T. F., De Castro, P., Tort, S., and Curno, M. (2016). Sex and Gender Equity in Research: rationale for the SAGER guidelines and recommended use. *Res. Integrity Peer Rev.* 1, 2. doi: 10.1186/s41073-016-0007-6

Hopewell, S., Loudon, K., Clarke, M. J., Oxman, A. D., and Dickersin, K. (2009). Publication bias in clinical trials due to statistical significance or direction of trial results. *Cochrane Database Syst. Rev.* 2009, Mr000006. doi: 10.1002/14651858.MR000006.pub3

Jackson, L., Leclerc, J., Erskine, Y., and Linden, W. (2005). Getting the most out of cardiac rehabilitation: a review of referral and adherence predictors. *Heart* 91, 10–14. doi: 10.1136/hrt.2004.045559

Kim, J. Y., Min, K., Paik, H. Y., and Lee, S. K. (2021). Sex omission and male bias are still widespread in cell experiments. *Am. J. Physiol. Cell Physiol.* 320, C742–C749. doi: 10.1152/ajpcell.00358.2020

Klein, S. L., Schiebinger, L., Stefanick, M. L., Cahill, L., Danska, J., de Vries, G. J., et al. (2015). Opinion: Sex inclusion in basic research drives discovery. *Proc. Nat. Acad. Sci. U.S.A.* 112, 5257–5258. doi: 10.1073/pnas.1502843112

Lindsey, M. L., LeBlanc, A. J., Ripplinger, C. M., Carter, J. R., Kirk, J. A., Hansell Keehan, K., et al. (2021). Reinforcing rigor and reproducibility expectations for use of sex and gender in cardiovascular research. *Am. J. Physiol. Heart Circul. Physiol.* 321, H819–H24. doi: 10.1152/ajpheart.00418.2021

Liu, K. R., Lew, L. A., McGarity-Shipley, E. C., Byrne, A. C., Islam, H., Fenuta, A. M., et al. (2021). Individual variation of follicular phase changes in endothelial function across two menstrual cycles. *Exp. Physiol.* 106, 1389–1400. doi: 10.1113/EP089482

McGregor, A. J., Hasnain, M., Sandberg, K., Morrison, M. F., Berlin, M., and Trott, J. (2016). How to study the impact of sex and gender in medical research: a review of resources. *Biol. Sex Differ.* 7, 46. doi: 10.1186/s13293-016-0099-1

Merriman, R., Galizia, I., Tanaka, S., Sheffel, A., Buse, K., and Hawkes, S. (2021). The gender and geography of publishing: a review of sex/gender reporting and author representation in leading general medical and global health journals. *BMJ Global Health.* 6, e005672. doi: 10.1136/bmjgh-2021-005672

Moreau, K. L., Hildreth, K. L., Meditz, A. L., Deane, K. D., and Kohrt, W. M. (2012). Endothelial function is impaired across the stages of the menopause transition in healthy women. *J. Clin. Endocrinol. Metab.* 97, 4692–4700. doi: 10.1210/jc.2012-2244

Moreau, K. L., Stauffer, B. L., Kohrt, W. M., and Seals, D. R. (2013). Essential role of estrogen for improvements in vascular endothelial function with endurance exercise in postmenopausal women. *J. Clin. Endocrinol. Metab.* 98, 4507–4515. doi: 10.1210/jc.2013-2183

National Institute for Health Research, (2020). *Improving Inclusion of Under-Served Groups in Clinical Research.* Guidance from INCLUDE project. National Institute for Health Research.

National Institutes of Health, (2017). *Inclusion of Women and Minorities as Participants in Research Involving Human Subjects.* National Institutes of Health: US Department of Health and Human Services.

Naylor, L. H., Green, D. J., Spence, A. L., Donker, S. C. M., and Thijssen, D. H. J. (2021). Is there an athlete's artery? A comparison of brachial and femoral artery structure and function in male strength, power and endurance athletes. *J. Sci. Med. Sport.* 24, 635–640. doi: 10.1016/j.jsams.2021.02.010

NIH: Grants and Funding (2017). *Inclusion of Women and Minorities as Participants in Research Involving Human Subjects.* NIH: Grants and Funding.

Paditsaeree, K., and Mitranun, W. (2018). Does combining elastic and weight resistance acutely protect against the impairment of flow-mediated dilatation in untrained men? *Artery Res.* 23, 1–8. doi: 10.1016/j.artres.2018.05.003

Papadakis, Z., Forsse, J. S., and Peterson, M. N. (2020). Acute partial sleep deprivation and high-intensity interval exercise effects on postprandial endothelial function. *Eur. J. Appl. Physiol.* 120, 2431–2444. doi: 10.1007/s00421-020-04468-5

Pelletier, R., Ditto, B., and Pilote, L. (2015). A composite measure of gender and its association with risk factors in patients with premature acute coronary syndrome. *Psychosom. Med.* 77, 517–526. doi: 10.1097/PSY.0000000000000186

Raitakari, O. T., and Celermajer, D. S. (2000). Flow-mediated dilatation. *Br. J. Clin. Pharmacol.* 50, 397–404. doi: 10.1046/j.1365-2125.2000.00277.x

Restaino, R. M., Holwerda, S. W., Credeur, D. P., Fadel, P. J., and Padilla, J. (2015). Impact of prolonged sitting on lower and upper limb micro- and macrovascular dilator function. *Exp. Physiol.* 100, 829–838. doi: 10.1113/EP085238

Santos-Parker, J. R., McQueen, M. B., Martens, C. R., Seals, D. R., and Santos-Parker, K. S. (2018). Habitual aerobic exercise and circulating proteomic patterns in healthy adults: relation to indicators of healthspan. *J. Appl. Physiol.* 125, 1646–1659. doi: 10.1152/japplphysiol.00458.2018

Schmitt, B. H., and Millard, R. T. (1988). Construct validity of the bem sex role inventory (BSRI): Does the BSRI distinguish between gender-schematic and gender-aschematic individuals? *Sex Roles* 19, 581–588. doi: 10.1007/BF00289737

Seals, D. R., Nagy, E. E., and Moreau, K. L. (2019). Aerobic exercise training and vascular function with ageing in healthy men and women. *J Physiol.* 597, 4901–4914. doi: 10.1113/JP277764

Shenouda, N., Priest, S. E., Rizzuto, V. I., and MacDonald, M. J. (2018). Brachial artery endothelial function is stable across a menstrual and oral contraceptive pill cycle but lower in premenopausal women than in age-matched men. *Am. J. Physiol. Heart Circ. Physiol.* 315, H366–H74. doi: 10.1152/ajpheart.00102.2018

Sims, S. T., and Heather, A. K. (2018). Myths and Methodologies: Reducing scientific design ambiguity in studies comparing sexes and/or menstrual cycle phases. *Exp. Physiol.* 103, 1309–1317. doi: 10.1113/EP086797

Stanhewicz, A. E., and Wong, B. J. (2020). Counterpoint: Investigators should not control for menstrual cycle phase when performing studies of vascular control that include women. *J. Appl. Physiol.* 129, 1117–1119. doi: 10.1152/japplphysiol.00427.2020

Supervía, M., Medina-Inojosa, J. R., Yeung, C., Lopez-Jimenez, F., Squires, R. W., Pérez-Terzic, C. M., et al. (2017). Cardiac rehabilitation for women: a systematic review of barriers and solutions. *Mayo Clin. Proc.* 92, 565–577. doi: 10.1016/j.mayocp.2017.01.002

Tannenbaum, C., Greaves, L., and Graham, I. D. (2016). Why sex and gender matter in implementation research. *BMC Med. Res. Methodol.* 16:145. doi: 10.1186/s12874-016-0247-7

Thijssen, D. H., Bruno, R. M., van Mil, A. C. C. M., Holder, S. M., Faita, F., Greyling, A., et al. (2019). Expert consensus and evidence-based recommendations for the assessment of flow-mediated dilation in humans. *Eur. Heart J.* 40, 2534–2547. doi: 10.1093/eurheartj/ehz350

Tri-Council Policy Statement, (2018). *Ethical Conduct for Research Involving Humans (TCPS 2) - Chapter 4: Fairness and Equity in Research Participation.* Government of Canada: Panel on Research Ethics.

Waclawovsky, G., Boll, L. F. C., Eibel, B., Schaun, M. I., Lehnen, A. M., Alegretti, A. P., et al. (2021). Individuals with controlled hypertension show endothelial integrity following a bout of moderate-intensity exercise: randomized clinical trial. *Sci. Rep.* 11, 8528. doi: 10.1038/s41598-021-87990-6

Wenner, M. M., and Stachenfeld, N. S. (2020a). Point: Investigators should control for menstrual cycle phase when performing studies of vascular control that include women. *J. Appl. Physiol.* 129, 1114–1116. doi: 10.1152/japplphysiol.00443.2020

Wenner, M. M., and Stachenfeld, N. S. (2020b). Rebuttal from Wenner. *J. Appl. Physiol.* 129, 1121. doi: 10.1152/japplphysiol.00621.2020

Will, T. R., Proaño, S. B., Thomas, A. M., Kunz, L. M., Thompson, K. C., Ginnari, L. A., et al. (2017). Problems and progress regarding sex bias and omission in neuroscience research. *eNeuro* 4, ENEURO.0278-17.2017. doi: 10.1523/ENEURO.0278-17.2017

Williams, J. S., Dunford, E. C., and MacDonald, M. J. (2020). Impact of the menstrual cycle on peripheral vascular function in premenopausal women: systematic review & meta-analysis. *Am. J. Physiol.* 319, H1327–H1337. doi: 10.1152/ajpheart.00341.2020

Williams, J. S., Stimpson, T. V., Tremblay, J. C., Fenuta, A. M., and Pyke, K. E. (2019). The influence of acute hyperglycemia on brachial artery flow-mediated dilatation in the early and late follicular phases of the menstrual cycle. *Exp. Physiol.* 104, 957–966. doi: 10.1113/EP087536

Wilson, R., Adams, M. L., and Pyke, K. E. (2020). Inclusion of female participants in cardiovascular research: A case study of Ontario NSERC- funded programs. *Appl. Physiol. Nutr. Metab.* 45, 911–914. doi: 10.1139/apnm-2019-0693

Witham, M. D., Anderson, E., Carroll, C., Dark, P. M., Down, K., Hall, A. S., et al. (2020). Developing a roadmap to improve trial delivery for under-served groups: results from a UK multi-stakeholder process. *Trials.* 21, 694. doi: 10.1186/s13063-020-04613-7

Woitowich, N. C., Beery, A., and Woodruff, T. (2020). A 10-year follow-up study of sex inclusion in the biological sciences. *Elife* 9, e56344. doi: 10.7554/eLife.56344

Yeboah, J., Folsom, A. R., Burke, G. L., Johnson, C., Polak, J. F., Post, W., et al. (2009). Predictive value of brachial flow-mediated dilation for incident cardiovascular events in a population-based study: the multi-ethnic study of atherosclerosis. *Circulation* 120, 502–509. doi: 10.1161/CIRCULATIONAHA.109.864801

61

Yoon, D. Y., Mansukhani, N. A., Stubbs, V. C., Helenowski, I. B., Woodruff, T. K., and Kibbe, M. R. (2014). Sex bias exists in basic science and translational surgical research. *Surgery* 156, 508–516. doi: 10.1016/j.surg.2014.07.001

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

62

CHAPTER 3: Impact of the menstrual cycle on peripheral vascular function in premenopausal women: systematic review & meta-analysis.

Jennifer S. Williams, Emily C. Dunford, Maureen J. MacDonald

Supplemental Material from this Chapter can be found here:
https://doi.org/10.5683/SP2/OYUH6P

SYSTEMATIC REVIEW | *Integrative Cardiovascular Physiology and Pathophysiology*

Impact of the menstrual cycle on peripheral vascular function in premenopausal women: systematic review and meta-analysis

Ⓞ **Jennifer S. Williams, Emily C. Dunford, and** Ⓞ **Maureen J. MacDonald**
Vascular Dynamics Laboratory, Department of Kinesiology, McMaster University, Hamilton, Ontario, Canada

Submitted 12 May 2020; accepted in final form 2 October 2020

Williams JS, Dunford EC, MacDonald MJ. Impact of the menstrual cycle on peripheral vascular function in premenopausal women: systematic review and meta-analysis. *Am J Physiol Heart Circ Physiol* 319: H1327–H1337, 2020. First published October 16, 2020; doi:10.1152/ajpheart.00341.2020.—Fluctuations in endogenous hormones estrogen and progesterone during the menstrual cycle may offer vasoprotection for endothelial and smooth muscle (VSM) function. While numerous studies have been published, the results are conflicting, leaving our understanding of the impact of the menstrual cycle on vascular function unclear. The purpose of this systematic review and meta-analysis was to consolidate available research exploring the role of the menstrual cycle on peripheral vascular function. A systematic search of MEDLINE, Web of Science, and EMBASE was performed for articles evaluating peripheral endothelial and VSM function across the natural menstrual cycle: early follicular (EF) phase versus late follicular (LF), early luteal, mid luteal, or late luteal. A meta-analysis examined the effect of the menstrual cycle on the standardized mean difference (SMD) of the outcome measures. Analysis from 30 studies ($n = 1,363$ women) observed a "very low" certainty of evidence that endothelial function increased in the LF phase (SMD: 0.45, $P = 0.0001$), with differences observed in the macrovasculature but not in the microvasculature (SMD: 0.57, $P = 0.0003$, $I^2 = 84\%$; SMD: 0.21, $P = 0.17$, $I^2 = 34\%$, respectively). However, these results are partially explained by differences in flow-mediated dilation [e.g., discrete (SMD: 0.86, $P = 0.001$) vs. continuous peak diameter assessment (SMD: 0.25, $P = 0.30$)] and/or menstrual cycle phase methodologies. There was a "very low" certainty that endothelial function was largely unchanged in the luteal phases, and VSM was unchanged across the cycle. The menstrual cycle appears to have a small effect on macrovascular endothelial function but not on microvascular or VSM function; however, these results can be partially attributed to methodological differences.

endothelial function; hormonal cycle; macrovascular; microvascular; smooth muscle function

INTRODUCTION

Cardiovascular disease (CVD) is a leading cause of death worldwide, and the sex-specific prevalence of CVD is an ongoing area of research (3, 57). While the reported prevalence of CVD is greater in men than in age-matched women, the prevalence in women increases dramatically after menopause (26, 43). It has been hypothesized that the loss of menstrual cycle endogenous sex hormone production may be, in part, responsible for this increase. Specifically, the influence of the sex hormone estrogen (17β-estradiol) has been thought to delay the progression of CVD, potentially through its positive impact on inflammation, oxidative stress, vasodilator production [e.g., nitric oxide (NO)], and directly on the remodeling of the vasculature (18).

Vascular function is determined by the function of both the endothelium and vascular smooth muscle (VSM) that react to stimuli by vasoconstricting or vasodilating to modulate the vascular tone. Specifically, endothelial function is integral for cardiovascular health, as dysfunction is a precursor to the development of atherosclerosis, an early step in the progression of CVD (67). One noninvasive method of assessing macrovascular endothelial function is through a standardized flow-mediated dilation (FMD) reactive hyperemia test (8). Brachial artery FMD is an independent predictor of CVD (23, 67) and correlates well with the more invasive coronary artery endothelial-dependent function test (41). Guidelines for FMD have been developed to standardize the methodology (13, 19, 59, 60), including two recommendations which specify that premenopausal women should be assessed during the early follicular phase of the menstrual cycle (*days 1–7* of the menstrual cycle) when endogenous sex hormones estrogen and progesterone are the lowest (19, 59). A recent update to the FMD guidelines continues to recommend that premenopausal

Correspondence: M. J. MacDonald (macdonmj@mcmaster.ca).

women should be examined during a standardized phase of the menstrual cycle (60). The inclusion of this guideline was based on early research by Hashimoto and colleagues (21), which reported increased FMD during the late follicular and luteal phases of the menstrual cycle, when endogenous hormone levels are elevated. Similarly, VSM function, as assessed using a standard nitroglycerine-mediated vasodilation test, was also observed to be elevated throughout the menstrual cycle in the same study (21). This study was further supported by research by Williams and colleagues (65), which reported fluctuations in macrovascular FMD assessments and microvascular responses to endothelial (acetylcholine) and vascular smooth muscle (nitroprusside) stimuli across the menstrual cycle. However, in ~20 years since these studies, numerous studies have been published with both supporting and conflicting results, leaving the impact of the menstrual cycle on vascular endothelial and VSM function unclear (1, 14, 21, 34, 51, 65). Recently, researchers have engaged in a point-counterpoint debate, providing opinion on whether the menstrual cycle should or should not be controlled in vascular studies (58, 63). This apparent discrepancy in the literature raises questions about the establishment of menstrual cycle-specific guidelines in FMD testing (13, 59, 60) and may be relevant in the establishment of future macrovascular and microvascular assessment guidelines.

As a result, we conducted the first systematic review and meta-analysis to consolidate available research exploring the role of the menstrual cycle on peripheral macrovascular and microvascular endothelial and smooth muscle functions in premenopausal women.

METHODS

This systematic review and meta-analyses were conducted in accordance with the Preferred Reporting Items for Systematic Review and Meta-Analysis (PRISMA) guidelines (38). This review was also registered with the International Prospective Register of Systematic Reviews (PROSPERO): Registration No. 2019-CRD42019124743. The investigators adhered to the prestudy protocol, apart from the integration of additional subgroup and sensitivity analysis detailed below. All supplemental figures and appendices are publicly available through the following link: https://doi.org/10.5683/SP2/OYUH6P.

Data Search Strategy

A systematic search of published studies in peer-reviewed journals was undertaken to explore the impact of phases of the menstrual cycle on endothelial function and VSM function in premenopausal women. The systematic search included studies identified through MEDLINE, Web of Science, and EMBASE, from inception until August 2019, updated in January 2020, and again in August 2020. The search strategy was developed in consultation with a University librarian. The search consisted of the following combinations of keywords: "menstrual cycle," "phase," "menstruation," "luteal," "follicular," "vasodilation," "vasorelaxation," "vascular endothelium-dependent dilation," "endothelium-independent dilation," "endothelium," "vascular," "flow-mediated dilation," "FMD," "nitroglycerin-mediated dilation," "NTG," "microvascular," "nitroprusside," "acetylcholine," "skin blood flow," and "arteries: brachial or femoral or popliteal or radial" (Supplemental Appendix S1 for MEDLINE sample search). Additional hand searches of identified original research article reference lists were also conducted to identify additional studies to be included in the search. Only studies in English were included; however, no other restrictions were placed on the search.

Eligibility Criteria

Study design. Original studies of any design, including but not limited to observational studies, randomized controlled trials, and cross-

sectional studies, were eligible for inclusion. The following were excluded: reviews (i.e., narrative, literature, and systematic reviews), case studies, commentaries, and conference abstracts.

Population. The population included premenopausal women who were naturally cycling (i.e., not using hormonal contraceptives) and tested at least twice in their cycle, as detailed below. Studies including multiple groups of women (i.e., postmenopausal, premenopausal on hormonal contraceptives) or a comparator group of men were included, but only if the naturally cycling group could be separated out to be considered in this analysis. Studies that combined mixed groups (i.e., combining premenopausal women with natural cycles with those using hormonal contraceptives) were excluded from this review.

Exposure and comparisons. To be included in this review, studies had to have performed vascular assessments during at least two phases of the menstrual cycle, including once during the early follicular phase when endogenous hormone levels are low. The early follicular phase was used as a "comparator" phase in exploring phasic differences in endothelial and VSM function across common comparison phases of the menstrual cycle (i.e., late follicular, early luteal, mid-luteal, and late luteal phases).

Outcomes. Studies were included in this review if they assessed endothelial function or VSM function in a major conduit artery (brachial, radial, femoral, and popliteal) or the microvasculature (e.g., forearm or leg skin blood flow). Macrovascular endothelial function was commonly assessed using an FMD test, whereas macrovascular smooth muscle function was assessed using a sublingual nitroglycerine-mediated dilation test. Microvascular endothelial function was often assessed using skin blood flow responses to postocclusive reactive hyperemia (PORH) or iontophoresis using acetylcholine (ACh) or bradykinin. Microvascular smooth muscle function was generally assessed using sodium nitroprusside (SNP).

Study Selection and Data Extraction

Study selection was performed by two independent investigators (J. S. Williams and E. C. Dunford). Titles and abstracts were determined through the search strategy and assessed by two reviewers. Studies determined for inclusion by one of the two reviewers was included in full-text review. Both investigators reviewed all full-text articles for eligibility, and discrepancies regarding inclusion/exclusion were solved through consensus. A third reviewer (KS) was available to solve discrepancies but was not needed.

Variables of interest were extracted by both reviewers into a preformatted spreadsheet for each study, following piloting by investigators (J. S. Williams and E. C. Dunford). The following information was extracted: citation (authors and year of publication) participant characteristics [n, health status, fitness level, age, body mass index (BMI), resting systolic and diastolic blood pressure (SBP, DBP), resting heart rate (HR), and health status], information regarding vascular assessments of endothelial function and VSM function, methodology used (vasodilator stimuli, cuff placement, and occlusion time (if applicable)), and the vascular region assessed. In addition, factors which may influence macrovascular assessments, including the method of assessing artery diameter (continuous edge detection vs. diameter at 1 min after cuff deflation), and baseline diameter and shear rate/stress changes across a cycle have been extracted. Relative changes in assessments of vascular function were used in the meta-analysis [e.g., percent FMD (%FMD), rather than absolute FMD], where available. Information regarding the menstrual cycle phases, methodology used to determine phase, and hormonal profile (estradiol and progesterone levels) were also recorded. Where multiple measures of microvascular function were assessed for PORH occlusion time, the longest of the occlusions (3 min) was used. Hemodynamic data (SBP, DBP, and HR) were reported as either baseline data identified in the original study, or during the early follicular/menstrual phase, if multiple phases were included for baseline hemodynamics.

If data were unclear, absent, or included in figures only, the primary and/or corresponding author was contacted by the investigators through email to seek this information, and data not reported following attempts to contact authors are noted in Supplemental Appendix S4 as not reported (NR). If original data were unable to be extracted, and authors were unresponsive, data in figures were extracted using the online software "WebPlotDigitizer."

Quality and Certainty of Evidence Assessment Using SAQOR and GRADE

Two investigators (J. S. Williams, E. C. Dunford) independently assessed each study for quality and certainty of evidence, and discrepancies were solved through consensus. In recognition of potential conflict of interest (i.e., investigator as an author on an included study), a third reviewer (KS) independently performed SAQOR and GRADE analysis on a random selection of six studies, including the study in question. A systematic appraisal for observational research (SAQOR) was performed to evaluate individual study quality of evidence (risk of bias) (45), as previously performed in recent meta-analyses examining vascular function in observational studies (33, 39, 55). The SAQOR was adapted for the purposes of this review, including the following assessments: *i*) the sample, *ii*) the comparison condition, *iii*) quality of exposure and outcome assessment, *iv*) confounding variables, and *v*) reporting of data (see Supplemental Appendix S2 for more details on questions). The SAQOR was categorized to "high," "moderate," "low," or "very low" quality. Additionally, the Grading of Recommendations Assessment, Develop-ment and Evaluation (GRADE) using GRADEPro was performed to assess the certainty of evidence for outcomes in the meta-analyses: endothelial function and VSM function. The certainty of evidence for each outcome was assessed using the following scale: "high," "moderate," "low," or "very low" certainty. According to the GRADE criteria, observational studies all started as "low" (nonrandomized controlled trial) and were downgraded or upgraded using the following established criteria. The certainty of outcome was downgraded due to *1*) risk of bias/study limitations, *2*) inconsistency in results, *3*) indirectness of results, *4*) imprecision, or *5*) reporting/publication bias. Bias was serious if ≥50% of weight of the pooled SMD contributing to each outcome analysis was from studies indicating high risk of bias in "very low" or "low" quality of evidence from SAQOR. Inconsistency was considered serious when heterogeneity assessed using an I^2 test, with ≥50%. Imprecision was considered serious if the outcome's 95% confidence interval crossed the line of no effect (i.e., both positive and negative CI values). To explore the presence of publication bias, funnel plots with the standard funnel of phase differences versus the study standard error were analyzed using visual inspection in analyses with at least 10 studies. Studies were upgraded if there was *1*) a large magnitude of effect (SMD = 0.8 or higher), *2*) a dose response, or *3*) confounding factors would reduce the effect or suggest a spurious effect, with the latter two criteria only upgraded if the outcome was not already downgraded.

Data synthesis and Analysis

Data synthesis. In cases where multiple groups or multiple outcomes (e.g., microvascular iontophoresis studies) were included in a single study, data for each group or condition were included separately in the meta-analyses, and the sample size was adjusted accordingly. Data presented as means ± SE were converted to means ± standard deviation (SD), using the following formula: SD = means ± SE × $\sqrt{n}$, where *n* is the sample size of the study. Data presented as mean (range) were converted with the formula SD = range/4. Data presented using 95% CI were converted using the following formula: SD = [(upper 95% − lower 95%)/2 × 95% *t* value] × $\sqrt{n}$. Finally, when using median and interquartile range (IQR), mean was estimated as median and IQR was converted to SD using the formula SD = IQR/1.35. If estradiol and progesterone hormone levels were reported in pg/mL or ng/mL, they were converted to pmol/L and nmol/L, respectively, using the following conversion factor: 1 pg/mL = 3.67 pmol/L estradiol and 1 ng/mL progesterone = 3.18 nmol/L.

Data analysis. The meta-analyses were performed using Review Manager (RevMan v5.3; Cochrane Collaboration, Oxford, UK). The outcome variables included standardized mean difference (SMD) in endothelial function and VSM function through the menstrual cycle phases including comparing assessments in *1*) the early follicular (EF) phase to the late follicular (LF) phase and *2*) the EF phase to the luteal phases [early luteal (EL), mid-luteal (ML), and late luteal (LL)]. Each SMD was weighted according to the inverse variance method and pooled with a random-effects model. The SMD summary statistic was chosen over a mean difference statistic, as it allowed for the standardization of different outcome variable methodologies for this meta-analysis [e.g., endothelial function assessed by FMD (with multiple methodologies), PORH, and iontophoresis]. A positive SMD corresponded with an increase in endothelial function and VSM function during different phases of the menstrual cycle. Significance for results was set to $P \leq 0.05$ and effect sizes were examined qualitatively according to Cohen's guidelines, with an SMD of 0.2, 0.5, and 0.8 denoting small, medium, and large effect sizes, respectively (12).

Subgroup and sensitivity analysis. Where heterogeneity was high ($I^2 \geq 50\%$), subgroup analysis was performed examining *1*) macrovasculature and microvasculature; *2*) macrovascular endothelial function methods that use a discrete measurement of diameter (e.g., 60 s after deflation) compared with continuous measurements to determine peak dilation; *3*) microvascular endothelial function methods comparing PORH and iontophoresis; and *4*) reporting of activity levels (e.g., active, inactive, and not reported). Subgroup analysis was also performed stratifying studies based on the number of steps in the three-step hormonal cycle method (e.g., menstrual cycle mapping/self-report, urinary ovulation prediction, and serum/plasma hormone measurement) (Supplemental Appendix S5) (49). Similarly, sensitivity analysis using the following parameters *1*) removing clinical populations from analysis (*n* = 4 studies) and *2*) removing studies testing upper limb (proximal) cuff placement compared with lower limb (distal) cuff placement (*n* = 1 study) or where cuff placement was unclear (*n* = 1 study).

Meta-regressions. Using the IBM Statistical Package for the Social Sciences (SPSS; v 24; Chicago, IL) and Wilson's SPSS metareg.sps macro, weighted meta-regressions were performed where more than 10 studies were available using the inverse variance of the dependent variable: SMD in endothelial function from EF and LF or EF and ML phases. The following independent variables were entered into the regression model: year of study publication, age, average menstrual cycle length, BMI, resting systolic and diastolic blood pressure, and the change in blood estradiol levels between phases. GraphPad (v 8; La Jolla, CA) was used to graph regressions.

RESULTS

Study Selection and Characteristics

The systematic review search resulted in the inclusion of 1,336 articles that subsequently underwent title and abstract screening, resulting in 78 potential articles (Fig. 1). Following a full text review for eligibility, 33 studies remained in the review (1, 2, 4–6, 10, 11, 14–16, 20, 21, 25, 27–29, 32, 34–36, 40, 42, 46, 48, 50, 51, 53, 54, 56, 62, 64, 65, and 68), with exclusions identified in the figure. One study was excluded from the meta-analysis due to challenges with extracting data (4), one study was excluded as it included a LF phase comparator instead of EF (46), and one study was excluded as it combined LF and ML phases in its comparison analysis (11). The majority of studies identified (*n* = 28) used an observational repeated measure within-subject design (OBS RM), whereas two studies used a cross-sectional study design. Twenty-eight studies included healthy participants (*n* = 1, 302) and two studies included clinical populations, specifically variant angina (*n* = 10) (28), and

PhD Thesis – J. Williams; McMaster University - Kinesiology
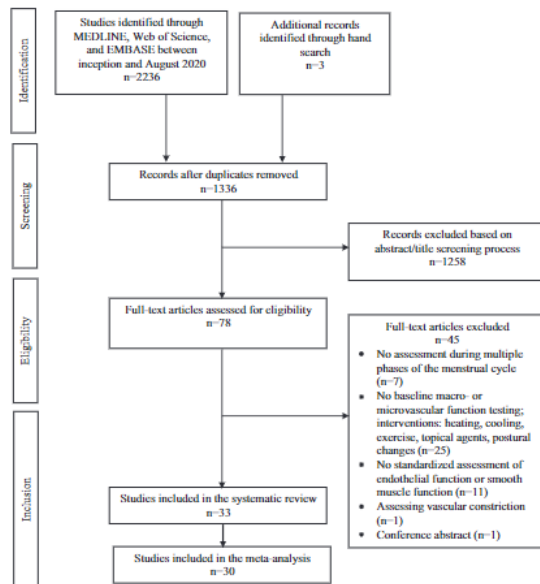
Fig. 1. Systematic review and meta-analysis flow diagram.

smokers ($n = 17$) (16). Two of the studies including healthy participants as controls also included a subgroup of smokers ($n = 13$) (25), and individuals with premenstrual syndrome ($n = 21$) (56), bringing the total of participants in clinical populations to 61. Participants ($n = 1,363$) were generally young (28.9 ± 6.6 yr), with regular menstrual cycles (average cycle length: 29.0 ± 1.4 days), exhibiting a healthy BMI and blood pressure (BMI: 23.0 ± 1.6 kg/m^2, systolic BP: 111.1 ± 7.7 mmHg, and diastolic BP: 69.6 ± 4.3 mmHg). Further study characteristics and extracted data from each study are provided in Supplemental Appendices S3 and S4.

Quality Analysis and GRADE

Using SAQOR, the majority of studies were classified as "moderate" quality ($n = 19$ studies), with a range from "low" ($n = 1$ study) to "high" ($n = 13$ studies) quality (see Supplemental Appendices S3 and S7). According to GRADE scoring criteria, observational studies began with a "low" certainty assessment. All outcomes were downgraded to "very low" certainty of evidence for one of the three reasons: *1*) serious risk of bias, *2*) inconsistency (i.e., high heterogeneity), or *3*) imprecision (i.e., 95% CI crossing over no effect line) (Supplemental Appendix S6). There was no evidence of publication bias (Supplemental Fig. S11, *a–f*). Only four analyses were upgraded from "very low" to "low" certainty of evidence for having a large effect size (Supplemental Appendix S6).

Endothelial Function: Endothelial-Dependent Dilation

Follicular phase. There was a "very low" certainty of evidence, with a small effect size, that endothelial function increased from the EF to LF phase [26 studies; $n = 1,304$ women; SMD: 0.45 (95% CI: 0.22, 0.69), $P = 0.0001$, downgraded for serious inconsistency; Fig. 2]. Heterogeneity was high ($I^2 = 78\%$),

so subgroup analysis was performed examining macrovascular versus microvascular measures of endothelial function. There remained a "very low" certainty of evidence, with a medium effect size, that the difference across EF to LF phases was driven by an increase in function in the macrovasculature [21 studies; $n = 1,168$ women; SMD: 0.57 (95% CI: 0.26, 0.87), $P = 0.0003$, downgraded for serious inconsistency] but not in the microvasculature [7 studies; $n = 136$ women; SMD: 0.21 (95% CI: -0.09, 0.52), $P = 0.17$, downgraded for serious inconsistency and imprecision]; however, subgroups were not statistically different ($P = 0.11$; Fig. 2). High heterogeneity remained for the macrovascular endothelial function ($I^2 = 84\%$) but not in the microvasculature ($I^2 = 34\%$), so further subgroup analysis was performed to examine differences in methodology used for macrovascular assessments: discrete versus continuous diameter assessment. There was a "low" certainty of evidence, with a large effect size, that endothelial function increased across the EF to LF phase in the discrete diameter assessment subgroup [11 studies; $n = 1,041$ women; SMD: 0.86 (95% CI: 0.42, 1.29), $P = 0.0001$, downgraded for serious inconsistency, but upgraded with a large effect; Fig. 3]. In contrast, there was a "very low" certainty of evidence that endothelial function was unchanged across the EF to LF phase in the continuous diameter assessment subgroup [10 studies; $n = 127$ women; SMD: 0.25 (95% CI: -0.22, 0.71), $P = 0.30$, downgraded for serious inconsistency and imprecision; Fig. 3].

Heterogeneity remained high for both subgroups (discrete subgroup: $I^2 = 89\%$; continuous subgroup: $I^2 = 68\%$), and subgroups trended to being significantly different ($P = 0.06$). Sensitivity analysis for removal of studies with proximal/unclear cuff placement or clinical populations did not alter the EF to LF results, apart from decreasing the effect size to medium, and thus the certainty of evidence to "very low," in the discrete macrovascular assessment with clinical population removal (SMD: 0.74, $P = 0.001$). Finally, subgroup analysis examining fitness (subgroups: sedentary, active, and not reported) did not explain the heterogeneity in the macrovascular endothelial function results (Supplemental Fig. S1).

Further subgroup analysis examining hormonal cycle assessments was performed. Studies meeting 0 or 3/3 hormonal cycle assessment criteria resulted in no changes to endothelial function across the EF to LF phases {[0 criteria: 2 studies; $n = 770$ women; SMD: 0.23 (95% CI: -0.31, 0.77), $P = 0.41$, $I^2 = 44\%$], [3 criteria: 6 studies; $n = 85$ women; SMD: 0.51 (95% CI: -0.29, 1.30), $P = 0.21$, $I^2 = 82\%$] Supplemental Fig. S2}. However, studies meeting only one or two of the criteria resulted in significant increases in endothelial function across follicular phases {[1 criteria: 9 studies; $n = 293$ women; SMD: 0.33 (95% CI: 0.02, 0.64), $P = 0.04$, $I^2 = 64\%$], [2 criteria: 9 studies; $n = 156$ women; SMD: 0.76 (95% CI: 0.14, 1.37), $P = 0.02$, $I^2 = 83\%$] Supplemental Fig. S2]}, though the subgroups were not significantly different ($P = 0.58$).

Luteal Phases

There was a "very low" certainty of evidence that endothelial function was unchanged across the EF to EL phases [6 studies; $n = 961$ women; SMD: -0.25 (95% CI: -0.51, 0.02), $P = 0.07$, downgraded for serious inconsistency and imprecision; Supplemental Fig. S3]. Heterogeneity was high ($I^2 = 56\%$), so subgroup analysis was performed examining the macrovascular versus

Study or Subgroup	Late Follicular Mean	SD	Total	Early Follicular Mean	SD	Total	Weight	Std. Mean Difference IV, Random, 95% CI
1.1.1 Macrovascular								
Rakobowchuk al (2013)	6.62	0.86	8	8.2	1.4	8	2.3%	-1.29 [-2.39, -0.18]
D'Urzo et al (2018)	6.4	3.1	12	7.9	4.3	12	3.1%	-0.39 [-1.20, 0.42]
Schnabel et al (2013)	12.1	4.9	121	14.1	6.4	121	4.6%	-0.35 [-0.60, -0.10]
Jochmann et al (2009) (1)	8.6	3	13	9.7	4.3	13	3.2%	-0.29 [-1.06, 0.49]
Williams et al (2019)	8.39	2.42	17	8.68	2.88	17	3.4%	-0.11 [-0.78, 0.57]
Luca et al (2016)	7.2	3.16	10	7.1	3.16	10	2.9%	0.03 [-0.85, 0.91]
Valtonen et al (2010)	0.284	0.276	761	0.259	0.359	761	4.8%	0.08 [-0.02, 0.18]
Shenouda et al (2018)	7.8	3.2	15	6.9	3.5	15	3.3%	0.26 [-0.46, 0.98]
Jochmann et al (2009) (2)	10.3	4.4	12	8.9	5.3	12	3.1%	0.28 [-0.53, 1.08]
Stamatelopoulos et al (2012) (3)	7.5	2.88	15	6.7	2.21	15	3.3%	0.30 [-0.42, 1.02]
Brandao et al (2014)	13.57	0.37	21	12.93	2.48	21	3.6%	0.35 [-0.26, 0.96]
Stamatelopoulos et al (2012) (4)	7.97	2.41	21	6.82	3.1	21	3.6%	0.41 [-0.21, 1.02]
Williams et al (2001) (5)	10	2.71	8	8.8	2.32	8	2.6%	0.45 [-0.55, 1.45]
English et al (1998)	10.9	6.26	20	8	4.92	20	3.6%	0.50 [-0.13, 1.14]
Garcia-Fernandez et al (2004)	16	5.73	17	13.17	3.28	17	3.4%	0.59 [-0.10, 1.28]
O'Brien et al (2020)	5.8	1.5	9	4.6	1.6	9	2.6%	0.74 [-0.23, 1.70]
Sorensen et al (2002)	12.5	6.5	10	7.2	4.4	10	2.7%	0.91 [-0.02, 1.85]
Harris et al (2012)	12.4	5.42	15	8	3.49	15	3.2%	0.94 [0.18, 1.70]
Sorensen et al (2006)	11.5	5.7	13	5.44	4.8	13	3.0%	1.11 [0.28, 1.95]
Hashimoto et al (1995)	18.2	3.34	17	11.22	2.39	17	2.8%	2.35 [1.45, 3.24]
Kawano et al (1996)	14.1	3.49	15	4.9	3.1	15	2.5%	2.71 [1.69, 3.74]
Adkisson et al (2010) (6)	5.12	0.51	8	3.32	0.43	8	1.3%	3.61 [1.87, 5.35]
Kawano et al (2001)	8.16	1.11	10	1.67	0.73	10	0.8%	6.62 [4.17, 9.07]
Subtotal (95% CI)			1168			1168	69.5%	0.57 [0.26, 0.87]
Heterogeneity: Tau² = 0.37; Chi² = 133.44, df = 22 (P < 0.00001); I² = 84%								
Test for overall effect: Z = 3.64 (P = 0.0003)								
1.1.2 Microvascular								
Ketel et al (2009)	50.36	73.14	18	88.75	50.62	18	3.5%	-0.60 [-1.27, 0.07]
Yvonne-Tee et al (2008) (7)	49.67	15.65	20	53.18	22.45	20	3.6%	-0.18 [-0.80, 0.44]
Yvonne-Tee et al (2008) (8)	47.66	21.38	20	48.5	21.69	20	3.6%	-0.04 [-0.66, 0.58]
Mayrovitz et al (2007)	1.22	0.52	10	1.14	0.41	10	2.9%	0.16 [-0.71, 1.04]
Yvonne-Tee et al (2008) (9)	46.64	16.05	20	43.55	15.34	20	3.6%	0.19 [-0.43, 0.81]
Williams et al (2001) (10)	3.3	1.55	7	2.7	0.77	7	2.4%	0.46 [-0.61, 1.53]
Adkisson et al (2010) (11)	29	5.4	7	26.3	4.2	7	2.4%	0.52 [-0.55, 1.59]
Adkisson et al (2010) (12)	25.8	5.9	8	21.5	4.3	8	2.5%	0.79 [-0.24, 1.82]
Chan et al (2001)	1,349.7	697.91	15	863.1	436.1	15	3.2%	0.81 [0.06, 1.56]
Arora et al (1998)	186	31	11	147	57	11	2.9%	0.82 [-0.06, 1.70]
Subtotal (95% CI)			136			136	30.5%	0.21 [-0.09, 0.52]
Heterogeneity: Tau² = 0.08; Chi² = 13.69, df = 9 (P = 0.13); I² = 34%								
Test for overall effect: Z = 1.36 (P = 0.17)								
Total (95% CI)			1304			1304	100.0%	0.45 [0.22, 0.69]
Heterogeneity: Tau² = 0.30; Chi² = 147.22, df = 32 (P < 0.00001); I² = 78%								
Test for overall effect: Z = 3.81 (P = 0.0001)								
Test for subgroup differences: Chi² = 2.59, df = 1 (P = 0.11); I² = 61.4%								

Footnotes
(1) Smokers
(2) Healthy Participants
(3) Healthy Participants
(4) Clinical PMS
(5) FMD Outcome (n=15/2)
(6) FMD Outcome (n=23/3)
(7) Age 41-50 cohort
(8) Age 31-40 cohort
(9) Age 21-30 cohort
(10) Microvascular Outcome (n=15/2)
(11) PORH Calf Outcome (n=23/3)
(12) PORH Forearm Outcome (n=23/3)

Fig. 2. Forest plot for endothelial function: early follicular vs. late follicular phases, with subgroup analysis: macrovascular vs. microvascular endothelial function. CI, confidence intervals (95%); IV, inverse-variance method; I², measure of heterogeneity; SD, standard deviation; std. mean difference, standard mean difference.

microvascular endothelial function. There was a "very low" certainty of evidence for both subgroups, with endothelial function remaining unchanged across the EF to EL phases in the macrovasculature {6 studies; $n = 939$ women; SMD: -0.27 [95% CI: -0.27 (-0.58, 0.03), $P = 0.08$, downgraded for serious inconsistency and imprecision]}, and in the microvasculature [2 studies; $n = 22$ women; SMD: -0.17 (95% CI: -0.78, 0.44), $P = 0.59$, downgraded for serious imprecision], with no difference between subgroups ($P = 0.76$). Sensitivity analysis for cuff placement resulted in nonsignificant differences between EF to EL phases ($P = 0.27$ macrovascular).

There was a "very low" certainty of evidence that endothelial function remained unchanged across the EF to ML phases [12 studies; $n = 183$ women; SMD: 0.37 (95% CI: -0.19, 0.92), $P = 0.19$, downgraded for serious inconsistency and imprecision; Fig. 4]. Given the high heterogeneity

($I^2 = 84\%$), subgroup analysis was performed examining the macrovasculature versus microvasculature. There remained a "very low" certainty of evidence for both subgroups, with the unchanged endothelial function detected in the macrovasculature [8 studies; $n = 125$ women; SMD: 0.69 (95% CI: -0.9, 1.48), medium effect size, $P = 0.08$, downgraded for serious inconsistency and imprecision], and in the microvasculature [4 studies; $n = 58$ women; SMD: -0.27 (95% CI: -0.19, 0.92) $P = 0.15$, downgraded for serious imprecision], with a significant difference between subgroups detected ($P = 0.03$). Heterogeneity remained high in the macrovasculature ($I^2 = 87\%$) but not in the microvasculature ($I^2 = 0\%$); as a result, further subgroup analysis was formed examining discrete versus continuous diameter assessments. There was a "low" and "very low" certainty of evidence for macrovascular discrete and continuous assessments, respectively. The

IMPACT OF MENSTRUAL CYCLE ON VASCULAR FUNCTION

Study or Subgroup	Late Follicular Mean	SD	Total	Early Follicular Mean	SD	Total	Weight	Std. Mean Difference IV, Random, 95% CI
1.2.1 Discrete Diameter Measurements								
Schnabel et al (2013)	12.1	4.9	121	14.1	6.4	121	6.2%	-0.35 [-0.60, -0.10]
Valtonen et al (2010)	0.284	0.276	761	0.259	0.359	761	6.4%	0.08 [-0.02, 0.18]
Stamatelopoulos et al (2012) (1)	7.5	2.88	15	6.7	2.21	15	4.7%	0.30 [-0.42, 1.02]
Brandao et al (2014)	13.57	0.37	21	12.93	2.48	21	5.1%	0.35 [-0.26, 0.96]
Stamatelopoulos et al (2012) (2)	7.97	2.41	21	6.82	3.1	21	5.1%	0.41 [-0.21, 1.02]
English et al (1998)	10.9	6.26	20	8	4.92	20	5.0%	0.50 [-0.13, 1.14]
Garcia-Fernandez et al (2004)	16	5.73	17	13.17	3.28	17	4.8%	0.59 [-0.10, 1.28]
Sorensen et al (2002)	12.5	6.5	10	7.2	4.4	10	4.0%	0.91 [-0.02, 1.85]
Sorensen et al (2006)	11.5	5.7	13	5.44	4.8	13	4.3%	1.11 [0.28, 1.95]
Hashimoto et al (1995)	18.2	3.34	17	11.22	2.39	17	4.1%	2.35 [1.45, 3.24]
Kawano et al (1996)	14.1	3.49	15	4.9	3.1	15	3.7%	2.71 [1.69, 3.74]
Kawano et al (2001)	8.16	1.11	10	1.67	0.73	10	1.2%	6.62 [4.17, 9.07]
Subtotal (95% CI)			1041			1041	54.9%	0.86 [0.42, 1.29]
Heterogeneity: Tau² = 0.43; Chi² = 102.08, df = 11 (P < 0.00001); I² = 89%								
Test for overall effect: Z = 3.85 (P = 0.0001)								
1.2.2 Continuous Diameter Measurements								
Rakobowchuk et al (2013)	6.62	0.86	8	8.2	1.4	8	3.5%	-1.29 [-2.39, -0.18]
D'Urzo et al (2018)	6.4	3.1	12	7.9	4.3	12	4.4%	-0.39 [-1.20, 0.42]
Jochmann et al (2009) (3)	8.6	3	13	9.7	4.3	13	4.5%	-0.29 [-1.06, 0.49]
Williams et al (2019)	8.39	2.42	17	8.68	2.88	17	4.9%	-0.11 [-0.78, 0.57]
Luca et al (2016)	7.2	3.16	10	7.1	3.16	10	4.2%	0.03 [-0.85, 0.91]
Shenouda et al (2018)	7.8	3.2	15	6.9	3.5	15	4.7%	0.26 [-0.46, 0.98]
Jochmann et al (2009) (4)	10.3	4.4	12	8.9	5.3	12	4.4%	0.28 [-0.53, 1.08]
Williams et al (2001)	10	2.71	8	8.8	2.32	8	3.8%	0.45 [-0.55, 1.45]
O'Brien et al (2020)	5.8	1.5	9	4.6	1.6	9	3.9%	0.74 [-0.23, 1.70]
Harris et al (2012)	12.4	5.42	15	8	3.49	15	4.6%	0.94 [0.18, 1.70]
Adkisson et al (2010)	5.12	0.51	8	3.32	0.43	8	2.1%	3.61 [1.87, 5.35]
Subtotal (95% CI)			127			127	45.1%	0.25 [-0.22, 0.71]
Heterogeneity: Tau² = 0.41; Chi² = 31.22, df = 10 (P = 0.0005); I² = 68%								
Test for overall effect: Z = 1.04 (P = 0.30)								
Total (95% CI)			1168			1168	100.0%	0.57 [0.26, 0.87]
Heterogeneity: Tau² = 0.37; Chi² = 133.44, df = 22 (P < 0.00001); I² = 84%								
Test for overall effect: Z = 3.64 (P = 0.0003)								
Test for subgroup differences: Chi² = 3.50, df = 1 (P = 0.06), I² = 71.4%								

Footnotes
(1) Healthy Participants
(2) Clinical PMS
(3) Smokers
(4) Healthy Participants

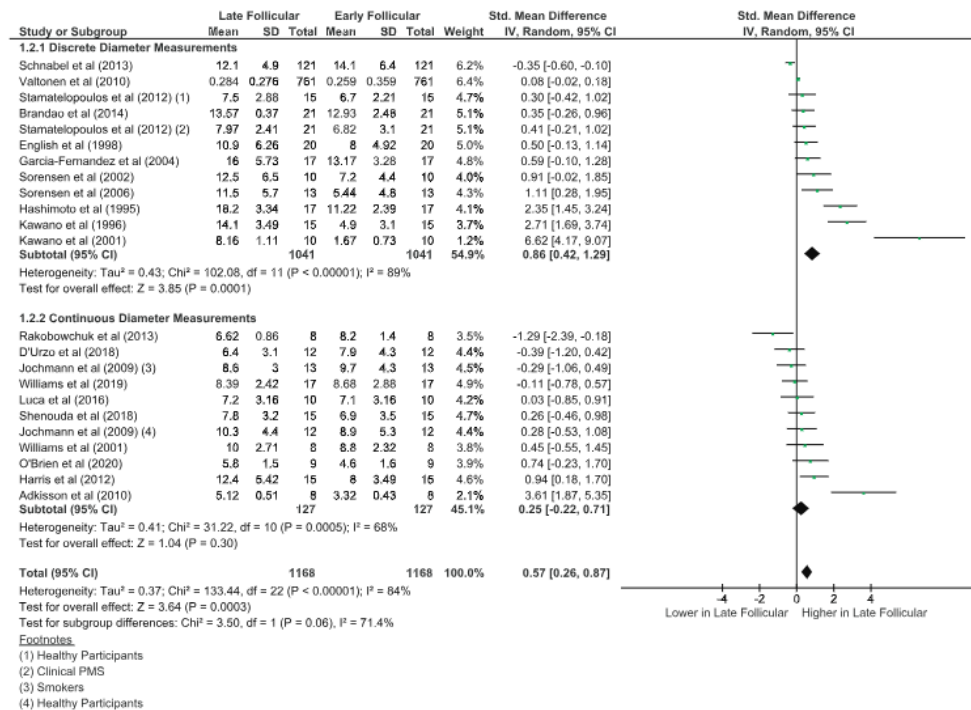Fig. 3. Forest plot for macrovascular endothelial function: early follicular vs. late follicular phases, with subgroup analysis: discrete vs. continuous diameter measurements. CI, confidence intervals (95%); IV, inverse-variance method; I², measure of heterogeneity; SD, standard deviation; std. mean difference, standard mean difference.

discrete diameter assessment subgroup observed a large effect size in the increase in endothelial function from EF to ML [4 studies; $n = 62$ women; SMD: 1.87 (95% CI: 0.30, 3.43), $P = 0.02$, downgraded for serious inconsistency, but upgraded for a large effect], while for the continuous diameter subgroup, there was no change in endothelial function observed across phases [4 studies; $n = 63$ women; SMD: -0.06 (95% CI: -0.75, 0.63), $P = 0.86$, downgraded for serious inconsistency and imprecision], with a significant difference between subgroups detected (Supplemental Fig. S4; $P = 0.03$). Heterogeneity remained high in the discrete ($I^2 = 92\%$) and continuous ($I^2 = 72\%$) subgroups. Sensitivity analysis with cuff placement did not change the results; however, removal of clinical populations resulted in a nonsignificant difference from EF to ML in the macrovascular ($P = 0.16$; Supplemental Fig. S5) and macrovascular discrete subgroups ($P = 0.12$). Similarly, subgroup analysis examining hormonal cycle assessments (Supplemental Fig. S6) and fitness assessments both resulted in unchanged endothelial function from EF to ML and did not explain the heterogeneity.

Finally, there was a "very low" certainty of evidence that endothelial function remained unchanged across the EF to LL phases [6 studies; $n = 977$ women; SMD: 0.04 (95% CI: -0.05, 0.13), $P = 0.38$, downgraded for serious risk of bias and imprecision; Supplemental Fig. S7], with low heterogeneity ($I^2 = 0\%$). Sensitivity analysis for removal of studies with proximal/unclear cuff placement or clinical populations did not alter the EF to LL results.

VSM Function: Endothelial-Independent Dilation

Follicular phase. There was "very low" certainty of evidence that VSM function remained unchanged from the EF to LF phases [13 studies; $n = 193$ women; SMD: 0.18 (95% CI: -0.07, 0.42), $P = 0.16$, downgraded for serious imprecision; Fig. 5], with low heterogeneity ($I^2 = 30\%$). Sensitivity analysis for removal of studies with proximal/unclear cuff placement or clinical populations did not alter the EF to LF results.

Luteal phases. There was "very low" certainty of evidence that VSM function remained unchanged from EF to EL phases [2 studies; $n = 35$ women; SMD: 0.09 (95% CI: -0.42, 0.61), $P = 0.72$, downgraded for serious imprecision; Supplemental Fig. S8], with low heterogeneity ($I^2 = 12\%$). Similarly, there was "very low" certainty of evidence that VSM function remained unchanged from EF to ML phases [7 studies; $n = 120$ women; SMD: 0.19 (95% CI: -0.08, 0.45), $P = 0.17$, downgraded for serious imprecision; Supplemental Fig. S9], with low heterogeneity ($I^2 = 7\%$). Finally, there was a "very low" certainty of evidence that VSM function remained unchanged from EF to LL phases [1 study, $n = 15$ women; SMD: -0.25 (95% CI: -0.97, 0.47), $P = 0.50$; Supplemental Fig. S10]. Sensitivity analysis for removal of studies with proximal/unclear cuff placement or clinical populations did not alter in any of the luteal phases.

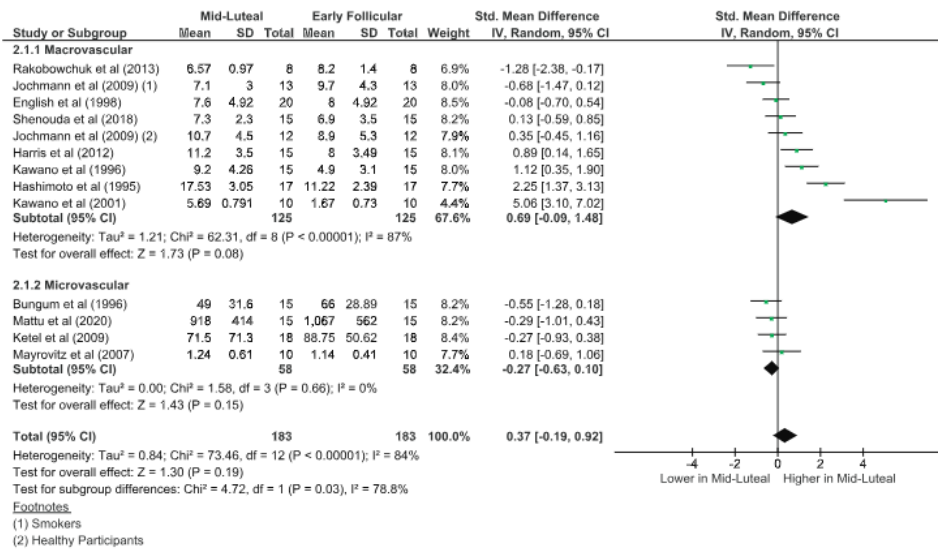| Study or Subgroup	Mid-Luteal Mean	SD	Total	Early Follicular Mean	SD	Total	Weight	Std. Mean Difference IV, Random, 95% CI	Std. Mean Difference IV, Random, 95% CI
2.1.1 Macrovascular									
Rakobowchuk et al (2013)	6.57	0.97	8	8.2	1.4	8	6.9%	-1.28 [-2.38, -0.17]	
Jochmann et al (2009) (1)	7.1	3	13	9.7	4.3	13	8.0%	-0.68 [-1.47, 0.12]	
English et al (1998)	7.6	4.92	20	8	4.92	20	8.5%	-0.08 [-0.70, 0.54]	
Shenouda et al (2018)	7.3	2.3	15	6.9	3.5	15	8.2%	0.13 [-0.59, 0.85]	
Jochmann et al (2009) (2)	10.7	4.5	12	8.9	5.3	12	7.9%	0.35 [-0.45, 1.16]	
Harris et al (2012)	11.2	3.5	15	8	3.49	15	8.1%	0.89 [0.14, 1.65]	
Kawano et al (1996)	9.2	4.26	15	4.9	3.1	15	8.0%	1.12 [0.35, 1.90]	
Hashimoto et al (1995)	17.53	3.05	17	11.22	2.39	17	7.7%	2.25 [1.37, 3.13]	
Kawano et al (2001)	5.69	0.791	10	1.67	0.73	10	4.4%	5.06 [3.10, 7.02]	
Subtotal (95% CI)			125			125	67.6%	0.69 [-0.09, 1.48]	
Heterogeneity: Tau² = 1.21; Chi² = 62.31, df = 8 (P < 0.00001); I² = 87%									
Test for overall effect: Z = 1.73 (P = 0.08)									
2.1.2 Microvascular									
Bungum et al (1996)	49	31.6	15	66	28.89	15	8.2%	-0.55 [-1.28, 0.18]	
Mattu et al (2020)	918	414	15	1,067	562	15	8.2%	-0.29 [-1.01, 0.43]	
Ketel et al (2009)	71.5	71.3	18	88.75	50.62	18	8.4%	-0.27 [-0.93, 0.38]	
Mayrovitz et al (2007)	1.24	0.61	10	1.14	0.41	10	7.7%	0.18 [-0.69, 1.06]	
Subtotal (95% CI)			58			58	32.4%	-0.27 [-0.63, 0.10]	
Heterogeneity: Tau² = 0.00; Chi² = 1.58, df = 3 (P = 0.66); I² = 0%									
Test for overall effect: Z = 1.43 (P = 0.15)									
Total (95% CI)			183			183	100.0%	0.37 [-0.19, 0.92]	
Heterogeneity: Tau² = 0.84; Chi² = 73.46, df = 12 (P < 0.00001); I² = 84%									
Test for overall effect: Z = 1.30 (P = 0.19)									
Test for subgroup differences: Chi² = 4.72, df = 1 (P = 0.03), I² = 78.8%									

Footnotes
(1) Smokers
(2) Healthy Participants

Fig. 4. Forest plot for endothelial function: early follicular vs. mid luteal phases, with subgroup analysis: macrovascular vs. microvascular endothelial function. CI, confidence intervals (95%); IV, inverse-variance method; I², measure of heterogeneity; SD, standard deviation; std. mean difference, standard mean difference.

Meta-regressions. The SMD in endothelial function between EF and LF phases was not significantly mediated by age ($R^2 = 0.014$, $P = 0.444$), menstrual cycle length ($R^2 = 0.031$, $P = 0.358$), BMI ($R^2 = 0.003$, $P = 0.756$), systolic blood pressure ($R^2 = 0.008$, $P = 0.654$), diastolic blood pressure ($R^2 = 0.063$, $P = 0.185$), or the change in estradiol levels from the EF to LF phases ($R^2 = 0.008$, $P = 0.575$) (Supplemental Fig. S12a). However, the SMD in endothelial function between EF and LF phases was significantly associated with the year of publication ($R^2 = 0.207$, $P = 0.0003$; Supplemental Fig. S12b). The SMD in endothelial function between EF and ML phases was not significantly mediated by any variable (Supplemental Fig. S12, b and c).

DISCUSSION

This is the first systematic review and meta-analysis to examine the impact of the menstrual cycle on vascular endothelial and VSM functions. Following the systematic review process, 29 studies assessing endothelial function and 14 studies assessing VSM function were analyzed and their data were extracted for subsequent meta-analyses. There was a "very low" certainty of evidence that endothelial function increased to a small degree in the EF to LF phases, driven by an increase in macrovascular endothelial function, with no effect observed in microvascular function. However, further subgroup analysis found that this effect may be the result of differences in FMD and/or hormonal cycle assessment methodologies, further supported by a negative association with the year of publication. In contrast, endothelial function was found to be largely unchanged in the luteal phases, and VSM also did not change across the menstrual cycle. Given the significant heterogeneity unexplained fully by subgroup analysis and meta-regressions, and "very low" certainty of evidence in the GRADE assessments, the results of this meta-analyses should be interpreted with caution. Taken al-

together, this review and meta-analysis present novel and timely findings, along with considerations for future directions for the further integration of menstrual cycle considerations in cardiovascular physiology research.

Prior to this review, there was no consensus on the influence of the menstrual cycle on the vascular function, given conflicting results from studies over the past 25 years (1, 14, 21, 34, 51, 65). The lack of consensus has brought about debate on whether the menstrual cycle should or should not be controlled for in vascular research and has likely prevented more widespread inclusion of women in studies (58, 63). For simplicity, the EF phase was used as a "baseline" for when endogenous estrogen and progesterone levels are the lowest in the natural menstrual cycle of premenopausal women, in line with current guidelines (13, 59). This was compared with phases of the cycle when hormone levels are elevated (e.g., LF = estrogen levels elevated; ML = estrogen and progesterone levels elevated). Through analysis of the studies identified in the systematic review, a medium effect of the menstrual cycle was identified on the macrovascular endothelial function during the LF phase when endogenous estradiol levels are elevated. However, subgroup analysis revealed that studies examining discrete diameter assessments (e.g., 60 s after cuff deflation) versus continuous diameter assessments (e.g., 3 min assessment to determine peak) found that studies using discrete assessments observed an increase across these phases, while those using continuous measures did not. Discrete diameter assessments do not account for the considerable interindividual variability in both the timing and response pattern that exists in FMD assessments. While several studies have not found menstrual cycle variation in the time to peak diameter, ranging from 35 to 40 s to ~100 s (14, 40, 42, 51), this analysis does not account for differences in the progression of dilation (i.e., diameter remaining elevated longer in one phase vs. another). Furthermore, subgroup analysis for endothe-

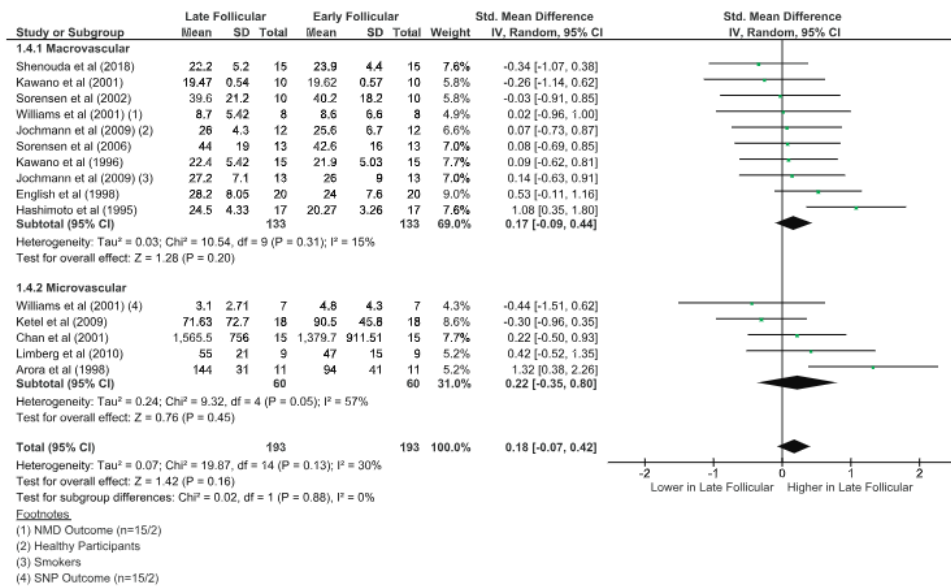H1334   IMPACT OF MENSTRUAL CYCLE ON VASCULAR FUNCTION

Fig. 5. Forest plot for smooth muscle function: early follicular vs. late follicular phases. CI, confidence intervals (95%); IV, inverse-variance method; I^2, measure of heterogeneity; SD, standard deviation; std. mean difference, standard mean difference.

lial function examining hormonal cycle assessments found that studies that used some (1–2 steps) of the criteria in the three-step method of hormonal cycle assessments were more likely to also observe an increase in FMD across phases, while assessments that used all three criteria were less likely to make these same observations (49). Given the progression of guidelines for methodologies, it is unsurprising that there was a significant negative relationship between the publication year and SMD in these phases, such that as methods became more refined, consistent, and precise over the past 25 years, SMD became smaller.

Analysis examining the luteal phases found that while there were no effects of phase on the EL or LL phases, there was a significant increase in macrovascular endothelial function in the ML phase for discrete, but not continuous, diameter assessments, similar to the LF phase. However, when sensitivity analysis was performed removing clinical populations, this increase was no longer present. While controversial, the lack of substantive changes in the luteal phase of the menstrual cycle may point to the antagonistic effects of progesterone on the small benefits attributed to estrogen alone, which have been previously discussed (15, 21). These results again point to minimal effects of the luteal phases of the menstrual cycle on the macrovascular function, particularly when differences in methodology and the health status of participants are also taken into consideration.

In contrast to the effects on the macrovasculature, there were no effects of the menstrual cycle on the microvascular function. It is possible that the vascular beds (macrovascular vs. microvascular) may be differentially susceptible to hormonal influence. Recent research by Jekell and colleagues (24) found weak relationships between macrovascular and microvascular endothelial function assessments in individuals with hypertension. This finding has been further supported by research in patients with rheumatoid arthritis, also demonstrating a lack of

relationship between macrovascular and microvascular function assessments (47). The authors consider that the lack of agreement between peripheral vascular assessments likely points to different regulatory mechanisms in each vascular bed (24, 47). Research examining the influence of vessel size on endothelial NO synthase (eNOS) protein content found that the content was the greatest in the larger conduit and resistance vessels, compared with smaller arterioles (30). Similarly, it has been previously supported through in vivo and in vitro research that estrogen modulates vascular endothelial and VSM functions through binding to estrogen receptors (ER), predominantly ERα, to exert both genomic and nongenomic effects linked to increased bioavailability of vasodilators, such as eNOS activation to increase NO production (7, 18, 37, 61, 66). Recently, one study identified that protein levels of ERs were higher in macrovascular than in microvascular endothelial cells (22). Taken altogether, macrovascular endothelial cells may be more susceptible to factors influencing NO bioavailability; however, further research is needed to explore the underlying mechanisms (e.g., ERα and β expressions and eNOS presence) potentially driving differences between the macrovascular and microvascular responses to the menstrual cycle.

As previously discussed, estrogen may modulate vascular function through estrogen receptors and influence the bioavailability of NO through eNOS activation (7, 18, 37, 61, 66). However, the meta-regressions from this review identified no relationships between the change in estradiol levels in the EF to LF or EF to ML phases and the SMD in vascular endothelial function between the same phases. It is possible that the significant heterogeneity in the SMD between studies may instead be explained by interindividual variability in ERα receptor expression, rather than the differences in estradiol levels, as previously proposed by Gavin and colleagues (17). This mechanistic hypothesis is supported by a strong relationship observed between

the downstream ERα receptor expression and endothelial function, as assessed by FMD (17). Further research is necessary to explore differences between participants in estradiol levels, ERα receptor expression, and its role on macrovascular and microvascular functions.

The accumulation of the identified small effect of the menstrual cycle on the macrovascular endothelial function may provide some support for the observations of reduced incidence of CVD in premenopausal women compared with age-matched men (44), and the loss in vasoprotection and subsequent increase in CVD observed following the menopausal transition (9, 26, 31, 43). Additionally, the findings of this meta-analysis may explain the discrepant findings of acute stimuli studies, which report both vasoprotection, or lack thereof, to acute stimuli (20, 34, 64). For example, research by Harris and colleagues (20) and Luca and colleagues (34) reported protection from a high-fat meal and ischemia-reperfusion injury, respectively, during the LF phase compared with the EF phase. However, these observations were not consistent with the research by Williams and colleagues (64) where there was no difference between EF and LF phases in the vascular response to an acute hyperglycemic insult. These studies, in combination with the findings of this review, support the idea that the small vasoprotective effect of elevated hormones during specific phases of the menstrual cycle may overcome the deleterious effect of some, but not all, acute stimuli. Further research exploring the influence of the menstrual cycle on vascular responses to acute stimuli is needed.

Recommendations for Researchers

Given the results of the subgroup analyses in this review, a discussion of best practice recommendations for researchers is warranted. While the menstrual cycle does not appear to have an effect on the microvascular endothelial function or VSM function, continued attention to examining the macrovascular endothelial function is needed. First, it is evident that differences in methodology—specifically examining the peak diameter at a discrete time point versus continuous assessment of diameter to identify the peak—may be, in part, responsible for phasic differences reported. Similarly, while cuff placement (proximal vs. distal) did not influence the results, this should be considered in future macrovascular endothelial function studies. Following of the current FMD guidelines may reduce heterogeneity between studies examining the effect of the menstrual cycle (60). Similarly, when assessments are performed across the menstrual cycle, using the three-step method of assessment (menstrual cycle tracking before testing, ovulation testing, and sex hormone assessment) may also reduce this heterogeneity (49). Utilization of these recommendations has been done in recent research by our group and others, where fluctuations across the cycle have not been observed when including all three methods of assessment and current FMD guidelines (34, 51, 64). Finally, this review highlights potential considerations for future guidelines, including standardizing menstrual cycle phases (e.g., LF and ML) and testing day(s) within each phase. For example, while some studies assessed the LF phase (when endogenous estrogen levels are elevated) as the 2–3 days preceding ovulation (1, 34, 65), while others characterized this phase as *days 7–14* of the menstrual cycle (50, 56, 62), which may have contributed to some of the heterogeneity observed. Similarly, researchers should continue to consider methods to decrease study bias, including randomization of phases tested and blinding of personnel to phases

during data collection and analysis, as cited in several studies (13, 52, 59).

Limitations

A limitation of this meta-analysis was the significant heterogeneity in both outcome variables and across all phases of the menstrual cycle assessed. While this heterogeneity was partially explained by differences in the vascular bed, heterogeneity remained high following subgroup analysis for FMD and menstrual cycle assessment methodologies. Similarly, heterogeneity was largely unexplained by baseline characteristics of participants (age, average menstrual cycle length, BMI, and blood pressure), as observed in the meta-regressions performed. Some of the heterogeneity may be explained by the variation between studies in how each phase of the menstrual cycle was assessed. The lack of standardization in menstrual cycle phase determination between studies may contribute significantly to the variability observed in the impact of phase on vascular function and is an important consideration for future directions, as detailed in the previous recommendations. Additionally, another limitation of this review was the poor certainty of evidence, identified through the GRADE criteria as "very low" for the majority of analysis, primarily a result of inconsistency and/or imprecision in studies analyzed. However, it is important to note that observational research, according to GRADE criteria, starts at a "low" certainty of evidence score, despite strong study design. Furthermore, quality analysis specific to observational research (SAQOR) was performed and assessed in all but one study with moderate or high-quality scores. Another limitation of this review was the lack of reporting of participant characteristics, especially information regarding fitness levels/exercise participation, supplementation with vitamins (e.g., vitamin C/E), and other medication use, which may have limited the ability to account for baseline characteristics to explain heterogeneity in meta-regressions. Finally, given that the majority of participants ($n = 1,302$) were healthy and young (28.9 ± 6.6 yr), this limited the generalizability of the findings of this review to predominantly healthy, premenopausal women and not high-risk populations with varying metabolic and cardiovascular conditions.

Conclusions and Future Directions

Based on the findings of this systematic review and meta-analysis, the menstrual cycle has a small effect on macrovascular endothelial function, as observed through an increase in SMD during the LF phase, but largely has no effect in the luteal phases. However, there is a "very low" certainty of evidence, and this small effect may be in part attributed to differences in FMD and hormonal assessment methodologies. Additionally, the menstrual cycle does not appear to influence VSM function or microvascular endothelial function. As a result, the effect of variations in hormone levels throughout the menstrual cycle may play a smaller role than previously proposed (21). Future research should continue to explore the influence of the menstrual cycle on vascular function under acute and chronic periods of vascular vulnerability: in acute cardiovascular and metabolic stimuli, and in older premenopausal participants, especially patients with varying metabolic disease states.

GRANTS

DISCLOSURES

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

AUTHOR CONTRIBUTIONS

J.S.W., E.C.D., and M.J.M. conceived and designed research; J.S.W. and E.C.D. analyzed data; J.S.W. and E.C.D. interpreted results of experiments; J.S.W. prepared figures; J.S.W. and E.C.D. drafted manuscript; J.S.W., E.C.D., and M.J.M. edited and revised manuscript; J.S.W., E.C.D., and M.J.M. approved final version of manuscript.

REFERENCES

1. **Adkisson EJ, Casey DP, Beck DT, Gurovich AN, Martin JS, Braith RW.** Central, peripheral and resistance arterial reactivity: fluctuates during the phases of the menstrual cycle. *Exp Biol Med (Maywood)* 235: 111–118, 2010. doi:10.1258/ebm.2009.009186.

2. **Arora S, Veves A, Caballaro AE, Smakowski P, LoGerfo FW.** Estrogen improves endothelial function. *J Vasc Surg* 27: 1141–1147, 1998. doi:10.1016/S0741-5214(98)70016-3.

3. **Bots SH, Peters SAE, Woodward M.** Sex differences in coronary heart disease and stroke mortality: a global assessment of the effect of ageing between 1980 and 2010. *BMJ Glob Health* 2: e000298, 2017. doi:10.1136/bmjgh-2017-000298.

4. **Bowyer L, Brown MA, Jones M.** Vascular reactivity in men and women of reproductive age. *Am J Obstet Gynecol* 185: 88–96, 2001. doi:10.1067/mob.2001.114502.

5. **Brandão HF, Serra PJ, Zanolla K, Cabral ACV, Geber S.** Variation of endothelial function during the menstrual cycle evaluated by flow-mediated dilatation of brachial artery. *Jornal Brasileiro de Reproducao Assistida* 18: 148–150, 2014. doi:10.5935/1518-0557.20140022.

6. **Bungum L, Kvernebo K, Oian P, Maltau JM.** Laser Doppler-recorded reactive hyperaemia in the forearm skin during the menstrual cycle. *Br J Obstet Gynaecol* 103: 70–75, 1996. doi:10.1111/j.1471-0528.1996.tb09517.x.

7. **Caulin-Glaser T, García-Cardeña G, Sarrel P, Sessa WC, Bender JR.** 17 beta-estradiol regulation of human endothelial cell basal nitric oxide release, independent of cytosolic Ca2+ mobilization. *Circ Res* 81: 885–892, 1997. doi:10.1161/01.RES.81.5.885.

8. **Celermajer DS, Sorensen KE, Gooch VM, Spiegelhalter DJ, Miller OI, Sullivan ID, Lloyd JK, Deanfield JE.** Non-invasive detection of endothelial dysfunction in children and adults at risk of atherosclerosis. *Lancet* 340: 1111–1115, 1992. doi:10.1016/0140-6736(92)93147-F.

9. **Celermajer DS, Sorensen KE, Spiegelhalter DJ, Georgakopoulos D, Robinson J, Deanfield JE.** Aging is associated with endothelial dysfunction in healthy men years before the age-related decline in women. *J Am Coll Cardiol* 24: 471–476, 1994. doi:10.1016/0735-1097(94)90305-0.

10. **Chan NN, MacAllister RJ, Colhoun HM, Vallance P, Hingorani AD.** Changes in endothelium-dependent vasodilatation and alpha-adrenergic responses in resistance vessels during the menstrual cycle in healthy women. *J Clin Endocrinol Metab* 86: 2499–2504, 2001.

11. **Clifton VL, Crompton R, Read MA, Gibson PG, Smith R, Wright IM.** Microvascular effects of corticotropin-releasing hormone in human skin vary in relation to estrogen concentration during the menstrual cycle. *J Endocrinol* 186: 69–76, 2005. doi:10.1677/joc.1.06030.

12. **Cohen J.** *Statistical Power Analysis for the Behavioral Sciences.* New York: Routledge, 1988.

13. **Corretti MC, Anderson TJ, Benjamin EJ, Celermajer D, Charbonneau F, Creager MA, Deanfield J, Drexler H, Gerhard-Herman M, Herrington D, Vallance P, Vita J, Vogel R; International Brachial Artery Reactivity Task Force.** Guidelines for the ultrasound assessment of endothelial-dependent flow-mediated vasodilation of the brachial artery: a report of the International Brachial Artery Reactivity Task Force. *J Am Coll Cardiol* 39: 257–265, 2002. doi:10.1016/S0735-1097(01)01746-6.

14. **D'Urzo KA, King TJ, Williams JS, Silvester MD, Pyke KE.** The impact of menstrual phase on brachial artery flow-mediated dilatation during

15. **English JL, Jacobs LO, Green G, Andrews TC.** Effect of the menstrual cycle on endothelium-dependent vasodilation of the brachial artery in normal young women. *Am J Cardiol* 82: 256–258, 1998.

16. **García-Fernández R, Pérez-Velasco JG, Concepción-Millán A, Sosa S, Navaroli F, García-Barreto D.** Estrogen does not prevent endothelial dysfunction caused by cigarette smoking. *Clin Cardiol* 27: 71–73, 2004. doi:10.1002/clc.4960270205.

17. **Gavin KM, Seals DR, Silver AE, Moreau KL.** Vascular endothelial estrogen receptor alpha is modulated by estrogen status and related to endothelial function and endothelial nitric oxide synthase in healthy women. *J Clin Endocrinol Metab* 94: 3513–3520, 2009. doi:10.1210/jc.2009-0278.

18. **Green DJ, Hopkins ND, Jones H, Thijssen DH, Eijsvogels TM, Yeap BB.** Sex differences in vascular endothelial function and health in humans: impacts of exercise. *Exp Physiol* 101: 230–242, 2016. doi:10.1113/EP085367.

19. **Harris RA, Nishiyama SK, Wray DW, Richardson RS.** Ultrasound assessment of flow-mediated dilation. *Hypertension* 55: 1075–1085, 2010. doi:10.1161/HYPERTENSIONAHA.110.150821.

20. **Harris RA, Tedjasaputra V, Zhao J, Richardson RS.** Premenopausal women exhibit an inherent protection of endothelial function following a high-fat meal. *Reprod Sci* 19: 221–228, 2012. doi:10.1177/1933719111418125.

21. **Hashimoto M, Akishita M, Eto M, Ishikawa M, Kozaki K, Toba K, Sagara Y, Taketani Y, Orimo H, Ouchi Y.** Modulation of endothelium-dependent flow-mediated dilatation of the brachial artery by sex and menstrual cycle. *Circulation* 92: 3431–3435, 1995. doi:10.1161/01.CIR.92.12.3431.

22. **Huxley VH, Kemp SS, Schramm C, Sieveking S, Bingaman S, Yu Y, Zaniletti I, Stockard K, Wang J.** Sex differences influencing micro- and macrovascular endothelial phenotype in vitro. *J Physiol* 596: 3929–3949, 2018. doi:10.1113/JP276048.

23. **Inaba Y, Chen JA, Bergmann SR.** Prediction of future cardiovascular outcomes by flow-mediated vasodilatation of brachial artery: a meta-analysis. *Int J Cardiovasc Imaging* 26: 631–640, 2010. doi:10.1007/s10554-010-9616-1.

24. **Jekell A, Kalani M, Kahan T.** The interrelation of endothelial function and microvascular reactivity in different vascular beds, and risk assessment in hypertension: results from the Doxazosin-ramipril study. *Heart Vessels* 34: 484–495, 2019. doi:10.1007/s00380-018-1265-7.

25. **Jochmann N, Müller S, Kuhn C, Gericke C, Baumann G, Stangl K, Stangl V.** Chronic smoking prevents amelioration of endothelial function in the course of the menstrual cycle. *Circ J* 73: 568–572, 2009. doi:10.1253/circj.CJ-08-0628.

26. **Kannel WB, Hjortland MC, McNamara PM, Gordon T.** Menopause and risk of cardiovascular disease: the Framingham study. *Ann Intern Med* 85: 447–452, 1976. doi:10.7326/0003-4819-85-4-447.

27. **Kawano H, Motoyama T, Kugiyama K, Hirashima O, Ohgushi M, Yoshimura M, Ogawa H, Okumura K, Yasue H.** Menstrual cyclic variation of endothelium-dependent vasodilation of the brachial artery: possible role of estrogen and nitric oxide. *Proc Assoc Am Physicians* 108: 473–480, 1996.

28. **Kawano H, Motoyama T, Ohgushi M, Kugiyama K, Ogawa H, Yasue H.** Menstrual cyclic variation of myocardial ischemia in premenopausal women with variant angina. *Ann Intern Med* 135: 977–981, 2001 [Erratum in *Ann Intern Med* 136: 253, 2002]. doi:10.7326/0003-4819-135-11-200112040-00009.

29. **Ketel IJ, Stehouwer CD, Serné EH, Poel DM, Groot L, Kager C, Hompes PG, Homburg R, Twisk JW, Smulders YM, Lambalk CB.** Microvascular function has no menstrual-cycle-dependent variation in healthy ovulatory women. *Microcirculation* 16: 714–724, 2009. doi:10.3109/10739680903199186.

30. **Laughlin MH, Turk JR, Schrage WG, Woodman CR, Price EM.** Influence of coronary artery diameter on eNOS protein content. *Am J Physiol Heart Circ Physiol* 284: H1307–H1312, 2003. doi:10.1152/ajpheart.00792.2002.

31. **Lieberman EH, Gerhard MD, Uehata A, Walsh BW, Selwyn AP, Ganz P, Yeung AC, Creager MA.** Estrogen improves endothelium-dependent, flow-mediated vasodilation in postmenopausal women. *Ann Intern Med* 121: 936–941, 1994. doi:10.7326/0003-4819-121-12-199412150-00005.

32. **Limberg JK, Eldridge MW, Proctor LT, Sebranek JJ, Schrage WG.** Alpha-adrenergic control of blood flow during exercise: effect of sex and menstrual phase. *J Appl Physiol (1985)* 109: 1360–1368, 2010. doi:10.1152/japplphysiol.00518.2010.

33. **Loader J, Montero D, Lorenzen C, Watts R, Méziat C, Reboul C, Stewart S, Walther G.** Acute hyperglycemia impairs vascular function in

healthy and cardiometabolic diseased subjects: systematic review and meta-analysis. *Arterioscler Thromb Vasc Biol* 35: 2060–2072, 2015. doi:10.1161/ATVBAHA.115.305530.

34. **Luca MC, Liuni A, Harvey P, Mak S, Parker JD.** Effects of estradiol on measurements of conduit artery endothelial function after ischemia and reperfusion in premenopausal women. *Can J Physiol Pharmacol* 94: 1304–1308, 2016. doi:10.1139/cjpp-2015-0589.

35. **Mattu AT, MacInnis MJ, Doyle-Baker PK, Murias JM.** Effects of the menstrual and oral contraceptive cycle phases on microvascular reperfusion. *Exp Physiol* 105: 184–191, 2020. doi:10.1113/EP088135.

36. **Mayrovitz HN, Brown-Cross D, Washington Z.** Skin tissue water and laser Doppler blood flow during a menstrual cycle. *Clin Physiol Funct Imaging* 27: 54–59, 2007. doi:10.1111/j.1475-097X.2007.00716.x.

37. **Miller VM, Duckles SP.** Vascular actions of estrogens: functional implications. *Pharmacol Rev* 60: 210–241, 2008. doi:10.1124/pr.107.08002.

38. **Moher D, Liberati A, Tetzlaff J, Altman DG; PRISMA Group.** Preferred reporting items for systematic reviews and meta-analyses: the PRISMA statement. *PLoS Med* 6: e1000097, 2009. doi:10.1371/journal.pmed.1000097.

39. **Montero D, Walther G, Diaz-Cañestro C, Pyke KE, Padilla J.** Microvascular dilator function in athletes: a systematic review and meta-analysis. *Med Sci Sports Exerc* 47: 1485–1494, 2015. doi:10.1249/MSS.0000000000000567.

40. **O'Brien MW, Johns JA, Al-Hinnawi A, Kimberly DS.** Popliteal flow-mediated dilatory responses to an acute bout of prolonged sitting between earlier and later phases of natural menstrual and oral contraceptive pill cycles. *J Appl Physiol (1985)* 129: 637–645, 2020. doi:10.1152/japplphysiol.00424.2020.

41. **Raitakari OT, Celermajer DS.** Flow-mediated dilatation. *Br J Clin Pharmacol* 50: 397–404, 2000. doi:10.1046/j.1365-2125.2000.00277.x.

42. **Rakobowchuk M, Parsloe ER, Gibbins SE, Harris E, Birch KM.** Prolonged low flow reduces reactive hyperemia and augments low flow mediated constriction in the brachial artery independent of the menstrual cycle. *PLoS One* 8: e55385, 2013. doi:10.1371/journal.pone.0055385.

43. **Reckelhoff JF, Fortepiani LA.** Novel mechanisms responsible for postmenopausal hypertension. *Hypertension* 43: 918–923, 2004. doi:10.1161/01.HYP.0000124670.03674.15.

44. **Rosenthal T, Oparil S.** Hypertension in women. *J Hum Hypertens* 14: 691–704, 2000. doi:10.1038/sj.jhh.1001095.

45. **Ross LE, Grigoriadis S, Mamisashvili L, Koren G, Steiner M, Dennis CL, Cheung A, Mousmanis P.** Quality assessment of observational studies in psychiatry: an example from perinatal psychiatric research. *Int J Methods Psychiatr Res* 20: 224–234, 2011. doi:10.1002/mpr.356.

46. **Rossi M, Di Maria C, Erba P, Galetta F, Carpi A, Santoro G.** Study of skin vasomotion during phollicular and luteal phase in young healthy women. *Clin Hemorheol Microcirc* 42: 107–115, 2009. doi:10.3233/CH-2009-1189.

47. **Sandoo A, Carroll D, Metsios GS, Kitas GD, Veldhuijzen van Zanten JJ.** The association between microvascular and macrovascular endothelial function in patients with rheumatoid arthritis: a cross-sectional study. *Arthritis Res Ther* 13: R99, 2011. doi:10.1186/ar3374.

48. **Saxena AR, Seely EW, Goldfine AB.** Cardiovascular risk factors and menstrual cycle phase in pre-menopausal women. *J Endocrinol Invest* 35: 715–719, 2012.

49. **Schaumberg MA, Jenkins DG, Janse de Jonge XAK, Emmerton LM, Skinner TL.** Three-step method for menstrual and oral contraceptive cycle verification. *J Sci Med Sport* 20: 965–969, 2017. doi:10.1016/j.jsams.2016.08.013.

50. **Schnabel RB, Biener MP, Wilde S, Sinning CR, Ojeda FM, Zeller T, Lubos E, Lackner KJ, Warnholtz A, Gori T, Espinola-Klein C, Blankenberg S, Munzel T, Wild PS.** Sex differences in noninvasive vascular function in the community. *J Hypertens* 31: 1437–1446, 2013. doi:10.1097/HJH.0b013e328360f755.

51. **Shenouda N, Priest SE, Rizzuto VI, MacDonald MJ.** Brachial artery endothelial function is stable across a menstrual and oral contraceptive pill cycle but lower in premenopausal women than in age-matched men. *Am J Physiol Heart Circ Physiol* 315: H366–H374, 2018. doi:10.1152/ajpheart.00102.2018.

52. **Sims ST, Heather AK.** Myths and methodologies: reducing scientific design ambiguity in studies comparing sexes and/or menstrual cycle phases. *Exp Physiol* 103: 1309–1317, 2018. doi:10.1113/EP086797.

53. **Sorensen MB, Collins P, Ong PJ, Webb CM, Hayward CS, Asbury EA, Gatehouse PD, Elkington AG, Yang GZ, Kubba A, Pennell DJ.** Long-term use of contraceptive depot medroxyprogesterone acetate in young women impairs arterial endothelial function assessed by cardiovascular magnetic resonance. *Circulation* 106: 1646–1651, 2002. doi:10.1161/01.CIR.0000030940.73167.4E.

54. **Sorensen MB, Franks S, Robertson C, Pennell DJ, Collins P.** Severe endothelial dysfunction in young women with polycystic ovary syndrome is only partially explained by known cardiovascular risk factors. *Clin Endocrinol (Oxf)* 65: 655–659, 2006. doi:10.1111/j.1365-2265.2006.02645.x.

55. **Sprung VS, Atkinson G, Cuthbertson DJ, Pugh CJ, Aziz N, Green DJ, Cable NT, Jones H.** Endothelial function measured using flow-mediated dilation in polycystic ovary syndrome: a meta-analysis of the observational studies. *Clin Endocrinol (Oxf)* 78: 438–446, 2013. doi:10.1111/j.1365-2265.2012.04490.x.

56. **Stamatelopoulos KS, Georgiopoulos G, Papaioannou T, Lambrinoudaki I, Kouzoupis A, Vlachopoulos C, Georgiou SP, Manios E, Alevizaki M, Papamichael CM, Sfikakis PP.** Can premenstrual syndrome affect arterial stiffness or blood pressure? *Atherosclerosis* 224: 170–176, 2012. doi:10.1016/j.atherosclerosis.2012.05.037.

57. **Stanhewicz AE, Wenner MM, Stachenfeld NS.** Sex differences in endothelial function important to vascular health and overall cardiovascular disease risk across the lifespan. *Am J Physiol Heart Circ Physiol* 315: H1569–H1588, 2018. doi:10.1152/ajpheart.00396.2018.

58. **Stanhewicz AE, Wong BJ.** Counterpoint: investigators should not control for menstrual cycle phase when performing studies of vascular control that include women. *J Appl Physiol (1985)* 129: 1117–1119, 2020. doi:10.1152/japplphysiol.00427.2020.

59. **Thijssen DH, Black MA, Pyke KE, Padilla J, Atkinson G, Harris RA, Parker B, Widlansky ME, Tschakovsky ME, Green DJ.** Assessment of flow-mediated dilation in humans: a methodological and physiological guideline. *Am J Physiol Heart Circ Physiol* 300: H2–H12, 2011. doi:10.1152/ajpheart.00471.2010.

60. **Thijssen DHJ, Bruno RM, van Mil ACCM, Holder SM, Faita F, Greyling A, Zock PL, Taddei S, Deanfield JE, Luscher T, Green DJ, Ghiadoni L.** Expert consensus and evidence-based recommendations for the assessment of flow-mediated dilation in humans. *Eur Heart J* 40: 2534–2547, 2019. doi:10.1093/eurheartj/chz350.

61. **Tostes RC, Nigro D, Fortes ZB, Carvalho MH.** Effects of estrogen on the vascular system. *Braz J Med Biol Res* 36: 1143–1158, 2003. doi:10.1590/s0100-879x2003000900002. 12937779

62. **Valtonen P, Punnonen K, Saarelainen H, Heiskanen N, Raitakari OT, Juonala M, Viikari JS, Alfthan G, Kähönen M, Laaksonen R, Lyyra-Laitinen T, Laitinen T, Heinonen S.** ADMA concentration changes across the menstrual cycle and during oral contraceptive use: the Cardiovascular Risk in Young Finns Study. *Eur J Endocrinol* 162: 259–265, 2010. doi:10.1530/EJE-09-0658.

63. **Wenner MM, Stachenfeld NS.** Point: investigators should control for menstrual cycle phase when performing studies of vascular control that include women. *J Appl Physiol (1985)* 129: 1114–1116, 2020. doi:10.1152/japplphysiol.00443.2020.

64. **Williams JS, Stimpson TV, Tremblay JC, Fenuta AM, Pyke KE.** The influence of acute hyperglycemia on brachial artery flow-mediated dilatation in the early and late follicular phases of the menstrual cycle. *Exp Physiol* 104: 957–966, 2019. doi:10.1113/EP087536.

65. **Williams MR, Westerman RA, Kingwell BA, Paige J, Blombery PA, Sudhir K, Komesaroff PA.** Variations in endothelial function and arterial compliance during the menstrual cycle. *J Clin Endocrinol Metab* 86: 5389–5395, 2001. doi:10.1210/jcem.86.11.8013.

66. **Xing D, Nozell S, Chen YF, Hage F, Oparil S.** Estrogen and mechanisms of vascular protection. *Arterioscler Thromb Vasc Biol* 29: 289–295, 2009. doi:10.1161/ATVBAHA.108.182279.

67. **Yeboah J, Folsom AR, Burke GL, Johnson C, Polak JF, Post W, Lima JA, Crouse JR, Herrington DM.** Predictive value of brachial flow-mediated dilation for incident cardiovascular events in a population-based study: the multi-ethnic study of atherosclerosis. *Circulation* 120: 502–509, 2009. doi:10.1161/CIRCULATIONAHA.109.864801.

68. **Yvonne-Tee GB, Rasool AH, Halim AS, Wong AR, Rahman AR.** Method optimization on the use of postocclusive hyperemia model to assess microvascular function. *Clin Hemorheol Microcirc* 38: 119–133, 2008.

CHAPTER 4: Influence of hormonal contraceptives on peripheral vascular function and structure in premenopausal females: a review.

Jennifer S. Williams, Maureen J. MacDonald

Supplemental Material from this Chapter can be found here:
https://doi.org/10.5683/SP2/VXJLGS

REVIEW

Integrative Cardiovascular Physiology and Pathophysiology

Influence of hormonal contraceptives on peripheral vascular function and structure in premenopausal females: a review

Jennifer S. Williams and Maureen J. MacDonald

Vascular Dynamics Laboratory, Department of Kinesiology, McMaster University, Hamilton, Ontario, Canada

Abstract

Hormonal contraceptives are one of the most widely used prescriptions for premenopausal women worldwide. Although the risk of venous and arterial cardiovascular events (e.g., deep vein thrombosis, arterial clotting) with hormonal contraceptives, specifically oral contraceptive pills, has been established, the literature on early risk indicators, such as peripheral vascular structure and function has yet to be consolidated. The purpose of this review is to summarize literature examining the impact of different hormonal contraceptives on vascular function and structure, including consideration of phasic differences within a contraceptive cycle, and to propose future directions for research. It is evident that hormonal contraceptive use appears to impact both macrovascular and microvascular endothelial function, with phasic differences in some contraceptive types dependent on progestin type, the ratio of ethinyl estradiol-to-progestin, and route of administration. However, hormonal contraceptives do not appear to impact smooth muscle function in the macrovasculature or microvasculature, arterial stiffness, or vascular structure. Underlying mechanisms for observed impacts and areas of future research are discussed. This review provides timely consolidation of research examining hormonal contraceptives and peripheral vascular function and structure and provides guidance on considerations for hormonal contraceptive use in study design.

endothelial function; macrovascular; microvascular; oral contraceptive pills; smooth muscle function

INTRODUCTION

Hormonal contraceptives, specifically oral contraceptive pills (OCPs), are one of the most widely used prescriptions for women between the ages of 15 and 49 worldwide, used by an estimated 151 million women (1). Approximately 9.1 million women in the United States, 13% of the population, use OCPs (2). Similarly, ~16% of Canadian women use OCPs, and the majority of women use a combination pill containing both ethinyl estradiol (EE) and a progestin (3); however, the use of OCPs is far more pervasive when factoring in lifetime use. In Australia, ~75% of women have used OCPs at some point in their lifetime (4). Similarly, ~80% of Swedish women are current or former users of OCPs (5). Across a woman's reproductive lifetime, many different types of OCPs and non-oral contraceptives may be used for a variety of purposes, including for the prevention of pregnancy, treating reproductive conditions, and relieving some symptoms of menopause (6–8). As a result, understanding the physiological effects of different types of hormonal contraceptives on the cardiovascular system at different stages of life is integral to women's health throughout the lifespan.

Over the course of the last 60 years, four generations of OCPs have been developed in a progressive effort to reduce adverse side effects. These generations differ through modifications in the dosage of EE and varying compositions of progestins. While progestins are similarly derived, each progestin has differing androgenicity (i.e., the ability to produce similar physiological responses as androgens, such as testosterone, dihydrotestosterone), with newer variants having low androgenicity or are anti-androgenic compared to older progestins (9). OCPs typically have a 28-day cycle formulation, involving a placebo or no-pill week, followed by 3 wk of either the same (monophasic) or increasing dosages (bi/triphasic) of synthetic hormones each week. Originally introduced in the 1960s, first-generation pills often contained the highest dosages of estrogen (150 µg estrogen mestranol) and progestins called "estranes", including norethindrone [acetate] or ethynodiol diacetate (10). In subsequent generations of OCPs, the dosage of EE was reduced to 50 µg, then to 20–35 µg, and the composition of the progestin was designed to reduce androgenicity (10). In the 1970s, second-generation pills were developed, containing "gonane" progestins, including levonorgestrel, or less commonly, norgestrel (10). In the 1990s, third-generation pills were developed, containing gonane progestins desogestrel or norgestimate, and, more recently, fourth-generation pills were developed containing drospirenone (10). In data reported from the Canada Health

Measures Survey in 2015, ~29% of women used second-generation OCPs (levonorgestrel), 44% used third-generation OCPs (norgestimate), and 16% used fourth-generation OCPs (drospirenone) (3). Another change in some pill formulations has been in the reduction of the 7-day placebo week to a shorter placebo week to reduce hormone withdrawal symptoms, often thereby extending the use of active pills by 2–5 days per cycle depending on the type of OCP (11, 12). Although the 7-day placebo week is still the most common formulation used and studied, the reduction of the placebo week in some formulations has important implications given the lack of follicular suppression and, thus, raised endogenous hormone levels in the 7-day placebo week versus shortened placebo week users (11, 12).

In addition to the development of different generations of OCPs, more recently, non-oral routes of contraceptives have been developed in an effort to increase adherence, and thus, effectiveness in pregnancy prevention, as well as to decrease side effects and increase affordability. These alternative contraceptives include the transdermal patch and vaginal ring, which function similarly to OCPs, by delivering 21 days of continuous synthetic estrogen and progesterone, followed by a 7-day withdrawal period. Longer-duration options for contraception include the intramuscular depot medroxyprogesterone acetate (DMPA) injection, which is injected intramuscularly into the upper arm and lasts ~12 wk, providing continuous progestin release. Lastly, hormonal intrauterine devices (IUDs), which provide a continuous low-dose release of progestin (levonorgestrel), are gaining prominence as a long-term hormonal contraceptive option lasting 3–7 years. In the United States, the proportion of women using long-term, reversible methods of contraception has doubled between 2008 and 2014, with likely increases with growing usage (13). Similarly, according to the US National Survey of Family Growth 2015–2017, just over 10% of women use long-acting reversible contraceptives (2). When determining the prescription of hormonal contraceptives, often arterial and thromboembolic risks are weighed alongside individual risk factors (e.g., smoking, obesity, and family history) in counselling women about contraceptives options. While controversial, there are data to suggest that cardiovascular risks are elevated in women taking OCPs, especially in those on first- and second-generation OCPs with higher estrogen dosages, individuals within their first year of use, and those older than 35 years old and/or smokers (14–16). OCP use has been associated with cardiovascular conditions, such as venous thrombosis, stroke, and myocardial infarction, despite the low-population attributable risk in young women (17, 18).

Given that hormonal contraceptives may influence the pathophysiology of vascular disease, determining their role in the early signs of vascular dysfunction and the associated structural vascular remodeling is key. The development of cardiovascular disease (CVD) is often preceded by atherosclerosis, stemming from alterations to the three layers of the artery that regulate vascular structure and function (19). Specifically, alterations in central and peripheral arterial stiffness of the tunica adventitia [e.g., pulse wave velocity (PWV), distensibility, and β-stiffness] (20), smooth muscle function of the tunica media (e.g., nitroglycerine-mediated dilation) (21), and endothelial function of the tunica intima (e.g., flow-mediated dilation) (22–24) have all been identified as early risk indicators in the progression of CVD (Fig. 1A) (20, 22). In examining research on early risk indicators, it is important to consider the clinically meaningful difference that increases in stiffness or impairments in vascular function can induce. For example, a recent meta-analysis found that for every 1% decrease in flow-mediated dilation, a measure of macrovascular endothelial function, there was a 13% (95% CI: 9%–17%) increase in the future risk of cardiovascular events (23). Similarly, an increase in central PWV, a measure of arterial stiffness, by 1 m/s is associated with a 15% (95% CI: 9–21%) increased risk of CVD mortality (25). Therefore, when examining vascular function and structure with hormonal contraceptive use, it is important to consider the clinical relevance of statistically significant differences in this review.

Some of the complexity associated with summarizing the available research in this area stems from the complexity of the contraceptives themselves. There are ~35 unique hormonal contraceptive options, each with their own synthetic hormone make-up and route of administration (e.g., oral, transdermal, vaginal, intramuscular); however, the majority of options are OCPs (10, 26). As a result, researchers have often grouped together results from women who use hormonal contraceptives, characterizing them as a single cohort in comparison to a naturally cycling comparator group (27–30). However, recent research has identified that the synthetic hormone make-up of contraceptives and their route of administration may impact early risk indicators for CVD (31–33). Additionally, one of the previously cited barriers to including women in cardiovascular research studies is the perceived complexity of controlling for the hormonal cycle phase (34). In considering study participants who use hormonal contraceptives, the question of whether to control for phase (e.g., placebo vs. active hormone phase; Fig. 1B) in contraceptives that have fluctuating hormone levels is unclear. While methodological recommendations have been made for examining or controlling for phasic differences in OCP, patch, and ring use, specifically in exercise science (35), further discussion is necessary, especially given the exponential increase in research examining sex differences and sex-specific research in women (36–38).

Therefore, the purpose of this review is threefold: 1) to summarize literature examining the impact of different generations of OCPs and non-oral contraceptives on vascular function and structure; 2) to clarify the impact of phasic differences in hormonal contraceptives—primarily oral contraceptives—on vascular function and structure; and 3) to propose future directions for research and provide guidance on the inclusion of women who use hormonal contraceptives in cardiovascular research studies, given the recent calls for a focus in this area (35, 39, 40).

INFLUENCE OF HORMONAL CONTRACEPTIVES ON ENDOTHELIAL FUNCTION

Macrovascular endothelial function is commonly assessed using a standard reactive hyperemia flow-mediated dilation (FMD) test, whereby a pneumatic cuff is inflated to supersystolic pressures (≥200 mmHg) for a period of 5 min and then
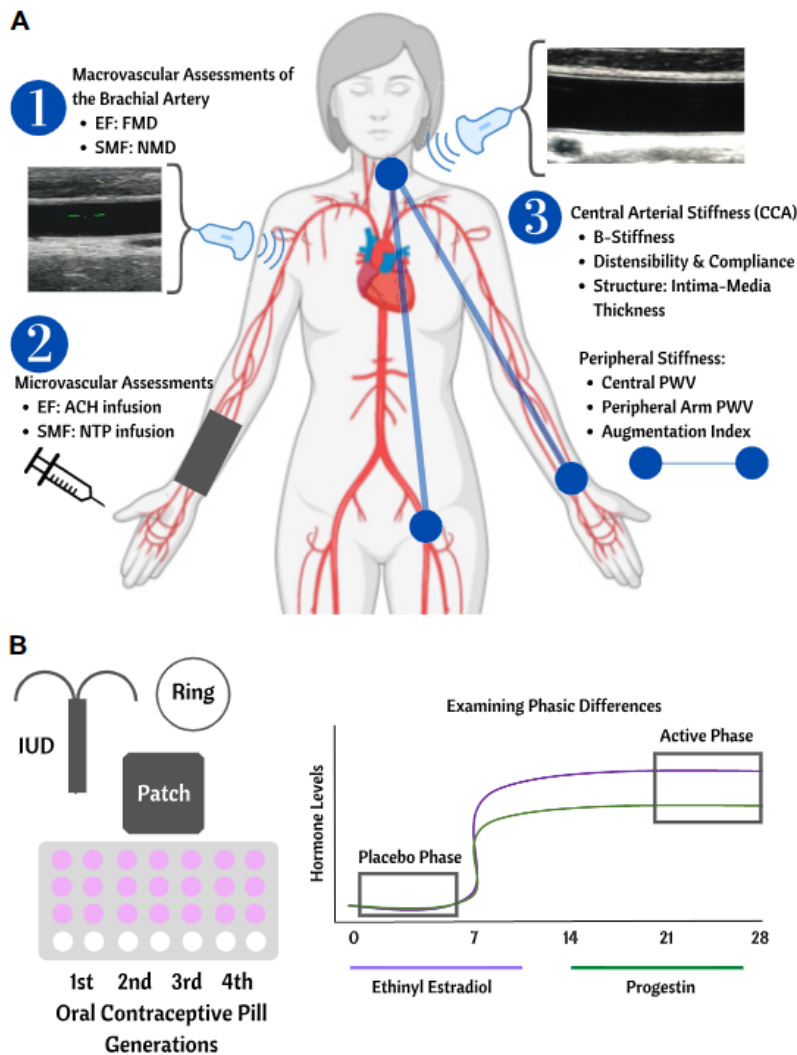
Figure 1. Methods assessing the influence of hormonal contraceptives (i.e., oral contraceptive pills, transdermal patch, vaginal ring, and intrauterine device) on vascular function and structure. *A*: methods commonly used for assessing the influence of hormonal contraceptives on vascular function and structure in humans. *1*) Macrovascular endothelial function (EF) assessment can utilize brachial artery flow-mediated dilation (FMD), while smooth muscle function (SMF) can use brachial artery nitroglycerine-mediated dilation (NMD). *2*) Microvascular EF assessment may involve acetylcholine (ACh)-induced dilation, and nitroprusside (NTP)-induced dilation for SMF. *3*) Arterial stiffness methods include the examination of the common carotid artery (CCA) β-stiffness, distensibility and compliance, and arterial structure: intima-media thickness. Peripherally, arterial stiffness is commonly assessed using pulse wave velocity (PWV) centrally at the carotid to femoral arteries, and peripherally in the arm from the carotid to radial arteries. Augmentation index is also used as an indicator of arterial stiffness. *B*: common hormonal contraceptive methods discussed in this review include four generations of oral contraceptive pills, transdermal patch, vaginal ring, and intrauterine device. When examining phasic differences, studies often examine differences between the placebo (no hormone) phase and during the last week of the active phase. The graphics used were developed on open-source platforms Canva.com and Biorender.com

deflated (41). Measurements of the proximal artery, often the brachial artery, are assessed using Doppler ultrasound before (baseline) and in the 3 min following cuff deflation, with the difference between baseline and peak diameter of the artery represented as a percentage change relative to baseline diameter (%FMD; Fig. 1) (41). Microvascular endothelial function is commonly assessed through either infusion or transdermal application of acetylcholine—an endothelium-specific vasodilator—and subsequent detection of changes in microvascular blood flow in the distal limb through laser-Doppler flowmetry or venous plethysmography (ACh-mediated dilation; Fig. 1) (42). As detailed below, hormonal contraceptives appear to impair endothelial function in some contraceptive options (OCP second generation, DMPA intramuscular injection), but not others (OCP fourth generation

and IUDs), which may be attributable to the progestin type, dosage, and/or route of administration (Table 1 and Supplemental Table S1, https://doi.org/10.5683/SP2/VXJLGS; all supplemental tables may be found at this site). Similarly, there are phasic differences in the impact of hormonal contraceptives on endothelial function, which appear to depend on both the ratio of EE dose to progestin dose and the route of administration (Table 1).

Macrovascular Endothelial Function

Early cross-sectional research on OCP use found no difference in %FMD between OCP users and naturally cycling controls; however, this study did not consider the differences in OCP type and dosage in this conclusion (29). Indeed, recent cross-sectional and intervention studies have found that

Table 1. *Summary of the impact of hormonal contraceptive use and phasic differences on macrovascular and micro-vascular endothelial function*

	Impact of HC Use on Endothelial Function	Impact of Phase on Endothelial Function(Placebo → Active Phase)
Combined EE/Progestin Contraceptive Options		
Second-generation OCPs (levonorgestrel) VLD: 20 mcg EE/100 mcg LNG LD: 30 mcg EE/150 mcg LNG	↓ %FMD [vs. self 6 mo (43), fourth-generation users (44), controls (45, 46)]	VLD: ↓ %FMD (33) LD: ↔ %FMD (32,33) LD: ↔ACh (32)
Third-generation OCPs (desogestrel) VLD: 20 mcg EE/150 mcg DSG LD: 30 mcg EE/150 mcg DSG	↔ACh [vs. self 6 mo (47)]	VDL: ↔ %FMD (31) LD: ↑ %FMD (31,32) LD: ↑ACh (32)
Fourth-generation OCPs (DRP, CMA)	↔ %FMD [vs. self 6 mo (43, 48)]	↑ %FMD (32, 49)↑ACh (32)
Ring (2.7 mg EE/11.7 mg etonogestrel – third generation)		↑ %FMD (50)
Progestin-Only Contraceptive Options		
DMPA intramuscular injection (containing first- generation pro-gestin, medroxyprogesterone acetate)	↓ %FMD [vs. controls (46, 51)]	↔ %FMD (48-h postinjection (51), across 9 wk postinjection (52))
IUD (containing second- generation progestin, levonorgestrel)	↔ %FMD [vs. self 3, 6, and 12 mo (53)]	
Progestin-only pills ("mini-pill")		

ACh, acetylcholine-mediated vasodilation (microvascular); CMA, chlormadinone acetate; DRP, drospirenone; DMPA, depot medroxy-progesterone acetate; DSG, desogestrel; EE, ethinyl estradiol; HC, hormonal contraceptive; IUD, intrauterine device; LD, low dose; LNG, levonorgestrel; OCP, oral contraceptive pill; %FMD, percent flow-mediated dilation (macrovascular); VLD, very low dose.

hormonal contraceptives appear to impair macrovascular endothelial function in second-generation OCP and DMPA intramuscular injection users, but not in fourth-generation OCP or IUD users. Research by Heidarzadeh et al. (45) observed lower %FMD in OCP users with the progestin levonorgestrel (second generation) than in naturally cycling controls (45). This finding has been confirmed in another cross-sectional study (46) and an intervention study following 6 mo of second-generation OCP use (43). In contrast, interventional research studies have found no change in %FMD following 6 mo of OCP use with the progestin drospirenone or chlormadinone acetate (both fourth generation) (43, 48). Few studies have compared contraceptive types; however, an early cross-sectional study by Friedman et al. (44) found that those using OCPs which contain estrane or gonane progestins (first and second generation) had lower %FMD than users of OCPs with drospirenone (fourth generation). Altogether, it has been well established that second- generation OCP use impairs endothelial function (FMD) in comparison to naturally cycling controls and later generations of OCPs.

The long-term effects of OCP use have been further supported by correlational research which found that the type of progestin in an OCP is an independent predictor of the FMD response; however, the researchers suggest that age may have been a confounder (44). In contrast, recent research from our laboratory by Shenouda et al. (54) found a negative correlation between duration of use and %FMD in second-generation OCPs, but not in third- and fourth-generation users, even after controlling for age. However, this finding is not completely supported by research by Heidarzadeh et al. (45), who found a nonsignificant negative relationship between %FMD and duration of use in second-generation OCP users, despite observing impaired function in OCP users versus naturally cycling controls. Although there is some evidence of a relationship between long-term OCP use and

endothelial dysfunction, further longitudinal research is necessary.

Exploring non-oral routes of administration, it is apparent that DMPA intramuscular injection use reduces, while IUD use does not appear to impact, endothelial function. Researchers found that DMPA users had lower %FMD than naturally cycling controls (46, 51) and a similar level of impairment as second-generation OCP users (46). Collectively, it appears that the chronic use of DMPA, a high-dose progestin-only, non-oral contraceptive (150 mg injection every 12 wk), has pronounced adverse effects on the vasculature long term (46, 51).

On the basis of the research presented by Lizarelli et al. (46) above, it could be hypothesized that IUD use would also result in impairment in endothelial function, given that second-generation OCP users had lower %FMD than controls, and IUDs use the same second-generation progestin (levonorgestrel) in its long-acting, non-oral composition. Contrary to this hypothesis, one study by Selim and Hussein (53) observed no change in %FMD with IUD use at 3, 6, or 12 mo postinsertion. This may be a result of differences in the route of administration (oral vs. vaginal) or the balance of estrogen and progesterone derivatives. For example, this study found that endogenous estradiol levels in blood remained the same with IUD use, while endogenous progesterone levels were reduced to compensate for increased exogenous progestin (levonorgestrel) levels. In contrast, OCP use dramatically reduces both endogenous estrogen and progesterone levels as they are replaced by exogenous hormones. The differential effects of exogenous EE and endogenous 17β estradiol on endothelial function may be responsible for differences observed in these contraceptive methods containing levonorgestrel (second-generation OCP vs. IUD). Similarly, OCP and DMPA use results in significantly higher progestin levels in the blood, compared to IUD use, which may be a contributing factor.

With respect to hormonal contraceptive phase and macrovascular endothelial function, early cross-sectional research discussed above found no differences in %FMD across four time points in the contraceptive cycle (29). In addition, research from our group by Shenouda et al. (54) has also observed no change in %FMD across a pill cycle. Similar findings have also been observed recently by O'Brien et al. (55). However, as these studies grouped OCP users together, it is possible that the types of contraceptives that participants were using may have masked phasic differences. For example, in the study by Shenouda et al. (54), approximately half of the participants were on a second-generation, very low-dose contraceptive [decreases %FMD, according to one study (33)], while the other half were on a low-dose third-generation [increases %FMD (31, 32)] or fourth-generation, low-dose OCP (increases %FMD), detailed further in the studies below (32).

Exploring second-generation OCPs (progestin = levonorgestrel), research by Torgrimson et al. (33) found no phasic differences in low-dose users (30 µg EE/150 µg LNG) but found a decrease in %FMD across phases in very low dose users (20 µg EE/100 µg LNG). This has been further supported by research by Thompson et al. (32), who also found no change in %FMD in second-generation users, noting that the participants were using low-dose OCPs. Similarly, research by Meendering et al. (31) observed increased %FMD in third-generation users with a low-dose composition [30 µg EE/150 µg desogestrel (DSG)], but no change in very low dose third-generation OCPs (20 µg EE/150 µg DSG), which has a 10 µg lower dosage of EE but the same amount of progestin. In a subset study of very low dose users, during the placebo pill phase, the provision of EE (10 µg) resulted in elevated %FMD compared to both the active and placebo phases. These findings provide evidence that EE may counteract the effects of progestin and improve endothelial function. Furthermore, research from Thompson et al. (32) identified increased %FMD in third- and fourth-generation users on low-dose (30 µg EE/150 µg DSG or 3 mg drospirenone) OCP composition in the active phase compared to the placebo phase. This observation has been supported by findings in fourth-generation OCP users with improved %FMD (49). The existing research suggests that progestins counteract the beneficial effects of EE alone, with some progestins having a more potent effect than others (e.g., levonorgestrel > drospirenone, desogestrel), hence, the phase differences between OCP generations and dosage levels. Therefore, the balance between EE and progestin type in the contraceptive is integral to understanding the effects on the endothelium.

While research on the acute effects of non-orally administered contraceptives on endothelial function is limited, some early research by Sorensen et al. (51) found that while %FMD was reduced in DMPA users compared with naturally cycling controls, there was no change in %FMD acutely (48 h) postinjection. Similarly, more recent work by Torgrimson et al. (52) has also found no change in %FMD across a 9-wk period with DMPA users. In contrast to the lack of phasic differences observed with the DMPA injection, research by Torgrimson et al. (50) has found an increase in %FMD in the active phase of using the vaginal ring. The ring uses a third-generation progestin, which in OCPs has been shown to increase %FMD (31, 32), with a much higher

ratio of EE:progestin than an OCP, which may explain this result, in line with prior studies.

Interestingly, research from the Minson group has further explored the independent effects of progestin and E₂ supplementation. In a study by Meendering et al. (56), participants were first put on a gonadotropin-releasing hormone antagonist (GnRHa) for 4 days to suppress endogenous sex hormone production. Following this period, participants took transdermal estradiol (0.1 mg/day) daily for 3 days, and then estradiol + progestin medroxyprogesterone acetate (MPA; 5 mg/day) daily for 3 days (56). The researchers found that %FMD was increased in the transdermal estradiol condition but was reduced back to GnRHa suppression levels following the provision of MPA (56). Follow-up research by the same group found that DMPA + E₂ supplementation through a 0.1-mg transdermal patch, 0.1-mg vaginal ring, or 2× daily 1-mg oral dosage all increased %FMD compared with DMPA alone (52). Furthermore, these researchers found that E₂ supplementation via the vaginal ring resulted in higher %FMD compared to E₂ supplementation orally (52). Estrogen supplementation appears to improve endothelial function, which is counteracted by the effects of progestin and depends, in part, by route of administration.

Overall, hormonal contraceptive use impairs endothelial function, as assessed by FMD, in second-generation OCP and DMPA intramuscular injection users, but not in fourth-generation OCP or IUD users. Similarly, there are phasic differences in the impact of hormonal contraceptives on FMD. Second-generation OCPs may either impair or not impact FMD, dependent on dosage level (very low vs. low dose), while third- and fourth-generation OCPs improve FMD.

Microvascular Endothelial Function

The microvasculature may respond differently to external stimuli than the macrovasculature (57, 58), and, as such, examining the literature on microvascular function separate from the macrovasculature will aid in understanding how hormonal contraceptives may differentially affect these vascular beds. Results from cross-sectional and interventional studies are mixed with respect to the effects of OCP use on ACh-mediated vasodilation. For example, two studies have observed no difference in ACh-mediated dilation with OCP use versus naturally cycling controls (OCP generation not specified) (59) or after 6 mo of use in third-generation OCP users (47). Similarly, a study by John et al. (60) cited no change in ACh-mediated dilation in OCP users (second and third generation) versus naturally cycling controls; however, there was a nonsignificant increase in dilation, which may have been underpowered. Research by Limberg et al. (28) observed that ACh-mediated vasodilation was elevated in OCP users compared with naturally cycling controls; however, OCP type was not disclosed. Therefore, it is likely that the discrepancies in the literature are a result of differences in OCP types; however, minimal studies have been conducted detailing information about OCP generation.

Exploring phasic differences, a study by Murphy et al. (59) did not observe differences across an OCP cycle; however, this study did not provide a description of OCP composition. In contrast, research by Thompson et al. (32) stratified on the basis of OCP generation found increased ACh-mediated

Table 2. *Summary of the impact of hormonal contraceptive use and phasic differences on macrovascular and microvascular smooth muscle function*

	Impact of HC Use on Smooth Muscle Function	Impact of Phase on Smooth Muscle Function (Placebo → Active Phase)
Combined EE/Progestin Contraceptive Options		
Second-generation OCPs (levonorgestrel) VLD: 20 mcg EE/100 mcg LNG LD: 30 mcg EE/150 mcg LNG		VLD: ↔ %NMD (33) LD: ↔ %NMD (33) ↔ NTP (32)
Third-generation OCPs (desogestrel) VLD: 20 mcg EE/150 mcg DSG LD: 30 mcg EE/150 mcg DSG		VLD: ↔ %NMD (31) LD: ↔ %NMD (31) ↔ NTP (32, 47)
Fourth-generation OCPs (DRP, CMA)	↔ %NMD [vs. self 6 mo (48)]	↔ NMD (49) ↔ NTP (32)
Ring (2.7 mg EE/11.7 mg etonogestrel – third generation)		↔ %NMD (50)
Progestin-Only Contraceptive Options		
DMPA intramuscular injection (containing first-generation progestin, medroxyprogesterone acetate)	↔ %NMD [vs. controls (51)]	↔ %NMD [48 h postinjection (51); across weeks of use (52)]
IUD (containing second-generation progestin, levonorgestrel)	↔ %NMD [vs. self 3, 6, and 12 mo (53)]	
Progestin-only pills ("mini-pill")		

CMA, chlormadinone acetate; DMPA, depot medroxyprogesterone acetate; DRP, drospirenone; DSG, desogestrel; EE, ethinyl estradiol; HC, hormonal contraceptive; IUD, intrauterine device; LD, low dose; LNG, levonorgestrel; %NMD, percent nitroglycerine-mediated dilation (macrovascular); OCP, oral contraceptive pill; NTP, nitroprusside-mediated dilation (microvascular); VLD, very low dose.

dilation in third- and fourth- generation OCP active phases versus placebo phases, with no phasic differences observed in second-generation users. It is possible that the discrepancies between these two studies are a result of the Murphy et al. (59) study including only participants using second-generation OCPs. This is further supported by recent work by Mattu et al. (61), who found no phasic differences in microvascular function in primarily second-generation OCP users. Overall, there are mixed results as to the impact of hormonal contraceptive use on microvascular endothelial function, with studies identifying either no change or improvements with OCP use. While phasic differences have been observed, with increased ACh-mediated dilation in third- and fourth-generation OCP users but not second-generation users across the hormonal cycle, further research is needed.

INFLUENCE OF HORMONAL CONTRACEPTIVES ON SMOOTH MUSCLE FUNCTION

Smooth muscle function can be assessed using methods to increase nitric oxide (NO) bioavailability, thereby increasing endothelium-independent dilation of both the macrovasculature and microvasculature. For macrovascular assessments of brachial artery smooth muscle function, 0.4 mg of nitroglycerine is typically sprayed sublingually, and brachial artery diameter measurements are acquired repeatedly using a Doppler ultrasound in the 10 min following administration (31, 33, 48, 50–54, 56). The difference in diameter between baseline and peak diameter in response to nitroglycerine administration is represented as a percent change from baseline (% nitroglycerine-mediated dilation, %NMD; Fig. 1). For microvascular assessments, nitroprusside is injected arterially (28, 47, 60) or is applied locally (32, 59), once or in graded dosages, and forearm blood flow is detected using strain gauge plethysmography or laser Doppler (Fig. 1). As detailed below, hormonal contraceptive use does not appear to impact smooth muscle function in either the macrovasculature or microvasculature (Table 2; Supplemental Table S2). Additionally, there do not appear to be any phasic differences in smooth muscle function with contraceptive use.

Macrovascular Smooth Muscle Function

While the majority of research on macrovascular smooth muscle function has focused on phasic differences and acute models of exogenous supplementation, detailed further in this section, two studies have examined the "longer-term" effects (<1 year) of contraceptive use in an interventional study design (48, 53). Researchers have found no impact of OCP use with the progestin drospirenone (fourth generation) following 6 mo of use, or IUD use with levonorgestrel (second generation)-based or copper-based following 3, 6, and 12 mo of use on smooth muscle function (48, 53). Furthermore, research by our group using a correlational design to examine the long-term effects of OCP use on smooth muscle function found no relationship between duration of OCP use and %NMD in a group of second-, third-, and fourth- generation OCP users (63). These studies provide support for the conclusion that hormonal contraceptive use appears not to influence macrovascular smooth muscle function.

Phasic differences in macrovascular smooth muscle function have been fairly well studied, including research on OCPs stratified by generation type, the DMPA intramuscular injection, and the vaginal ring (31, 33, 50, 51, 54). Researchers have found no phasic differences in smooth muscle function in second- and third-generation OCP users on very low or low-dose formulations, or in fourth-generation users (31, 33, 49). Further work from our laboratory has confirmed these findings, observing no phasic differences in smooth muscle function in OCP users on varying second-, third-, and fourth-

81

generation progestins (54). Exploring non-oral routes of administration, researchers have also found no differences in smooth muscle function 48 h postinjection of DMPA or across vaginal ring contraceptive phases (50, 51).

Well-designed research from the Minson group examined the independent roles of exogenous sex hormones and their route of administration on smooth muscle function in two recent studies detailed in full earlier (52, 56). Briefly, researchers examined *1*) the impact of endogenous suppression (via GnRHa) and the addition of exogenous estradiol and estradiol + MPA (40) and *2*) the impact of estradiol supplementation via transdermal patch, vaginal ring, or oral pill in DMPA users on smooth muscle function (52). With both interventions, smooth muscle function was unchanged (52, 56). Overall, it is evident that macrovascular smooth muscle function appears to be unaffected by hormonal contraceptive use, including phasic differences.

Microvascular Smooth Muscle Function

There has been minimal research conducted examining the effect of hormonal contraceptive use on smooth muscle function in the microvasculature. In the available studies, no impact on smooth muscle function was observed (32, 47, 59, 60) in all but one study (28). Research by Thompson et al. (32) observed no phasic differences in OCP users with levonorgestrel (second generation), desogestrel (third generation), or drospirenone (fourth generation) progestins. Furthermore, exploring differences in OCP users versus naturally cycling controls, early research by John et al. (60) found no difference between OCP users (second and third generation) and controls in smooth muscle function response to an infusion of nitroprusside. The same finding was confirmed by Murphy et al. [(59) no description of OCP types] and Virdis et al. (47) in third-generation OCP users, compared with naturally cycling controls.

However, recent research by Limberg et al. (28) found that smooth muscle function was enhanced in a dose-dependent manner, such that the highest dosages of nitroprusside were significantly different between OCP users and naturally cycling controls. Interestingly, Limberg et al. (28) proposed that the differences between studies may be attributable to differences in day of the hormonal cycle studied: *days 1–5* in this study when hormone levels were lower in their study, compared with *day 12* (60), the follicular phase (47), or the active pill phase (59) when hormone levels are elevated. The authors also propose that given findings by John et al. (60) observing reduced forearm blood flow in OCP users versus naturally cycling controls in response to an inhibitor of nitric oxide synthase (L-NMMA), it is possible that smooth muscle of OCP users is more responsive than smooth muscle of controls (28, 60). However, the dosages of nitroprusside were substantially different between studies, with the level at which significant differences were observed between OCP users and controls being at an infusion rate nearly 3.5 times that of the previous study, which found no differences (60). Using the 0.5 µg/100 g/min (~2,945 ng/min and ~2,775 ng/min infusion in OCP users and controls, respectively) infusion rate from this study (28) and comparing this to the highest infusion rate in the study by John et al. (60), which is 3,200 ng/min, the findings are comparable: no difference between groups. As a result, it is plausible that OCP use improves microvascular smooth muscle function, but only when assessed at supraphysiological levels of nitroprusside infusion. Overall, there appears to be no impact of hormonal contraceptives on macrovascular and microvascular smooth muscle function, including no impact of phasic differences within a hormonal contraceptive cycle.

INFLUENCE OF HORMONAL CONTRACEPTIVES ON ARTERIAL STIFFNESS

Local arterial stiffness of the common carotid artery (CCA), including assessments of β-stiffness, distensibility, and compliance, and peripheral arterial stiffness, including assessments of central (carotid-femoral) and peripheral (carotid-radial) PWV and augmentation index, are often used as early indicators in the atherosclerotic progression to CVD (Fig. 1) (25, 62). Importantly, increased arterial stiffness is an independent predictor of CVD progression (25).

There is limited research on the impact of hormonal contraceptive use on arterial stiffness. Examining local stiffness of the CCA, research by Franceschini et al. (43) observed no impact of second- or fourth-generation OCP use on CCA β-stiffness. Similarly, Lizarelli et al. (46) also observed no impact of second-generation OCP use or DMPA injection on CCA β-stiffness or distensibility. Examining peripheral stiffness, a cross-sectional study found no difference in augmentation index but a small increase in central PWV in OCP users compared with naturally cycling controls (27). However, it is important to note that the statistically significant increase in central PWV was $+0.1 \pm 0.7$ m/s, which is unlikely to be clinically meaningful, as a 1 m/s difference has been associated with a 15% increase in CV mortality (25, 27). The type of OCP and duration of use were not published, and the contraceptive phase was not controlled for; these critiques have been highlighted in-depth in a recent editorial regarding this study (63). Furthermore, research from our group by Priest et al. (64) assessed CCA β-stiffness and central PWV across an OCP cycle (second-, third-, and fourth-generation OCPs) and natural menstrual cycle in a group of women and did not observe differences between OCP users and naturally cycling controls. This study also found no relationship between OCP duration of use and β-stiffness or central PWV, indicating that OCP use may not have a long-term effect on arterial stiffness (64).

Examining hormonal contraceptive phasic differences on arterial stiffness, researchers have observed no differences in central and peripheral arm PWV, augmentation index, and CCA β-stiffness (Supplemental Table S3) (30, 32, 64). Research by Thompson et al. (32) used a digital volume pulse device to determine a measure of stiffness and reflection of pulse waveforms as an assessment of global stiffness in the arterial tree in four groups of participants on OCPs containing levonorgestrel (second generation), desogestrel (third generation), and drospirenone (fourth generation), and naturally cycling controls (32). Researchers observed no phasic differences in stiffness, assessed in the placebo and active pill phases of the OCP cycle (32). Similarly, research by Priest et al. (64) detailed earlier performed subgroup analysis on second-generation versus third- and fourth-generation OCP users, finding again no phasic differences in assessed CCA

β-stiffness and central PWV. This is supported by research by Yu et al. (30), who assessed central and peripheral arm PWV and augmentation index in participants who were on varying OCP formulations (30). Although the analysis was not separated by OCP type, researchers found no phasic differences in any stiffness measurement (30). Overall, there appears to be no major differences between hormonal contraceptive types or across phases within a hormonal contraceptive cycle in measures of arterial stiffness.

INFLUENCE OF HORMONAL CONTRACEPTIVES ON ARTERIAL STRUCTURE

Consistent with alterations in arterial stiffness of the CCA, structural changes, specifically the thickening of the intima-media (IMT), is associated with the development of atherosclerosis and is an independent predictor of CVD and cerebrovascular conditions. A meta-analysis found that a 0.1-mm increase in IMT was associated with a 12–17% increased risk of myocardial infarction and an 18–21% increased risk of stroke (65).

Research on the impact of hormonal contraceptives on arterial structure is limited, and there are opposing results from the three existing studies (Supplemental Table S3). Research by Heidarzadeh et al. (45) found that IMT was elevated in second-generation OCP users, who had been using OCPs for an average of 4.5 yr. This study also observed a nonsignificant positive relationship between IMT and duration of OCP use (45). However, researchers commented that 4.5 yr may not be sufficient enough of a range to examine long-term effects, and, therefore, further longitudinal research studies are needed (45).

Similarly, interventional research by Franceschini et al. (43) assessed females following 6 mo of OCP use, during their active pill phase, on either an OCP with levonorgestrel (second generation) or chlormadinone acetate (fourth generation), and found an approximate 10% increase in IMT in the second-generation OCP group only. In contrast, earlier research by the same laboratory group also assessed females on a second-generation OCP with levonorgestrel, compared with a progestin-only injection group and a naturally cycling control group, finding no difference between groups for CCA IMT (46). Contraceptive users in this study were of similar age and health status as the prior two studies, and they had been using their respective contraceptives for ~3 years (46). Therefore, it is unclear why there is an apparent difference between study conclusions, and further research is necessary to elucidate the impact of second-generation OCP use on CCA IMT. Taken altogether, hormonal contraceptive use may impact arterial structure of second-generation OCP users (elevated CCA IMT) to a small degree compared to naturally cycling controls (45), present after only 6 mo of use (43).

PROPOSED UNDERLYING MECHANISMS

This review has highlighted that hormonal contraceptives appear to have long-term and shorter-term, phasic impacts on endothelial cells, but not on surrounding vascular smooth muscle or structural connective tissues. Specifically, endothelial function appears to be depressed in second-generation OCP and DMPA intramuscular injection users but is preserved in fourth- generation and IUD users. Similarly, there are phasic differences in endothelial function, with impaired or unaltered function in second-generation OCP users (impaired in lower dosage of EE), unaltered or improved function in third-generation OCP users (unaltered in lower dosage of EE) and improved function in fourth-generation OCP users. There exists limited research on underlying mechanisms responsible for the opposing effects of EE and progestins on endothelial cells. It is evident in the literature, however, that there is a mechanistic balance between the vasoprotective effects of estrogen and the antagonistic effects of differing progestins, dependent on their relative androgenicity. While sex hormones have profound influence on the mechanisms that underlie vascular control, including fluid regulation and the renin-angiotensin-aldosterone system, lipid metabolism, and cardiac function, among others, this section will primarily focus on vascular endothelial cell pathways and atherosclerotic plaque development.

It has been extensively discussed that estrogen has a positive effect on the cardiovascular system, offering protection against atherosclerotic development and improved vascular responsiveness (66–70). Estrogen appears to enhance endothelial function through increased NO bioavailability via the increased expression and activation of endothelial nitric oxide synthase (eNOS) (66). This activation of the eNOS-NO pathway is largely dependent on estrogen's interaction with estrogen receptors (ERs), predominantly ERα (71), on vascular endothelial cells, and not localized in smooth muscle (66). In cellular models, stimulation of endothelial cells with physiological levels of estrogen appears to increase NO, through an ER-eNOS mediated pathway (72, 73). Extending this research to animal models, researchers have observed impaired basal NO release in the aorta of ER deficient mice, demonstrating a mechanistic role of ER in NO production (74). In humans, an early case study in a male with an ERα gene mutation found that he had substantially impaired FMD, but intact NMD, indicative of endothelial-specific dysfunction associated with ERα's role in NO bioavailability (75). Further supporting the role of ERα in endothelial function, in premenopausal females, researchers have found increased ERα expression in the late follicular phase of the menstrual cycle when endogenous estrogen levels are elevated, and significant positive relationships exist between ERα expression, eNOS, and FMD (76). In contrast to the role of endogenous estradiol, it has been proposed that exogenous EE specifically exerts antioxidant effects on endothelial cells, decreasing superoxide anion production and thus preventing NO degradation, rather than acting on upregulation of eNOS activity or protein expression (77).

There is minimal literature examining the mechanisms underlying the endothelial cell impacts of hormonal contraceptives, and their composition of EE and progestin. In early research by John et al (60), which included a limited group of OCP users from across multiple generations, there was an increase in basal NO production in OCP users compared with naturally cycling controls. However, the authors noted that this may be a compensatory mechanism to increase basal NO, thus accommodating for increased vulnerability of the vasculature to deleterious stimuli (60). In contrast, research by Merki-Feld et al. (78) found no change in plasma NO levels following 3 mo of either second-generation OCP

(levonorgestrel) or third- generation OCP (desogestrel) use. However, this study may not have been sufficient in duration to permit observations of long-term alterations in NO bioavailability, observed by others. For example, research by Fallah et al. (79) identified decreased NO levels in second-generation OCP users, who had been using OCPs for a longer duration (range: 3–36 mo), compared to naturally cycling controls. This latter study aligns with research previously discussed in this review, finding impaired endothelial function in second-generation OCP users compared with naturally cycling controls (43–46). However, basal NO production offers little insight into the mechanisms responsible for modulating endothelial function, notably, alterations in eNOS mRNA and protein expression, enzyme activity, and receptor interactions.

Research by Zerr-Fouineau et al. (80) has identified impaired endothelial cell eNOS mRNA and protein expression in cells treated with estrogen + levonorgestrel or MPA, compared with estrogen alone; however, NO production was only impaired in MPA and not levonorgestrel treatment groups. This research supports the notion that certain progestins—specifically levonorgestrel (e.g., second-generation OCPs) and MPA (e.g., DMPA injections)—may reduce endothelial function via impairments to the eNOS-NO pathway, as seen in prior literature examined in this review (43–46, 51). In contrast, research by Simoncini and colleagues (81) has also found that drospirenone (fourth-generation progestin), but not MPA, increases eNOS activity and NO production in a dose-dependent manner in endothelial cells. Additionally, with the provision of estrogen, drospirenone does not alter the estrogen-induced increase in eNOS activity and NO production, but the provision of MPA antagonizes this increase, reducing NO bioavailability (81). This research provides preliminary cellular mechanistic support for human observational findings detailed previously in this review, with no change in endothelial function over time in fourth-generation OCP users compared with naturally cycling controls (43, 48), but phasic improvements with the active pill phase, where drospirenone has been found to be permissive to the benefits of EE on endothelial function (32, 49).

Examining the effect of contraceptive use on arterial structure, there is evidence that EE treatment alone, and with progestins, reduces atherosclerotic development. For example, rabbits on a cholesterol-fed diet, meant to increase the development of atherosclerotic plaques, experienced a reduction in atherosclerosis with EE treatment (82). Similarly, cholesterol-fed rabbits experienced reductions in atherosclerotic plaque development in EE-alone or EE + various progestins (e.g., desogestrel, third generation; levonorgestrel, second generation) treatments, with no difference between groups (83). These animal model results align with findings cited earlier in this review of the minimal effects of short-term OCP use on CCA IMT (45, 46).

In addition to mechanistic research examining vascular NO production, the metabolism of hormonal contraceptives and its impact on hormone levels is an emerging area of research. For example, recent work by Lazorwitz et al. (2019) identified three potential genetic variants influencing the concentration of contraceptive hormone etonogestrel (progestin), in addition to two nongenetic factors: body mass index and duration of implant use (84). However, the impact of genetic variants, duration of contraceptive use, and obesity (85) on hormonal contraceptive metabolism and vascular impact has not been explored.

Overall, while estrogen influences endothelial function through the ERα-eNOS-NO pathway, various progestins—specifically, levonorgestrel and MPA—may counteract the effects of estrogen and reduce NO bioavailability, thereby impairing endothelial function. However, further research is necessary to examine the independent and combined effects of EE and progestins on underlying mechanisms, with attention to creating animal models that mimic contraceptives with non-oral routes of administration (e.g., transdermal patch, IUD). Additionally, further research with human models is needed to connect cellular findings with human vascular outcomes, such as using serum exposure on human umbilical endothelial cell cultures, in combination with macrolevel vascular function and structure assessments.

CONCLUSIONS AND FUTURE DIRECTIONS

Hormonal contraceptives, which are used by premenopausal women worldwide, appear to influence specific components of the vasculature. It seems that hormonal contraceptives modulate macrovascular endothelial function, depending on the progestin type, dose of EE to progestin, and the route of administration of the contraceptive. Specifically, endothelial function appears to be impaired in second-generation OCP and DMPA injection users, but not in fourth-generation OCP or IUD users. When phasic differences are examined, it appears that endothelial function is impaired or remains unaltered in the active phase in second-generation OCP users, depending on the EE dosage, whereas phasic improvements in endothelial function are observed in third- and fourth-generation OCP users. While research on microvascular endothelial function is limited, the available data suggest that the microvasculature could respond similarly to the macrovasculature in response to hormonal contraceptive use. In contrast, hormonal contraceptives do not appear to impact macrovascular and microvascular smooth muscle function or assessments of arterial stiffness and structure. This research on the influence of hormonal contraceptives on early risk factors for CVD may help to explain the pathogenesis of CVD in women taking hormonal contraceptives.

Despite these advances in research on hormonal contraceptives, there are significant gaps in our understanding of their impact on vascular structure and function, which warrant future investigation. These gaps and proposed research directions are indicated in Fig. 2, inspired by a recent review by Seals et al. (86). Briefly, a major gap in our understanding of the impact of hormonal contraceptives on the vasculature is the long-term impacts and connection to the pathogenesis of CVD. Hormonal contraceptive users often utilize contraceptives for many years, yet minimal research has been conducted prospectively on vascular remodeling throughout use. Similarly, the majority of research has been conducted in healthy, young adults, and thus, it is presently not known whether the impact of hormonal contraceptives on vascular function is influenced by age or comorbidities. Additionally, as described further below, a major limitation to several studies examining the impact of hormonal contraceptives is the
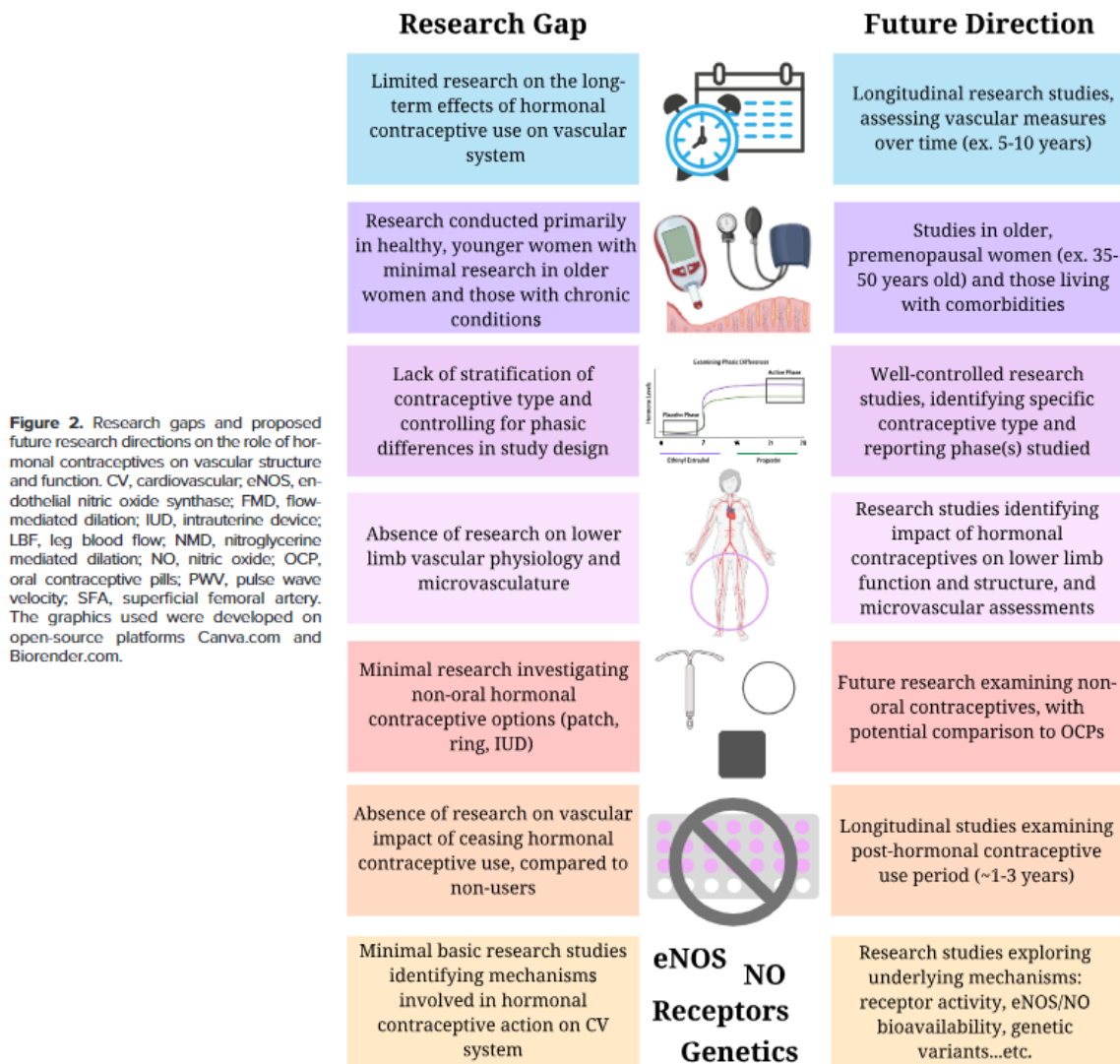
INFLUENCE OF CONTRACEPTIVES ON PERIPHERAL VASCULATURE

Figure 2. Research gaps and proposed future research directions on the role of hormonal contraceptives on vascular structure and function. CV, cardiovascular; eNOS, endothelial nitric oxide synthase; FMD, flow-mediated dilation; IUD, intrauterine device; LBF, leg blood flow; NMD, nitroglycerine mediated dilation; NO, nitric oxide; OCP, oral contraceptive pills; PWV, pulse wave velocity; SFA, superficial femoral artery. The graphics used were developed on open-source platforms Canva.com and Biorender.com.

lack of stratification of different contraceptive types and the concern for reporting and/or controlling for phases of testing. Further research is needed to validate limited study findings, with a focus on newer generations of OCPs and non-oral options. Considerations for future study design are outlined briefly below. Furthermore, there is a paucity of research examining impacts on the microvasculature, and no research on lower limb physiology. Given that OCP use increases the risk of deep vein thrombosis in the leg, there is an evident need for increased understanding of the impact of OCP use on the lower limb vasculature in humans (87). Similar to the lack of long-term research identified earlier, there is no research examining the effects of hormonal contraceptive cessation on vascular

function or structure. For example, if impairments in endothelial function are observed with second-generation OCP use, does this impairment revert back following a period of cessation? If so, what is the associated time-course? Finally, minimal research has been conducted on the mechanisms underlying the impact of hormonal contraceptives on the vasculature. Studies elucidating the independent and combined effects of EE and progestins are needed to understand their interactions with mechanistic pathways, such as interactions with estrogen and progesterone receptors, eNOS content and activity, and NO bioavailability, alongside the influence of genetic variants in the metabolism and subsequent hormone levels of contraceptives.

85

CONSIDERATIONS FOR FUTURE STUDY DESIGN

While holistic research endeavors are needed to confirm these conclusions, the research presented in this review does provide some guidance for researchers seeking to integrate women who use hormonal contraceptives into vascular physiology studies; specifically, that hormonal contraceptive type and phase studied appears to modulate both macrovascular and microvascular endothelial function, but not smooth muscle function or assessments of arterial stiffness and structure. When designing research studies, specifically, studies examining endothelial function, researchers would be encouraged to consider the following recommendations. First, it is recommended that researchers report details regarding the hormonal contraceptive types from participants included in the study (e.g., EE dosage and progestin type), duration of use for contraceptive users, and the phase of the hormonal cycle that was tested. Second, participants should be stratified on the basis of contraceptive type or only seek participants on a single contraceptive type, to avoid collapsing all contraceptive users into one group. Third, participants should be tested in a specific phase of the hormonal cycle, such as during their placebo pill withdrawal phase, to most closely match with the early follicular phase of the natural menstrual cycle, as suggested by Sims and Heather (35) and in the recent FMD guidelines (41). Specifically, it is recommended that testing occur midway through the placebo week to account for differing half-lives of OCPs on the first day of hormone withdrawal and the lack of suppression of endogenous hormones in the latter days of the placebo week or the impact of longer placebo weeks (11, 35). Finally, researchers should consider the use of a naturally cycling control group, within a standardized phase of the menstrual cycle (e.g., early follicular phase) when studying the effects of hormonal contraceptives cross-sectionally.

DISCLOSURES

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

AUTHOR CONTRIBUTIONS

J.S.W. and M.J.M. conceived and designed research; J.S.W. prepared figures; J.S.W. and M.J.M. drafted manuscript; J.S.W. and M.J.M. edited and revised manuscript; J.S.W. and M.J.M. approved final version of manuscript.

REFERENCES

1. **United Nations: Department of Economic and Social Affairs.** *Contraceptive Use by Method 2019: Data Booklet.* New York: United Nations, 2019.

2. **Daniels K, Abma J.** Current contraceptive status among women aged 15–49: United States. *NCHS Data Brief* 388: 1–8, 2018.

3. **Rotermann M, Dunn S, Black A.** Oral contraceptive use among women aged 15 to 49: results from the Canadian Health Measures Survey. *Health Rep* 26: 21–28, 2015.

4. **Yusuf F, Siedlecky S.** Patterns of contraceptive use in Australia: analysis of the 2001 National Health Survey. *J Biosoc Sci* 39: 735–744, 2007. doi:10.1017/S0021932006001738.

5. **Margolis KL, Adami HO, Luo J, Ye W, Weiderpass E.** A prospective study of oral contraceptive use and risk of myocardial infarction among Swedish women. *Fertil Steril* 88: 310–316, 2007. doi:10.1016/j.fertnstert.2006.11.206.

6. **Badawy A, Elnashar A.** Treatment options for polycystic ovary syndrome. *Int J Women's Health* 3: 25–35, 2011. doi:10.2147/IJWH.S11304.

7. **Kaunitz AM.** Oral contraceptive use in perimenopause. *Am J Obstet Gynecol* 185: S32–S37, 2001. doi:10.1067/mob.2001.116525.

8. **Schindler AE.** Non-contraceptive benefits of oral hormonal contraceptives. *Int J Endocrinol Metab* 11: 41–47, 2012. doi:10.5812/ijem.4158.

9. **Darney PD.** The androgenicity of progestins. *Am J Med* 98: S104–S110, 1995. doi:10.1016/S0002-9343(99)80067-9.

10. **Shufelt CL, Bairey Merz CN.** Contraceptive hormone use and cardiovascular disease. 53: 221–231, 2009. [Erratum in *J Am Coll Cardiol* 53: 904, 2009]. doi:10.1016/j.jacc.2008.09.042.

11. **Schlaff WD, Lynch AM, Hughes HD, Cedars MI, DI S.** Manipulation of the pill-free interval in oral contraceptive pill users: the effect on follicular suppression. *Am J Obstet Gynecol* 190: 943–949, 2004. doi:10.1016/j.ajog.2004.02.012.

12. **Sulak PJ, Kuehl TJ, Ortiz M, Shull BL.** Acceptance of altering the standard 21-day/7-day oral contraceptive regimen to delay menses and reduce hormone withdrawal symptoms. *Am J Obstet Gynecol* 186: 1142–1149, 2002. doi:10.1067/mob.2002.122988.

13. **Kavanaugh ML, Jerman J.** Contraceptive method use in the United States: trends and characeristics between. *Contraception* 97: 14–21, 2018. doi:10.1016/j.contraception.2017.10.003.

14. **Dunn N, Thorogood M, Faragher B, de Caestecker L, MacDonald TM, McCollum C, Thomas S, Mann R, Lidegaard O.** Oral contraceptives and myocardial infarction: results of the MICA case-control study. *BMJ* 318: 1579–1583, 1999. doi:10.1136/bmj.318.7198.1579.

15. **Kaminski P, Szpotanska-Sikorska M, Wielgos M.** Cardiovascular risk and the use of oral contraceptives. *Neuro Endocrinol Lett* 34: 587–589, 2013.

16. **Tanis BC, van den Bosch MA, Kemmeren JM, Cats VM, Helmerhorst FM, Algra A, van der Graaf Y, Rosendaal FR.** Oral contraceptives and the risk of myocardial infarction. *JM* 345: 1787–1793, 2001. doi:10.1056/NEJMoa003216.

17. **Heinemann LA, Lewis MA, Thorogood M, Spitzer WO, Guggenmoos-Holzmann I, Bruppacher R.** Case-control study of oral contraceptives and risk of thromboembolic stroke: results from International Study on Oral Contraceptives and Health of Young Women. *BMJ* 315: 1502–1504, 1997. doi:10.1136/bmj.315.7121.1502.

18. **Lidegaard Ø, Løkkegaard E, Svendsen AL, Agger C.** Hormonal contraception and risk of venous thromboembolism: national follow-up study. *BMJ* 339: b2890, 2009. doi:10.1136/bmj.b2890.

19. **Hadi HAR, Carr CS, Al Suwaidi J.** Endothelial dysfunction: cardiovascular risk factors, therapy, and outcome. *Vasc Health Risk Manag* 1: 183–198, 2005.

20. **Mitchell GF, Hwang S-J, Vasan RS, Larson MG, Pencina MJ, Hamburg NM, Vita JA, Levy D, Ej B.** Arterial stiffness and cardiovascular events: the Framingham Heart Study. *Circulation* 121: 505–511, 2010. doi:10.1161/CIRCULATIONAHA.109.886655.

21. **Bennett MR, Sinha S, Owens GK.** Vascular Smooth Muscle Cells in Atherosclerosis. *Circ Res* 118: 692–702, 2016. doi:10.1161/CIRCRESAHA.115.306361.

22. **Green DJ, Jones H, Thijssen D, Cable NT, Atkinson G.** Flow-mediated dilation and cardiovascular event prediction: does nitric oxide matter? *Hypertension* 57: 363–369, 2011. doi:10.1161/HYPERTENSIONAHA.110.167015.

23. **Inaba Y, Chen JA, Bergmann SR.** Prediction of future cardiovascular outcomes by flow-mediated vasodilatation of brachial artery: a meta-analysis. *Int J Cardiovasc Imaging* 26: 631–640, 2010. doi:10.1007/s10554-010-9616-1.

24. Yeboah J, Folsom AR, Burke GL, Johnson C, Polak JF, Post W, Lima JA, Crouse JR, Herrington DM. Predictive value of brachial flow-mediated dilation for incident cardiovascular events in a population-based study: the multi-ethnic study of atherosclerosis. *Circulation* 120: 502–509, 2009. doi:10.1161/CIRCULATIONAHA.109.864801.

25. Vlachopoulos C, Aznaouridis K, Stefanadis C. Prediction of cardiovascular events and all-cause mortality with arterial stiffness: a systematic review and meta-analysis. *J Am Coll Cardiol* 55: 1318–1327, 2010. doi:10.1016/j.jacc.2009.10.061.

26. Stewart M, Black K. Choosing a combined oral contraceptive pill. *Aust Prescr* 38: 6–11, 2015. doi:10.18773/austprescr.2015.002.

27. Hickson SS, Miles KL, McDonnell BJ, Cockcroft Y, Wilkinson JR, McEniery IB; Carmel M. Use of the oral contraceptive pill is associated with increased large artery stiffness in young women: The ENIGMA Study. *J Hypertens* 29: 1155–1159, 2011. doi:10.1097/HJH.0b013e328346a5af.

28. Limberg JK, Peltonen GL, Johansson RE, Harrell JW, Kellawan JM, Eldridge MW, Sebranek JJ, Walker BJ, Schrage WG. Greater β-adrenergic receptor mediated vasodilation in women using oral contraceptives. *Front Physiol* 7: 215, 2016. doi:10.3389/fphys.2016.00215.

29. Valtonen P, Punnonen K, Saarelainen H, Heiskanen N, Raitakari OT, Juonala M, Viikari JSA, Alfthan G, Kahonen M, Laaksonen R, Lyyra-Laitinen T, Laitinen T, Heinonen S. ADMA concentration changes across the menstrual cycle and during oral contraceptive use: the Cardiovascular Risk in Young Finns Study. *Eur J Endocrinol* 162: 259–265, 2010. doi:10.1530/EJE-09-0658.

30. Yu A, Giannone T, Scheffler P, Doonan RJ, Egiziano G, Gomez YH, Papaioannou TG, Daskalopoulou SS. The effect of oral contraceptive pills and the natural menstrual cYCLe on arterial stiffness and hemodynamICs (CYCLIC). *J Hypertens* 32: 100–107, 2014. doi:10.1097/HJH.0000000000000012.

31. Meendering JR, Torgrimson BN, Miller NP, Kaplan PF, Minson CT. Ethinyl estradiol-to-desogestrel ratio impacts endothelial function in young women. *Contraception* 79: 41–49, 2009. doi:10.1016/j.contraception.2008.07.025.

32. Thompson AK, Przemska A, Vasilopoulou D, Newens KJ, Williams CM. Combined oral contraceptive pills containing desogestrel or drospirenone enhance large vessel and microvasculature vasodilation in healthy premenopausal women. *Microcirculation* 18: 339–346, 2011. doi:10.1111/j.1549-8719.2011.00094.x.

33. Torgrimson BN, Meendering JR, Kaplan PF, Minson CT. Endothelial function across an oral contraceptive cycle in women using levonorgestrel and ethinyl estradiol. *Am J Physiol Heart Circ Physiol* 292: H2874–H2880, 2007. doi:10.1152/ajpheart.00762.2006.

34. Wilson R, Adams ML, Pyke KE. Inclusion of female participants in cardiovascular research: a case study of Ontario NSERC-funded programs. *Appl Physiol Nutr Metab* 45: 911–914, 2020. doi:10.1139/apnm-2019-0693.

35. Sims ST, Heather AK. Myths and methodologies: reducing scientific design ambiguity in studies comparing sexes and/or menstrual cycle phases. *Exp Physiol* 103: 1309–1317, 2018. doi:10.1113/EP086797.

36. Aulakh AK, Anand SS. Sex and gender subgroup analyses of randomized trials. *Women's Health Issues* 17: 342–350, 2007. doi:10.1016/j.whi.2007.04.002.

37. Raeisi-Giglou P, Volgman AS, Patel H, Campbell S, Villablanca A, Hsich E. Advances in cardiovascular health in women over the past decade: guideline recommendations for practice. *J Womens Health (Larchmt)* 27: 128–139, 2018. doi:10.1089/jwh.2016.6316.

38. Rich-Edwards JW, Kaiser UB, Chen GL, Manson JE, Goldstein JM. Sex and gender differences research design for basic, clinical, and population studies: essentials for investigators. *Endocrine Rev* 39: 424–439, 2018. doi:10.1210/er.2017-00246.

39. Heart and Stroke Foundation of Canada. *Ms. Understood. Women's hearts are victims of a system that is ill-equipped to diagnose, treat, and support them.* Ottawa, ON, Canada: Heart and Stroke Foundation, 2018.

40. Ritz SA, Antle DM, Cote J, Deroy K, Fraleigh N, Messing K, Parent L, St-Pierre J, Vaillancourt C, Mergler D. First steps for integrating sex and gender considerations into basic experimental biomedical research. *FASEB J* 28: 4–13, 2014. doi:10.1096/fj.13-233395.

41. Thijssen DHJ, Bruno RM, van Mil A, Holder SM, Faita F, Greyling A, Zock PL, Taddei S, Deanfield JE, Luscher T, Green DJ, Ghiadoni L. Expert consensus and evidence-based recommendations for the assessment of flow-mediated dilation in humans. *Eur Heart J* 40: 2534–2547, 2019. doi:10.1093/eurheartj/ehz350.

42. Flammer AJ, Anderson T, Celermajer DS, Creager MA, Deanfield J, Ganz P, Hamburg NM, Lüscher TF, Shechter M, Taddei S, Vita JA, Lerman A. The assessment of endothelial function: from research into clinical practice. *Circulation* 126: 753–767, 2012. doi:10.1161/CIRCULATIONAHA.112.093245.

43. Franceschini SA, Vieira CS, Martins WP, França JB, Ferriani RA. Effects of combined oral contraceptives containing levonorgestrel or chlormadinone on the endothelium. *Contraception* 87: 766–772, 2013. doi:10.1016/j.contraception.2012.09.023.

44. Friedman J, Cremer M, Jelani QU, Huang X, Jian J, Shah S, Katz SD. Oral contraceptive use, iron stores and vascular endothelial function in healthy women. *Contraception* 84: 285–290, 2011. doi:10.1016/j.contraception.2011.01.012.

45. Heidarzadeh Z, Asadi B, Saadatnia M, Ghorbani A, Fatehi F. The effect of low-dose combined oral contraceptive pills on brachial artery endothelial function and common carotid artery intima-media thickness. *J Stroke Cerebrovasc Dis* 23: 675–680, 2014. doi:10.1016/j.jstrokecerebrovasdis.2013.06.007.

46. Lizarelli PM, Martins WP, Vieira CS, Soares GM, Franceschini SA, Ferriani RA, Patta MC. Both a combined oral contraceptive and depot medroxyprogesterone acetate impair endothelial function in young women. *Contraception* 79: 35–40, 2009. doi:10.1016/j.contraception.2008.07.024.

47. Virdis A, Pinto S, Versari D, Salvetti G, Bernini G, Fruzzetti F, Genazzani AR, Taddei S, Salvetti A. Effect of oral contraceptives on endothelial function in the peripheral microcirculation of healthy women. *J Hypertens* 21, 2003. doi:10.1097/00004872-200312000-00015.

48. Giribela CR, Melo NR, Silva RC, Hong VM, Guerra GM, Baracat EC, Fm C-C. A combined oral contraceptive containing drospirenone changes neither endothelial function nor hemodynamic parameters in healthy young women: a prospective clinical trial. *Contraception* 86: 35–41, 2012. doi:10.1016/j.contraception.2011.08.017.

49. Meendering JR, Torgrimson BN, Miller NP, Kaplan PF, Minson CT. A combined oral contraceptive containing 30 mcg ethinyl estradiol and 3.0 mg drospirenone does not impair endothelium-dependent vasodilation. *Contraception* 82: 366–372, 2010. doi:10.1016/j.contraception.2010.03.009.

50. Torgrimson BN, Meendering JR, Miller NP, Kaplan PF, Minson CT. Endothelial function, endothelin-1, and fibrinogen in young women using the vaginal contraceptive ring. *Fertil Steril* 92: 441–447, 2009. doi:10.1016/j.fertnstert.2008.06.054.

51. Sorensen MB, Collins P, Ong PJL, Webb CM, Hayward CS, Asbury EA, Gatehouse PD, Elkington AG, Yang GZ, Kubba A, Pennell DJ. Long-term use of contraceptive depot medroxyprogesterone acetate in young women impairs arterial endothelial function assessed by cardiovascular magnetic resonance. *Circulation* 106: 1646–1651, 2002. doi:10.1161/01.CIR.0000030940.73167.4E.

52. Torgrimson BN, Meendering JR, Kaplan PF, Minson CT. Depot-medroxyprogesterone acetate and endothelial function before and after acute oral, vaginal, and transdermal estradiol treatment. *Hypertension* 57: 819–824, 2011. doi:10.1161/HYPERTENSIONAHA.110.163386.

53. Selim MF, Hussein AF. Endothelial function in women using levonorgestrel-releasing intrauterine system (LNG-IUS). *Contraception* 87: 396–403, 2013. doi:10.1016/j.contraception.2012.12.008.

54. Shenouda N, Priest SE, Rizzuto VI, MacDonald MJ. Brachial artery endothelial function is stable across a menstrual and oral contraceptive pill cycle but lower in premenopausal women than in age-matched men. *Am J Physiol Heart Circ Physiol* 315: H366–H374, 2018. doi:10.1152/ajpheart.00102.2018.

55. O'Brien MW, Johns JA, Al-Hinnawi A, Kimmerly DS. Popliteal flow-mediated dilatory responses to a bout of prolonged sitting between earlier and later phases of natural menstrual and contraceptive pill cycles. *J Appl Physiol (1985)* 129: 637–645, 2020. doi:10.1152/japplphysiol.00424.2020

56. Meendering JR, Torgrimson BN, Miller NP, Kaplan PF, Minson CT. Estrogen, medroxyprogesterone acetate, endothelial function, and biomarkers of cardiovascular risk in young women. *Am J Physiol-Heart Circ Physiol* 294: H1630–H1637, 2008. doi:10.1152/ajpheart.01314.2007.

57. Jekell A, Kalani M, Kahan T. The interrelation of endothelial function and microvascular reactivity in different vascular beds, and risk

assessment in hypertension: results from the Doxazosin-ramipril study. *Heart Vessels* 34: 484–495, 2019. doi:10.1007/s00380-018-1265-7.

58. Sandoo A, Carroll D, Metsios GS, Kitas GD, Veldhuijzen van Zanten JJ. The association between microvascular and macrovascular endothelial function in patients with rheumatoid arthritis: a cross-sectional study. *Arthritis Res Ther* 13: R99, 2011. doi:10.1186/ar3374.

59. Murphy MSQ, Vignarajah M, Smith GN. Increased microvascular vasodilation and cardiovascular risk following a pre-eclamptic pregnancy. *R2*: e12217, 2014. doi:10.14814/phy2.12217.

60. John S, Jacobi J, Schlaich MP, Delles C, Schmieder RE. Effects of oral contraceptives on vascular endothelium in premenopausal women. *Am J Obstet Gynecol* 183: 28–33, 2000. doi:10.1016/S0002-9378(00)37848-6.

61. Mattu AT, MacInnis MJ, Doyle-Baker PK, Murias JM. Effects of the menstrual and oral contraceptive cycle phases on microvascular reperfusion. *Exp Physiol* 105: 184–191, 2020. doi:10.1113/EP088135.

62. Oren A, Vos LE, Uiterwaal CSPM, Grobbee DE, Bots ML. Cardiovascular risk factors and increased carotid intima-media thickness in healthy young adults: the atherosclerosis risk in young adults (ARYA) study. *Arch Int Med* 163: 1787–1792, 2003. doi:10.1001/archinte.163.15.1787.

63. Sandoval Y-HG, Yu A, Daskalopoulou SS. Use of oral contraceptives and arterial stiffness. *J Hypertens* 29, 2011. doi:10.1097/HJH.0b013e32834b6247.

64. Priest SE, Shenouda N, MacDonald MJ. Effect of sex, menstrual cycle phase, and monophasic oral contraceptive pill use on local and central arterial stiffness in young adults. *Am J Physiol Heart Circ Physiol* 315: H357–H365, 2018. doi:10.1152/ajpheart.00039.2018.

65. Lorenz MW, Markus HS, Bots ML, Rosvall M, Sitzer M. Prediction of clinical cardiovascular events with carotid intima-media thickness: a systematic review and meta-analysis. *Circulation* 115: 459–467, 2007. doi:10.1161/CIRCULATIONAHA.106.628875.

66. Chambliss KL, Shaul PW. Estrogen modulation of endothelial nitric oxide synthase. *Endocr Rev* 23: 665–686, 2002. doi:10.1210/er.2001-0045.

67. Mendelsohn ME. Genomic and nongenomic effects of estrogen in the vasculature. *Am J Cardiol* 90: F3–F6, 2002. doi:10.1016/S0002-9149(02)02418-9.

68. Mendelsohn ME, Karas RH. The protective effects of estrogen on the cardiovascular system. *The. JM* 340: 1801–1811, 1999. doi:10.1056/NEJM199906103402306.

69. Miller VM, Mulvagh SL. Sex steroids and endothelial function: translating basic science to clinical practice. *Trends Pharmacol Sci* 28: 263–270, 2007. doi:10.1016/j.tips.2007.04.004.

70. Usselman CW, Stachenfeld NS, Bender JR. The molecular actions of oestrogen in the regulation of vascular health. 101: 356–361, 2016. doi:10.1113/EP085148.

71. Couse JF, Lindzey J, Grandien K, Gustafsson C-A, Korach KS. Tissue distribution and quantitative analysis of estrogen receptor-α (ERα) and estrogen receptor-β (ERβ) messenger ribonucleic acid in the wild-type and ERα-knockout mouse. *Endocrinology* 138: 4613–4621, 1997. doi:10.1210/endo.138.11.5496.

72. Caulin-Glaser T, García-Cardeña G, Sarrel P, Sessa WC, Bender JR. 17β-estradiol regulation of human endothelial cell basal nitric oxide release, independent of cytosolic Ca^{2+} mobilization. *Circ Res* 81: 885–892, 1997. doi:10.1161/01.RES.81.5.885.

73. Nevzati E, Shafighi M, Bakhtian KD, Treiber H, Fandino J, Fathi AR. Estrogen induces nitric oxide production via nitric oxide synthase activation in endothelial cells. *Acta Neurochir Suppl* 120: 141–145, 2015. doi:10.1007/978-3-319-04981-6_24.

74. Rubanyi GM, Freay AD, Kauser K, Sukovich D, Burton G, Lubahn DB, Couse JF, Curtis SW, Korach KS. Vascular estrogen receptors and endothelium-derived nitric oxide production in the mouse aorta. Gender difference and effect of estrogen receptor gene disruption. *CI* 99: 2429–2437, 1997. doi:10.1172/JCI119426.

75. Sudhir K, Chou TM, Messina LM, Hutchison SJ, Korach KS, Chatterjee K, Rubanyi GM. Endothelial dysfunction in a man with disruptive mutation in oestrogen-receptor gene. *Lancet* 349: 1146–1147, 1997. doi:10.1016/S0140-6736(05)63022-X.

76. Gavin KM, Seals DR, Silver AE, Moreau KL. Vascular endothelial estrogen receptor α is modulated by estrogen status and related to endothelial function and endothelial nitric oxide synthase in healthy women. *J Clin Endocrinol Metab* 94: 3513–3520, 2009. doi:10.1210/jc.2009-0278.

77. Arnal JF, Clamens S, Pechet C, Negre-Salvayre A, Allera C, Girolami JP, Salvayre R, Bayard F. Ethinylestradiol does not enhance the expression of nitric oxide synthase in bovine endothelial cells but increases the release of bioactive nitric oxide by inhibiting superoxide anion production. *Proc Natl Acad Sci U S A* 93: 4108–4113, 1996. doi:10.1073/pnas.93.9.4108.

78. Merki-Feld GS, Rosselli M, Dubey RK, Jäger AW, Keller PJ. Long-term effects of combined oral contraceptives on markers of endothelial function and lipids in healthy premenopausal women. *Contraception* 65: 231–236, 2002. doi:10.1016/S0010-7824(01)00312-2.

79. Fallah S, Nouroozi V, Seifi M, Samadikuchaksaraei A, Aghdashi EM. Influence of oral contraceptive pills on homocysteine and nitric oxide levels: as risk factors for cardiovascular disease. *J Clin Lab Anal* 26: 120–123, 2012. doi:10.1002/jcla.21492.

80. Zerr-Fouineau M, Jourdain M, Boesch C, Hecker M, Bronner C, Schini-Kerth VB. Certain progestins prevent the enhancing effect of 17β-estradiol on NO-mediated inhibition of platelet aggregation by endothelial cells. *Arterioscler Thromb Vasc Biol* 29: 586–593, 2009. doi:10.1161/ATVBAHA.108.178004.

81. Simoncini T, Fu XD, Caruso A, Garibaldi S, Baldacci C, Giretti MS, Mannella P, Flamini MI, Sanchez AM, Genazzani AR. Drospirenone increases endothelial nitric oxide synthesis via a combined action on progesterone and mineralocorticoid receptors. *Hum Reprod* 22: 2325–2334, 2007. doi:10.1093/humrep/dem109.

82. Sulistiyani Adelman SJ, Chandrasekaran A, Jayo J, St. Clair RW. Effect of 17α-dihydroequilin sulfate, a conjugated equine estrogen, and ethynylestradiol on atherosclerosis in cholesterol-fed rabbits. *Arterioscler Thromb Vasc Biol* 15: 837–846, 1995. doi:10.1161/01.ATV.15.7.837.

83. Alexandersen P, Haarbo J, Zandberg P, Jespersen J, Skouby SO, Christiansen C. Lack of difference among progestins on the anti-atherogenic effect of ethinyl estradiol: a rabbit study. *Hum Reprod* 18: 1395–1403, 2003. doi:10.1093/humrep/deg286.

84. Lazorwitz A, Aquilante CL, Oreschak K, Sheeder J, Guiahi M, Teal S. Influence of genetic variants on steady-state etonogestrel concentrations among contraceptive implant users. *Obstet Gynecol* 133, 2019. doi:10.1097/AOG.0000000000003189.

85. Edelman AB, Cherala G, Stanczyk FZ. Metabolism and pharmacokinetics of contraceptive steroids in obese women: a review. *Contraception* 82: 314–323, 2010. doi:10.1016/j.contraception.2010.04.016.

86. Seals DR, Nagy EE, Moreau KL. Aerobic exercise training and vascular function with ageing in healthy men and women. *J Physiol* 597: 4901–4914, 2019. doi:10.1113/JP277764.

87. van Hylckama Vlieg A, Helmerhorst FM, Vandenbroucke JP, Doggen CJ, Rosendaal FR. The venous thrombotic risk of oral contraceptives, effects of oestrogen dose and progestogen type: results of the MEGA case-control study. *BMJ* 339: b2921, 2009. doi:10.1136/bmj.b2921.

CHAPTER 5: Menstrual cycle and oral contraceptive pill phase largely do not influence vascular function, arterial stiffness, and associated cellular regulation in young, healthy premenopausal females.

Jennifer S. Williams, Jem L. Cheng, Jenna C. Stone, Michael J. Kamal, Joshua M. Cherubini, Gianni Parise, Maureen J. MacDonald

Abstract

Background: Historical exclusion of females in research has been, in part, due to the perceived influence of natural menstrual (NAT) and oral contraceptive pill (OCP) cycles on vascular outcomes. Previous research identified that NAT and OCP cycle phases may influence brachial artery endothelial function, however, findings are mixed. Limited research has examined arterial stiffness, smooth muscle and lower limb endothelial function. The purpose of this study was to investigate the influence of NAT and OCP cycles on cardiovascular outcomes and associated cellular regulation.

Methods: Forty-nine premenopausal females (n=17 NAT, n=17 2nd generation OCP, n=15 3rd generation OCP) participated in two randomized order experimental visits in the low (LH: early follicular/placebo) and high (HH: mid-luteal/active) hormone phases of their cycle. Brachial (BA) and femoral artery (SFA) endothelial function were assessed using a flow-mediated dilation (FMD) test, smooth muscle function was assessed using a nitroglycerine-mediated dilation test, and arterial stiffness was assessed centrally at the carotid artery and peripherally using pulse wave velocity. Cultured female human endothelial cells were exposed for 24h to serum obtained during the experimental visits to examine endothelial nitric oxide synthase (eNOS) and estrogen receptor alpha (ERα) protein content.

Results: BA FMD was elevated in the HH *versus* LH phase, regardless of group (HH: 7.7±3.5%, LH: 7.0±3.3%, p=0.02). Baseline BA diameter was decreased in the HH phase; accounting for differences in diameter with allometric scaling resulted in no effect of phase on %FMD$_{scaled}$ (HH: 7.6±2.6%, LH: 7.1±2.6%, p=0.052). SFA FMD, smooth

muscle function in the BA and SFA arteries, carotid artery and peripheral arterial stiffness, and eNOS and ERα protein content were unaffected by group or phase.

Conclusions: NAT and OCP cycles have no influence on vascular outcomes and ERα-eNOS pathway, apart from a small effect on BA endothelial function partially explained by differences in baseline artery diameter.

Keywords: vascular function, endothelial function, smooth muscle function, sex hormones, female inclusion

What is new?

- Comprehensive evaluation of cardiovascular system in naturally cycling and 2nd and 3rd generation oral contraceptive pill users indicates no major influence of hormonal cycling or contraceptive use on endothelial function and smooth muscle function in the upper and lower limbs, arterial stiffness, or underlying cellular mechanisms.

- Study findings challenge the historical exclusion of female participants on the basis of potentially confounding hormonal cycles on vascular outcomes.

What are the clinical implications?

- Oral contraceptive pill use (2nd and 3rd generation) does not appear to largely influence early risk factors for cardiovascular disease, including endothelial function and arterial stiffness, in young, healthy females.

- Researchers including females in cardiovascular trials may not need to control for oral contraceptive pill use or hormonal cycle phase; though considerations may depend on the research question, population and method of hormonal contraceptive examined.

Introduction

Hormonal contraceptives – specifically oral contraceptive pills (OCPs) – are the most widely utilized prescription for women between the ages of 15 and 49 worldwide, and are used by an estimated 151 million women.[1] Hormonal contraceptives are debated as a source of increased risk for arterial and venous cardiovascular events [2, 3], and understanding their influence on vascular function and structural remodelling is an area of ongoing research. Research examinining acute and chronic increases in central and peripheral arterial stiffness, smooth muscle dysfunction, and endothelial dysfunction[4, 5] of peripheral arteries provides insight into factors which may influence the incidence and progression of cardiovascular disease (CVD).[5, 6]

Reviews published by our group have identified that fluctuations in endogenous hormones with the natural menstrual cycle and exogenous hormones with OCP use influences endothelial function, but not smooth muscle function, and does not profoundly impact arterial stiffness or vascular structure.[7, 8] For example, a meta-analysis by our group found a small effect of the menstrual cycle on macrovascular endothelial function.[7] Mechanistically, there is also cellular evidence by Gavin *et al* (2009) that estrogen receptor alpha (ERα) is elevated alongside increased17β-estradiol levels during the menstrual cycle, which is positively associated with endothelial function.[9] Similarly, this study also identified a positive relationship between ERα and endothelial nitric oxide synthase (eNOS), an enzyme that produces the potent vasodilator nitric oxide (NO), which is involved in endothelial function responses.[9]

However, the impact of OCPs on the peripheral vasculature has only recently been examined in a series of well-designed studies from one primary research center,[10-]

[14] much of which has yet to be replicated. Research from this group has identified that

the within-cycle phase endothelial response to oral contraceptives depends largely on

the balance between exogenous ethinyl estradiol (EE) and progestin, and the type of

progestin (which varies based on generation of OCP). For example, research by

Torgrimson *et al* (2007) found that for 2nd generation OCPs with the progestin

levonorgestrel, endothelial function was unchanged in contraceptive users with 30mg of

EE (low dose), but was impaired in users with 20mg of EE (very low dose EE; same

dosage of progestin) across a hormonal cycle.[13] However, smooth muscle function was

unaltered in both groups.[13] Similarly, this group also found that in users of 3rd generation

OCPs containing the the progestin desogestrel, low dose (30 mcg ethinyl estradiol/150

mcg desogestrel) users experienced an improvement in endothelial function, while very

low dose (20 mcg ethinyl estradiol/150 mcg desogestrel) users experienced no phasic

difference; neither group experienced alterations in smooth muscle function.[11] These

findings have been supported by one other study, which found improved endothelial

function in low dose 3rd and 4th generation OCPs with the progestins desogestrel and

drospirenone, respectively, but no change in 2nd generation OCPs across the hormonal

cycle.[15]

Recent research from our lab group has further explored sex-based differences

in vascular function and arterial stiffness, with attention to the hormonal cycle.

Specifically, research by Shenouda *et al* (2018) identified no phasic differences in

endothelial function or smooth muscle function across the natural menstrual cycle or

hormonal contraceptive cycle of OCP users.[16] Similarly, research on the same group of

participants by Priest *et al* (2018) also identified no phasic differences in central and

peripheral arterial stiffness.[17] While these studies did not separate OCP users by generation, and thus progestin type, exploratory analysis identified a negative relationship between duration of OCP use and endothelial function in the 2nd but not the 3rd/4th generation group.[16] This study may provide evidence of a negative impact of 2nd generation OCP use that is aligned with prior findings[18, 19]; however, further research that separates OCP generations is needed to thoroughly define their associated vascular consequences.

Several gaps in our understanding of the influence of oral contraceptives on vascular physiology have been identified and thereby warrant further investigation. To our knowledge, there is no literature on the effects of contraceptive use on lower limb arterial endothelial and smooth muscle cells, both looking at differences across groups and phases and in the duration of OCP use. Similarly, there is a paucity of literature examining different OCP generations and both central and peripheral arterial stiffness. Finally, there is no literature examining the connection between *in vivo* vascular assessments and *in vitro* cellular mechanisms, particularly the ERα-eNOS pathway, in females across hormonal cycles.

Therefore, the purpose of this study is: (1) to comprehensively examine the influence of within-cycle phase differences of hormonal contraceptives (ex. 2nd and 3rd generation OCPs) and the natural menstrual cycle on arterial stiffness and endothelial and smooth muscle function in the upper and lower limbs; and (2) to explore the duration of OCP use on endothelial function and central arterial stiffness. We hypothesized that within-cycle phase increases in endothelial, but not smooth muscle, function or arterial stiffness would be seen in 3rd generation OCP users and NAT, but

not 2nd generation OCP users. Additionally, we hypothesized that 3rd generation OCP users would have increased endothelial function, but not smooth muscle function or arterial stiffness, compared to 2nd generation OCP users. Finally, we hypothesized that only the 2nd generation OCP users would experience negative effects of longer OCP duration of use on endothelial function and central arterial stiffness. Overall, this study provides novel and timely insight into the role of hormonal contraceptives on peripheral vascular physiology, especially given the calls for increased inclusion of female participants in human research.[20, 21]

Methods

Participant Recruitment & Ethics

Fourty-nine premenopausal females between the ages of 18 and 45 were recruited from the McMaster University and Hamilton communities from 2020-2022, through posters, online advertisements, and recruitment from prior lab studies. Sixty-two participants were initially eligible to participate in this study. However, due to COVID-19 pandemic-related closures resulting in delays in testing, twelve participants were unable to begin the study, and one participant was unable to complete the three testing visits; as a result, fourty-nine participants completed the study.

Sample size calculations were determined using a power simulation,[22] with an anticipated increase of relative %FMD of 1.5% between phases for OCP3, no change between phases for NAT, and a 1.5% decrease in OCP2, with an average SD of 1.2% based on previous studies.[15, 16, 19, 23] Sample size calculations indicated that 15 participants were needed to reach a power of >90%, so participants were recruited to each of the following groups: naturally cycling participants (n=17), 2nd generation OCPs

containing the progestin levonorgestrel (OCP2; n=17), and 3rd generation OCPs containing the progestins desogestrel or norgestimate (OCP3; n=15). This sample size is in agreement with other similar studies of OCP use and vascular function (n=7-20)[11, 15, 16, 19, 24]. All participants also participated in a previously published study examining substrate oxidation at rest and during exercise.[25]

Prior to commencing the study, volunteers attended a screening visit to become familiar with the lab environment and for an eligibility assessment. Volunteers completed a medical screening form and were excluded if they reported: cardiovascular or metabolic diseases, taking vasoactive medications, were smokers, were pregnant (currently or within the last year) or had undergone reproductive surgeries. Further, NAT participants were excluded if they had used hormonal contraceptives within 6 months of participating, if their menstrual cycles were irregular (one missed cycle in the last 6 months), or if the cycle was outside of a 'normal range' (21-35 day cycle length), as this may be indicative of hormonal cycle irregularity or other reproductive conditions. Volunteers were familiarized to the study protocol, including the cuff inflation on the distal forearm and lower thigh, and brachial artery and superficial femoral artery images and velocity signals were acquired to ensure adequate image and signal quality. Participants were then scheduled for testing around their hormonal cycle above and excluded if they were unable to complete the study visits.

This study was approved by the Hamilton Integrated Research Ethics Board (#7827) and conforms to the standards set by the Declaration of Helsinki, apart from clinical trial registration. Written informed consent was obtained from all volunteers prior to participating. This observational study was also conducted and written in compliance

with the Strengthening the Reporting of Observational Studies in Epidemiology

(STROBE) statement guidelines.[26]

Study Protocol

All study visits took place in the Vascular Dynamics Laboratory at McMaster

University, in a temperature and humidity controlled, quiet room. Following the

familiarization visit, participants took part in three testing visits: one cardiorespiratory

testing visit and two vascular assessment visits. The cardiorespiratory testing visit

occurred at least 72h prior to the first vascular assessment visit, where participants

completed a maximal exercise test to assess cardiorespiratory fitness. Anthropometric

measures were also taken during this visit. Participants then took part in two vascular

testing visits across their hormonal cycle (low hormone (LH) and high hormone (HH)

phases), with testing occurring at the same time of day in the morning for both visits, to

control for diurnal variation in vascular function.[27] The initial visit condition (i.e., LH or

HH phase) was randomized to control for potential order effects. Participants in the NAT

group tracked their hormonal cycle for at least two cycles prior to participating to assist

in estimating and scheduling testing dates. For NAT participants, the LH phase was

considered to be during their early follicular phase (days 2-6 following onset of

menstruation) and their HH phase was in the mid-luteal phase approximately 7 days

following a positive ovulation test (BFP Ovulation Tests, Fairhaven Health, Bellingham,

WA).[15, 21] For participants in both OCP groups (OCP2, OCP3), the LH phase was

considered to be during their placebo pill/no pill withdrawal week, during days 2-7 of

their hormonal cycle [21] and the HH phase was during the last 7 days of their active pills

(days 22-28) [15, 21]. Participants completed at least a 6h overnight fast, 12h without

alcohol or caffeine, and 24h without moderate to vigorous physical activity prior to each vascular testing visit.[28]

During each vascular testing visit, participants rested in the supine position for 10 minutes. Blood samples were then acquired for hormone measures as described below. Following a 5-minute rest, baseline hemodynamic measures were acquired. Central and peripheral stiffness measures [carotid artery stiffness, and pulse wave velocity (PWV) respectively], FMD, and NMD measurements were acquired in that order, with 10-minute rest periods between each test to allow for return to baseline arterial diameter (Figure 1).[29] An assessment of body composition was made during the LH visit.

Experimental Procedures

Anthropometric Assessments & DXA Scan. Anthropometric measurements of height, weight, waist circumference, and hip circumference were assessed during the first experimental visit (cardiorespiratory fitness session). Body composition was also assessed using a dual-energy X-ray absorptiometry (DXA) scan, taking place during the LH visit. The DXA scan provided information about body fat mass, body fat percentage, and lean body mass.

Cardiorespiratory Fitness. At least 72h prior to commencing the vascular testing sessions, participants completed an incremental exercise test to exhaustion on a cycle ergometer (Lode Excalibur Sport V 2.0, Groningen, Netherlands or Kettler Ergo Race, Kettler, Virginia Beach VA) to determine peak oxygen uptake ($\dot{V}O_2peak$), in line with current guidelines.[30] A metabolic cart with an online gas collection system (Quark CPET metabolic cart, COSMED, Italy) was used to measure oxygen consumption ($\dot{V}O_2$) and carbon dioxide production ($\dot{V}CO_2$), and heart rate (HR) was monitored continuously with

a HR monitor (Polar A3, Lake Success, NY). The test began with a 2-minute warm-up at

50 watts (or less if the participant was unable to perform a warm-up at this intensity),

after which the power was increased by 1 watt every two seconds (n=37) or 5 watts

every ten seconds (n=12) until volitional exhaustion or the point at which pedal cadence

fell below 60 rpm, as described previously.[31] Following exhaustion, participants were

instructed to cool down for two minutes at 50 watts or less. The $\dot{V}O_2$peak test was

considered succesful if at least two of the following four criteria were met: (1) perceived

exertion was ≥17 (BORG scale 6-20, (2) HR was within 10 beats per minute of 208-

0.7*age,[32] (3) respiratory exchange ratio (RER) was >1.1[33]; and (4) a plateau in $\dot{V}O_2$

was reached despite an increase in work.[34, 35] All participants achieved $\dot{V}O_2$peak.

$\dot{V}O_2$peak was defined as the highest oxygen consumption achieved over a 30s period

(typically achieved at the end of the test).

Blood Analysis. Following 10 minutes of supine rest at the start of each testing visit, a

standard venipuncture procedure was performed to acquire ~30mL of venous blood in

serum tubes (BD Vacutainer, Red BD Hemogard Closure, Franklin Lakes, NJ) and one

4mL plasma tube (BD Vacutainer, Purple EDTA Hemogard Closure, Franklin Lkaes,

NJ). To assess for viscosity from the plasma tube, two capillary tubes were filled and

spun at 3,500 rpm at 15°C for 10min (Sorvall Legend XTR Centrifuge, Thermo

Scientific, Waltham, MA), and were then read on a manual viscometer reader (Adams

Micro-hematocrit Reader, Clay-Adams New York). Viscosity data is missing for two

participants (n=1 NAT LH phase and n=1 NAT HH phase), due to breakage of capillary

tubes. The rest of the tubes were set aside for ~60min to allow for clotting prior to

spinning at 4,000 rpm at 4°C for 10min. Blood serum was aliquoted into 1.5mL

polypropylene tubes (Falcon Corning Science, Corning, NY), and frozen at -20°C (n=12)

or -80°C (n=37) until ready for batch analysis. Serum analysis was conducted using

ELISAs for the following tests: serum estradiol (Competitive Immunoassay, Ortho Vitros

Microwell), serum progesterone (Competitive Immunoassay, Ortho Vitros Microwell),

serum levonorgestrel in OCP2 users (Competitive Immunoassay, ThermoFisher

Scientific). One participant's estradiol levels were excluded as they were deemed to be

an outlier (OCP2 active phase: 716 pmol/L; beyond 3 SD from the mean).

Hemodynamic Assessments. Resting heart rate (HR) and blood pressure (BP) were

assessed using the average of the last two of three measurements obtained with an

automated BP assessment device (GE Dinamap ProSeries, Batesville IN). If the second

and third systolic BP measurements were not within 5 mmHg of each other, a fourth

measurement was obtained, and the last two measures averaged. Continuous HR was

assessed throughout the study using a single-lead ECG (ML123, AD Instruments,

Colorado Springs, CO), and BP was assessed using a beat-to-beat finger blood

pressure device calibrated to heart level (Finometer MIDI, Finapres Medical Systems,

Amsterdam, The Netherlands), which was placed on the right hand.

Arterial Stiffness (PWV). PWV, as a measure of regional arterial stiffness, was

assessed using applanation tonometry. Micromanometer-tipped pressure probes (SPT-

301, Millar Instruments) were used to detect pressure waveforms from the skin surface

overlying an artery. Pressure waveforms were then band-pass filtered at 5-30 Hz to

determine the foot of each pulse for later calculation of pulse transit time (LabChart 7,

AD Instruments, Colorado Springs, CO). Distance between measurement sites was

taken as the straight distance over the surface of the body using a measuring tape

pulled taut. Tonometers were placed on the carotid and femoral arterial sties for determination of central PWV, calculated using the following formula: central PWV = (0.8*carotid-femoral distance)/carotid-femoral pulse transit time.[36] Waveforms were also collected for peripheral leg PWV at the femoral and dorsalis pedis arteries, and for peripheral arm PWV at the carotid and radial arteries. Peripheral leg PWV was calculated using the formula: leg PWV = (femoral – dorsalis pedis distance)/femoral-dorsalis pedis transit time. Peripheral arm PWV was calculated using the formula: arm PWV = ((radial – suprasternal notch distance – (carotid – suprasternal notch distance))/carotid-radial pulse transit time.[37, 38] Measurements were reported as the average of two sets of 10 continuous heart cycles.

Carotid Artery Stiffness (β-Stiffness, Distensibility, Compliance) and IMT. Common carotid artery β-Stiffness, distensibility, compliance, and intima media thickness (IMT) were calculated from ultrasound derived diameter measurements[39] of the left carotid artery over 10 heart cycles, with pressures determined from measurements obtained using applanation tonometry at the right carotid artery, calibrated to finger cuff blood pressure measures described above. The β-stiffness index was calculated using the formula: β-stiffness = $\ln(SBP/DBP)/[LD_{max} – LD_{min}/LD_{min})$, using the maximum ($LD_{max}$) and minimum ($LD_{min}$) carotid artery diameters in each heart cycle.[17] Distensibility was calculated as: distensibility = $(\pi(LD_{max}/2)^2 – \pi(LD_{min}/2)^2)/(\pi(LD_{max}/2)^2)(PP)$, where LD_{max} is the maximal diameter, LD_{min} is the minimum diameter, and PP is the pulse pressure, which is the difference between systolic and diastolic pressures calculated at the carotid artery. Carotid artery compliance was assessed using the following formula for the same 10 heart cycles: compliance = $LD_{max} – LD_{min}/PP$. Finally, carotid artery IMT was

measured as the distance between the lumen-intima border and media-adventitia

border.

FMD. Participants took part in simultaneous arm and leg reactive hyperemia FMD

(FMD) tests to assess macrovascular endothelial function of the left brachial (BA) and

left superficial femoral arteries (SFA), respectively. In line with current guidelines,[28]

which have improved reproducibility of FMD[40, 41], pneumatic blood pressure cuffs were

placed around the forearm and lower thigh and were rapidly and simultaneously inflated

to suprasystolic pressure (~200 mmHg) for five minutes to occlude blood flow to the

distal arteries. Two identical Doppler ultrasound machines (Vivid Q, GE Medical

Systems, Horten, Norway) attached to 12 MHz linear array probes were used to

continuously measure artery diameter and blood velocity before cuff inflation (baseline;

30s), following 4 minutes of cuff inflation (occlusion; 30s) and in the three minutes

immediately following cuff deflation. Simultaneous assessments of the BA and SFA

were conducted in duplex mode, with an insonation angle corrected to 68°,[42] and heart

rate was collected using single-lead ECG for the duration of the test. Images were

stored in a Digital Imaging and Communications in Medicine (DICOM) format, and end-

diastolic frames were extracted and compiled (Sante DICOM Editor, v. 3.1.20,

Santesoft, Athens, Greece). DICOM files were then analyzed using a semi-automated

edge tracking software (Artery Measurement System (AMS) II, version 1.141,

Gothenburg, Sweden).[39] Analysis was conducted by one member of the research team

(JSW), blinded to group and phase to reduce bias. Baseline diameter was determined

as an average of the diameters in the 30s prior to inflation, and peak diameter was

determined as the largest 5-heart cycle average of diameters in the 3 minutes following

deflation. FMD was reported as both an absolute change (AbsFMD) and percentage change (%FMD) in diameter: FMD = peak diameter – baseline diameter; %FMD = (peak diameter – baseline diameter)/baseline diameter x 100%. Mean blood velocity (MBV) measures simultaneously collected with brightness mode ultrasound images were extracted as AVI files, and analyzed using a pixel-based tracking software (Measurements from Arterial Ultrasound Imaging; Hedgehog Medical, Waterloo, ON, Canada). MBV was similarly averaged into 5-heart cycle average time bins and used to calculate shear rate (SR), as described previously [SR = (MBV*8)/diameter].[43] The time to peak diameter and SR areas under the curve to the time of peak diameter are reported. All BA FMD images were included in the final dataset, however, one participant was excluded (n=1 NAT) from the SFA FMD analysis due to poor image quality. Similarly, one participant's BA deflation SR AUC was excluded (n=1 NAT) due to disruption in the blood flow signal at deflation; the same rationale was used to exclude n=3 NAT (2 LH, 1 HH) for SFA deflation SRAUC.

NMD. An NMD test was performed to assess macrovascular smooth muscle cell function, or endothelium-independent dilation. Following a 30s baseline image of the BA and SFA was obtained, participants received a sublingual 0.4mg dose of nitroglycerine. Following administration, 30s video clips were taken every minute for 10 minutes, using the same ultrasound settings as described for FMD. Absolute NMD (AbsNMD) and relative %NMD were calculated similarly to FMD with the peak as the highest 30s average diameter acquired. Analysis was conducted by one member of the research team (JSW), blinded to group and phase to reduce bias. A total of six participants (2 from each of NAT, OCP2, and OCP3) did not complete the two NMD tests across the

two phases, due to contraindications for the test (i.e., low blood pressure and hematocrit, n=4)[44] or did not consent to this test (n=2). One additional NAT participant's LH visit in the SFA only was removed from the dataset due to poor image quality.

Serum Cell Exposure Experiments. Female human umbilical vein endothelial cells (HUVEC) were pooled from four individual cell lines to create a female-only, mixed ethnic cell line (American Type Culture Collection, PCS-CC-2517, Lots: 0000230618 (Caucasian), 0000246083 (Hispanic), 0000315288 (Black), 451Z020 (Asian). Cells were grown in T75 flasks using full phenol-red free [to avoid estrogenic effects of phenol red[45]] endothelial cell growth medium (ECGM-2; PromoCell C-22216), supplemented with growth medium supplement mix (PromoCell C-39216). An additional 2% fetal bovine serum was added (4% concentration overall; Invitrogen 12483020), and 1% of 10,000 units/mL penicillin and 10,000 mg/mL streptomycin (Invitrogen, 15140122). Cells were cultured in an incubator in a humidifier at 5% CO_2 and 37°C. Once ready for experiments, cells were grown using the same procedure as above, with all experiments taking place at passages 4-7. Cells were plated at 300,000 cells/well into 6-well plates (ThermoFisher, 140675) for 24 hours to allow for adherence with full phenol-red free media. Cells were then deprived of serum in serum-free media for 4 hours to remove any hormonal effects of the cell media, and were then exposed to endothelial basal media supplemented with 10% human serum for 24h obtained in each of the testing visits, as previously reported by Brunt et al (2019).[46]

Following a 24h serum exposure period, cells were lysed using a buffer solution containing RIPA buffer (ThermoFisher, 89900), phosphatase inhibitor cocktail (Sigma Aldrich, P5726) and protease inhibitor tablets (ThermoFisher, A32963). Cell lysates

were sonicated (5 x 5s, 100% power; Fisher Scientific) and centrifuged at 14000 xg for 15 minutes to remove cell debris. Total protein concentration was determined using a BCA assay (ThermoFisher Scientific, 23225) and working samples were prepared using Laemmli sample buffer (BioRad, 161.737). Equal amounts of working sample (10 µg) were loaded into pre-cast polyacrylamide gels (BioRad, 4568083) with the addition of the Kaleidoscope™ ladder (BioRad, 1610375) for molecular weight determination. Proteins were transferred onto a nitrocellulose membrane (BioRad, 1704270), and a ponceau stain for total protein content was acquired (Sigma-Aldrich, P7170). All membranes were blocked for 1 hour in 5% bovine serum albumin (BioShop, ALB007) prepared in Tris-Buffered Saline (BioRad, 1706435) with 0.1% Tween 20 (TBST). After blocking, membranes were incubated overnight with primary antibodies prepared in TBST at 4°C. Membranes were then washed in TBST and incubated with anti-rabbit or anti-mouse IgG conjugates with horseradish peroxidase secondary antibodies for 1 hour at room temperature. Signals were detected using enhanced chemiluminescence (BioRad), and images were quantified using Image Lab (V6.1, BioRad). The following antibodies were used: ERα at 1:500 dilution (F-10, Santa Cruz Biotechnology, sc-8002 mouse) and eNOS at 1:1000 dilution (Cell Signalling, 32027S rabbit).

Statistical Analysis

All analyses were performed using the Statistical Package for Social Sciences (SPSS; IBM) version 26 (IBM, Chicago, IL, USA). All figures were made using GraphPad Prism (La Jolla, CA). All data are reported as mean ± SD, with statistical significance set to $p \leq 0.05$. A one-way analysis of variance (ANOVA) for the three groups (NAT, OCP2, OCP3) was used to compare baseline characteristics, including age, anthropometric

measures, cardiorespiratory fitness, and body composition. A linear mixed model (compound symmetry covariance structure) with factors phase (within-group: LH, HH) and group (between-groups; NAT, OCP2, OCP3) was used to examine blood measures, HR, BP, PWV, carotid artery stiffness, FMD, NMD, and cell exposure proteins. For FMD and NMD, measures of resting diameter, peak diameter, absolute and relative % for each were are assessed, as well as time to peak, baseline SR and SRAUC for FMD, in line with current guidelines.[28] Post-hoc *t*-tests with a Bonferroni correction to account for multiple comparisons were used to examine significant main effects or interactions. For levonorgestrel in OCP2 only, a paired t-test was used to compare across LH and HH phases. Associations between duration of use for OCP and vascular measures, and cellular protein content and brachial and femoral artery %FMD was assessed using Pearson's correlations, where the average across phases was used given the lack of changes across phases. Additional exploratory analysis examining the duration of use for OCP and carotid artery IMT, β-stiffness, distensibility, and compliance using Pearson's correlations.

Results

Participant Characteristics & Hormonal Cycling

Participant characteristics and hormonal cycling details are included in Table 1. There were no differences across NAT, OCP2, and OCP3 groups for participant characteristics. OCP2 participants used daily monophasic pills (Brands: Alesse [(n=5), Alysena (n=12)] containing 0.02 mg ethinyl estradiol and 0.1 mg levonorgestrel (21 days), with a 7 day placebo week. OCP3 participants used either daily monophasic pills (n=7, Marvelon/Mirvala/Freya, daily 0.03 mg ethinyl estradiol, 0.15 mg desogestrel) or one of three triphasic pill combinations with testing in the highest active week: Linessa

(n=3, 0.025 mg ethinyl estradiol, 0.15 mg desogestrel), Tricira Lo (n=3, 0.025 mg ethinyl estradiol, 0.25 mg norgestimate) or Tri-Jordyna (n=2, 0.035 mg ethinyl estradiol, 0.25 mg norgestimate).

Blood Analysis: Hematocrit & Sex Hormones

There was no main effect of group (p=0.36) or phase (p=0.28), or a group*phase interaction (p=0.09) for hematocrit levels (NAT LH: 42±3%, HH: 42±2%; OCP2 LH: 43±2%, HH: 42±3%; OCP3 LH: 41±4%, HH: 41±3%). There was an interaction between group and phase for both estradiol and progesterone levels, such that sex hormones were elevated in the HH phase in the NAT group only compared to LH phase (Figure 1B). There was no difference between phases for estrogen and progesterone in the OCP groups (Figure 1B). Finally, levonorgestrel was elevated in the HH phase compared to LH phase in OCP2 (LH: 79±62 pg/mL, HH: 284±177 pg/mL, p=0.01).

Resting Hemodynamics

There was a main effect of phase on resting HR, such that HR was elevated by ~2bpm in the HH phase compared to the LH phase (HH: 63±8 bpm, LH: 61±8 bpm, p=0.04; Table 2). All other hemodynamic measures were not different across group or phase (Table 2).

Arterial Stiffness

There was no effect of group or phase on measures of carotid artery stiffness (distensibility, compliance, β-stiffness) or IMT (Table 2). Similarly, there was no effect of group or phase on central or peripheral leg or arm PWV (Figure 2).

Endothelial Function (FMD)

For brachial artery FMD, there was a main effect of phase on baseline diameter (p=0.03; Table 3), such that diameter was lower in the HH phase compared to the LH phase. While there was no difference in peak diameter across groups or phases, there was a subsequent elevation in AbsFMD (p=0.04; Table 3) and %FMD (p=0.02; Figure 3A) in the HH phase compared to LH phase. However, after allometrically scaling for baseline diameter, this phasic difference was no longer significant (p=0.052, Figure 3B). Examining SR, there was an interaction between group and phase for baseline SR (p=0.049), such that no phasic differences were apparent in NAT or OPC2, but OCP3 had a significantly lower baseline SR in the HH phase compared to LH phase (p=0.01; Table 3).

For superficial femoral artery FMD, there was a main effect of phase on baseline artery diameter (Table 4), such that diameter was lower in the HH phase compared to the LH phase. In addition, there was a main effect of group on time to peak diameter, but with no significant post-hoc comparisons. However, there was no effect of group or phase on any other outcome, including peak artery diameter, SR, SR AUC, AbsFMD (Table 4) or unscaled and scaled SFA %FMD (Figures 3E & F, respectively). There was a significant positive relationship between BA %FMD and SFA %FMD (r=0.34, p=0.02).

Smooth Muscle Function (NMD)

For brachial artery NMD, there was no effect of phase or group on any smooth muscle function measurement (baseline diameter, peak diameter, or AbsNMD; Table 3), including %NMD, scaled or allometrically scaled (Figures 3C & D respectively). Similarly, for superficial femoral artery NMD, there was no effect of phase or group on any outcome (Table 4; Figures 3G & H).

Serum Exposure Protein Analysis

There was no effect of group or phase for serum exposure on eNOS protein content or ERα protein content (Figure 4). In addition, there was no relationship between average eNOS (r=-0.07, p=0.65) or ERα (r=-0.01, p=0.94) protein content and BA %FMD. Similarly, there was no relationship between average eNOS (r=-0.09, p=0.55) or ERα (r=-0.17, p=0.25) protein content and SFA %FMD.

Impact of Duration of OCP Use on Cardiovascular Outcomes

There was no effect of duration of OCP use on BA %FMD (r=0.10, p=0.58) or SFA %FMD (r=0.16, p=0.39). Examining OCP subgroups, there was also no effect of duration of use in either OCP2 (BA %FMD: r=-0.08, p=0.77; SFA %FMD: r=0.25, p=0.34) or OCP3 (BA %FMD: r=0.30, p=0.28; SFA %FMD: r=0.07, p=0.79). Similarly, there was no effect of duration of OCP use on central PWV in either OCP2 (r=0.46, p=0.07) or OCP3 (r=0.06, p=0.83). After visual inspection of the OCP2 duration of use and central PWV relationship revealed a potential outlier, removal of one participant resulted in a further non-significant effect of duration of OCP use on central PWV in OCP2 (r=0.32, p=0.23).

Examining carotid artery measurements, there was no effect of duration of OCP2 use on IMT (r=0.20, p=0.45), however, there was an initial effect of duration of use on distensibility (r=0.53, p=0.03), β-stiffness (r=-0.53, p=0.03), and compliance (r=0.57, p=0.02). However, after visual inspection identified an outlier, removal of one participant resulted in no effect of duration of use on distensiblilty (r=0.17, p=0.54), β-stiffness (r=-0.06, p=0.83), or compliance (r=0.14, p=0.62). Similarly, there was no effect of duration

of OCP3 use on carotid artery IMT (r=0.10, p=0.72), distensibility (r=-0.37, p=0.18), β-stiffness (r=-0.05, p=0.87) and compliance (r=-0.13, p=0.64).

Table 1. Participant Characteristics & Hormonal Cycling Details

	NAT	OCP2	OCP3	Main Effects
Age (years)	21 ± 2	21 ± 2	21 ± 3	p = 0.85
Height (cm)	164.1 ± 8.3	163.7 ± 7.0	164.4 ± 5.8	p = 0.96
Weight (kg)	67.0 ± 14.3	59.2 ± 9.0	61.8 ± 7.6	p = 0.11
BMI (kg/m^2)	24.9 ± 4.9	22.1 ± 3.2	22.8 ± 2.2	p = 0.08
Waist: Hip Ratio (A.U.)	0.77 ± 0.05	0.78 ± 0.05	0.76 ± 0.04	p = 0.41
Lean Body Mass (kg)	41.2 ± 4.7	38.8 ± 4.1	39.5 ± 3.7	p = 0.25
Fat Mass (kg)	23.1 ± 11.5	18.0 ± 6.0	20.2 ± 6.5	p = 0.22
%Fat Mass	33.2 ± 9.1	29.9 ± 5.7	32.1 ± 7.3	p = 0.43
$\dot{V}O_2$Peak (ml/kg/min)	38.2 ± 8.9	40.1 ± 6.9	38.5 ± 7.0	p = 0.74
Average Menstrual Cycle Length (days)	29 ± 2	--	--	--
Average Ovulation Date (days)	16 ± 3	--	--	--
Length of Time on Contraception (months)	--	40 ± 28 (3.3 ± 2.3 years)	29 ± 23 (2.4 ± 1.9 years)	--
Average Hours Since Pill Consumption at Testing (Active Phase) (hours)	--	11 ± 8	13 ± 7	--
Low Hormone Testing Date (Early Follicular/Placebo Phase; days since menses)	4 ± 2	5 ± 1	4 ± 1	--
High Hormone Testing Date (Mid-Luteal/Active Phase; days since menses)	22 ± 3 7 ± 1 days since ovulation	25 ± 2	24 ± 2	--

Table Caption: Mean ± SD. NAT = naturally cycling; OCP2 = oral contraceptive pill 2nd generation users; OCP3 = oral contraceptive pill 3rd generation users; A.U. = arbitrary units; BMI = body mass index.

Table 2. Resting Hemodynamics & Carotid Artery Stiffness

	NAT		OCP2		OCP3		Main Effects & Interactions
	LH	HH	LH	HH	LH	HH	
Resting Heart Rate (bpm)	63 ± 8	63 ± 8*	60 ± 7	61 ± 7*	60 ± 8	64 ± 8*	G: p = 0.62 **P: p = 0.04** G*P: p = 0.27
Resting Systolic Blood Pressure (mmHg)	103 ± 8	104 ± 6	106 ± 10	105 ±10	107 ± 5	105 ± 4	G: p = 0.44 P: p = 0.40 G*P: p = 0.46
Resting Diastolic Blood Pressure (mmHg)	62 ± 6	63 ± 6	65 ± 8	63 ± 9	63 ± 6	62 ± 6	G: p = 0.67 P: p = 0.20 G*P: p = 0.12
Resting Mean Arterial Pressure (mmHg)	77 ± 6	78 ± 5	80 ± 9	78 ± 9	79 ± 5	78 ± 4	G: p = 0.66 P: p = 0.19 G*P: p = 0.21
Carotid Artery Compliance (cm^2 x $mmHg^{-1}$)	0.15 ± 0.05	0.15 ± 0.05	0.13 ± 0.04	0.13 ± 0.06	0.13 ± 0.04	0.13 ± 0.04	G: p = 0.30 P: p = 0.69 G*P: p = 0.87
Carotid Artery Distensibility ($x10^3$ $mmHg^{-1}$)	5.6 ± 2.0	5.9 ± 2.1	5.2 ± 1.5	5.5 ± 2.5	5.3 ± 1.4	5.2 ± 1.9	G: p = 0.68 P: p = 0.51 G*P: p = 0.76
Carotid Artery β-Stiffness (A.U.)	5.0 ± 1.8	5.1 ± 1.6	5.2 ± 1.0	5.4 ± 1.3	5.0 ± 1.4	5.6 ± 1.2	G: p = 0.76 P: p = 0.18 G*P: p = 0.54
Carotid Artery Intima Media Thickness (mm)	0.41 ± 0.06	0.40 ± 0.05	0.41 ± 0.06	0.41 ± 0.06	0.38 ± 0.06	0.39 ± 0.06	G: p = 0.44 P: p = 0.91 G*P: p = 0.40

Table Caption: Mean ± SD. NAT = naturally cycling; OCP2 = oral contraceptive pill 2nd generation users; OCP3 = oral contraceptive pill 3rd generation users; LH = low hormone; HH = high hormon; G = group; P = phase; A.U. = arbitrary units; mmHg = millimeters of mercury. *Main effect of phase, such that heart rate was higher in the HH phase compared to LH phase.

Table 3. Vascular Function Assessments: Brachial Artery FMD, NMD

	NAT		OCP2		OCP3		Main Effects & Interactions
	LH	HH	LH	HH	LH	HH	
Brachial Artery FMD							
Baseline Diameter (mm)	3.65 ± 0.30	3.63 ± 0.32*	3.54 ± 0.53	3.52 ± 0.49*	3.38 ± 0.33	3.33 ± 0.31*	G: p = 0.13 **P: p = 0.03** G*P: p = 0.55
Peak Diameter (mm)	3.86 ± 0.32	3.85 ± 0.31	3.81 ± 0.50	3.83 ± 0.46	3.62 ± 0.31	3.59 ± 0.31	G: p = 0.14 P: p = 0.41 G*P: p = 0.53
AbsFMD (mm)	0.21 ± 0.10	0.22 ± 0.07^	0.27 ± 0.10	0.30 ± 0.11^	0.24 ± 0.10	0.26 ± 0.11^	G: p = 0.08 **P: p = 0.04** G*P: p = 0.51
Time to Peak (s)	51 ± 18	45 ± 15	52 ± 17	54 ± 22	46 ± 16	49 ± 15	G: p = 0.49 P: p = 0.92 G*P: p = 0.44
Baseline SR (s^{-1})	153.3 ± 90.1	132.4 ± 76.4	185.3 ± 75.3	212.5 ± 99.3	241.5 ± 131.0	168.6 ± 75.7#	**G: p = 0.04** P: p = 0.17 **G*P: p = 0.05**
SR AUC to Peak (x10^3 s^{-1})	22.5 ± 21.5	16.8 ± 10.8	24.8 ± 15.8	32.7 ± 32.5	27.4 ± 24.3	27.9 ± 20.7	G: p = 0.31 P: p = 0.77 G*P: p = 0.24
Brachial Artery NMD							
Baseline Diameter (mm)	3.58 ± 0.30	3.54 ± 0.32	3.52 ± 0.53	3.51 ± 0.51	3.26 ± 0.33	3.26 ± 0.33	G: p = 0.12 P: p = 0.35 G*P: p = 0.74
Peak Diameter (mm)	4.35 ± 0.39	4.31 ± 0.40	4.28 ± 0.58	4.31 ± 0.54	4.10 ± 0.32	4.06 ± 0.32	G: p = 0.28 P: p = 0.35 G*P: p = 0.36
AbsNMD (mm)	0.77 ± 0.22	0.76 ± 0.19	0.77 ± 0.16	0.79 ± 0.18	0.84 ± 0.11	0.80 ± 0.14	G: p = 0.68 P: p = 0.75 G*P: p = 0.33

Table Caption: Mean ± SD. NAT = naturally cycling; OCP2 = oral contraceptive pill 2nd generation users; OCP3 = oral contraceptive pill 3rd generation users. Abs = absolute; SR = shear rate; AUC = area under the curve.; LH = low hormone; HH = high hormon; G = group; P = phase. *Baseline diameter was lower in the HH phase compared to the LH phase. ^AbsFMD was higher in the HH phase compared to LH phase. #Baseline SR was lower in the HH phase compared to LH phase of OCP3.

Table 4. Vascular Function Assessments: Superficial Femoral Artery FMD, NMD

	NAT		OCP2		OCP3		Main Effects & Interactions
	LH	HH	LH	HH	LH	HH	
Superficial Femoral Artery FMD							
Baseline Diameter (mm)	5.31 ± 0.56	5.33 ± 0.48*	5.51 ± 0.58	5.43 ± 0.48*	5.26 ± 0.53	5.10 ± 0.50*	G: p = 0.29 **P: p = 0.02** G*P: p = 0.07
Peak Diameter (mm)	5.68 ± 0.61	5.69 ± 0.53	5.86 ± 0.59	5.83 ± 0.47	5.59 ± 0.47	5.44 ± 0.43	G: p = 0.20 P: p = 0.07 G*P: p = 0.14
AbsFMD (mm)	0.37 ± 0.14	0.36 ± 0.15	0.36 ± 0.14	0.40 ± 0.16	0.33 ± 0.17	0.34 ± 0.20	G: p = 0.73 P: p = 0.55 G*P: p = 0.29
Time to Peak (s)	56 ± 25	63 ± 21	83 ± 33	82 ± 36	83 ± 44	83 ± 38	**G: p = 0.03** P: p = 0.64 G*P: p = 0.82
Baseline SR (s^{-1})	150.3 ± 98.9	134.6 ± 72.4	137.5 ± 68.3	138.2 ± 51.7	134.9 ± 61.2	129.3 ± 68.4	G: p = 0.91 P: p = 0.38 G*P: p = 0.68
SR AUC to Peak ($\times 10^3\, s^{-1}$)	35.7 ± 33.4	50.6 ± 45.9	59.6 ± 39.3	61.3 ± 42.0	63.0 ± 58.3	61.8 ± 52.9	G: p = 0.31 P: p = 0.46 G*P: p = 0.62
Superficial Femoral Artery NMD							
Baseline Diameter (mm)	5.32 ± 0.52	5.28 ± 0.53	5.26 ± 0.63	5.26 ± 0.55	5.25 ± 0.34	5.23 ± 0.40	G: p = 0.99 P: p = 0.84 G*P: p = 0.85
Peak Diameter (mm)	5.95 ± 0.56	5.93 ± 0.48	5.96 ± 0.61	5.96 ± 0.64	5.87 ± 0.40	5.91 ± 0.44	G: p = 0.94 P: p = 0.62 G*P: p = 0.90
AbsNMD (mm)	0.63 ± 0.28	0.65 ± 0.24	0.70 ± 0.20	0.69 ± 0.22	0.62 ± 0.24	0.68 ± 0.23	G: p = 0.81 P: p = 0.60 G*P: p = 0.58

Table Caption: Mean ± SD. NAT = naturally cycling; OCP2 = oral contraceptive pill 2nd generation users; OCP3 = oral contraceptive pill 3rd generation users. Abs = absolute; SR = shear rate; AUC = area under the curve.; LH = low hormone; HH = high hormone; G = group; P = phase. *Baseline diameter was lower in the HH phase compared to the LH phase.
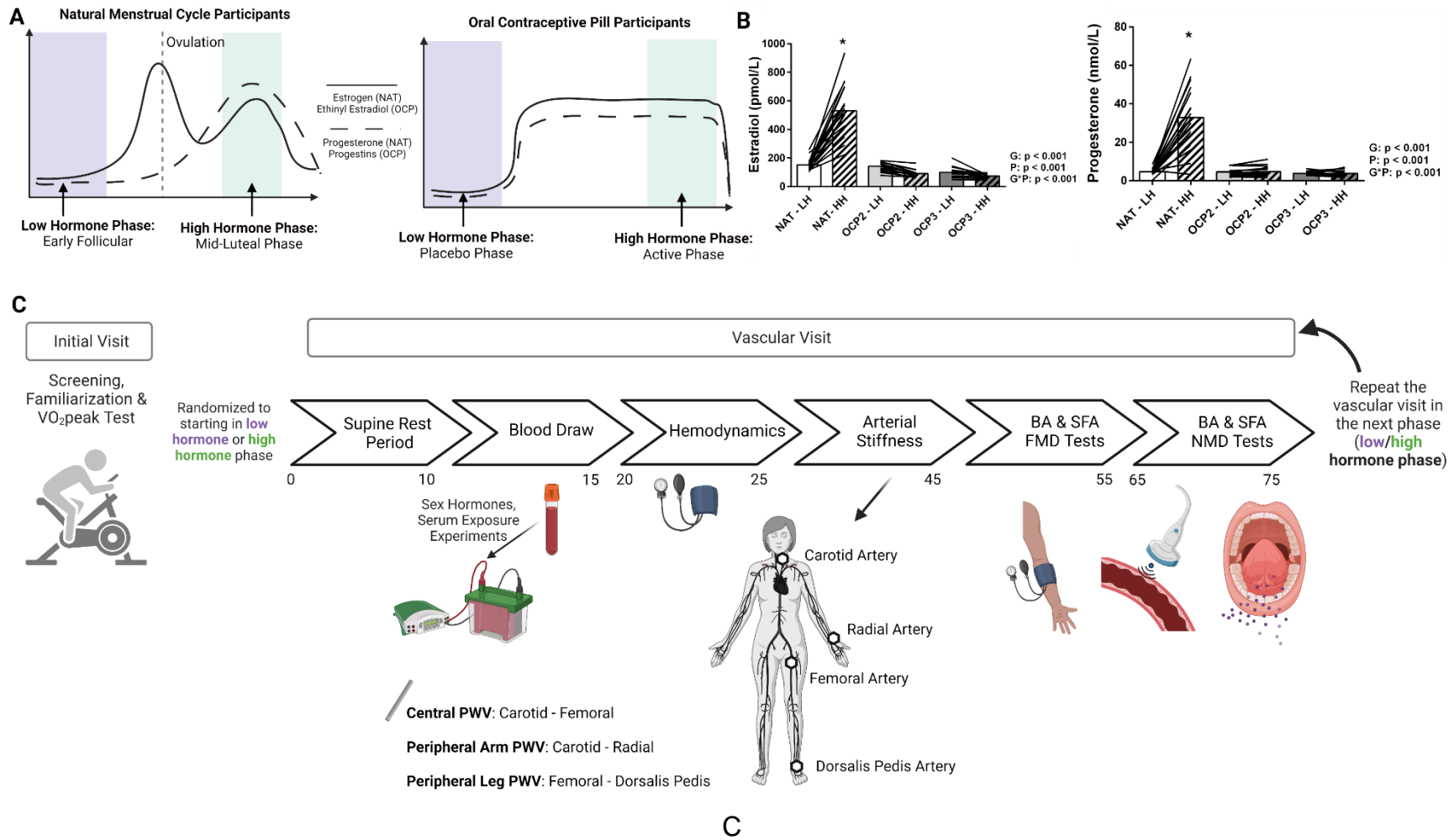
Figure 1. Hormonal Cycling Phase Testing Periods & Sex Hormone Confirmation and Study Protocol. (A) Hormonal cycling phase testing. Participants were tested in the LH phase (NAT = early follicular, OCP2/3 = placebo) and HH phase (NAT = mid-luteal, OCP2/3 = active) in a randomized order. This figure demonstrates a schematic of expected endogenous and exogenous hormone levels. **(B) Endogenous 17β-Estradiol and Progesterone Levels**. There was an interaction between group and phase for both endogenous estradiol and progesterone, such that these sex hormones were elevated in the HH phase compared to LH phase of NAT only; OCP2 and OCP3 experienced a suppression of endogenous hormones during the active phase of the cycle, compared to NAT. **(C) Study Protocol.** Prior to participating in the vascular testing sessions, participants completed records of hormonal cycling (including tracking for >2 months if NAT), and completed a cardiorespiratory fitness test. During each vascular testing visit, participants first rested supine for 10 minutes, followed by a standard blood draw. Participants then rested, and hemodynamic measures of heart rate and blood pressure were taken after 5 minutes. Central and peripheral arterial stiffness of the common carotid artery (CCA) and pulse wave velocity of the central and peripheral vasculature was assessed. This was followed by three vascular function tests: a reactive hyperemia flow-mediated dilation (FMD) test of the brachial (BA) and superficial femoral (SFA) arteries and a nitroglycerine-mediated dilation (NMD) test of the BA and SFA; tests were separated each by at least 10 minutes of rest. Figure created in Biorender.com.
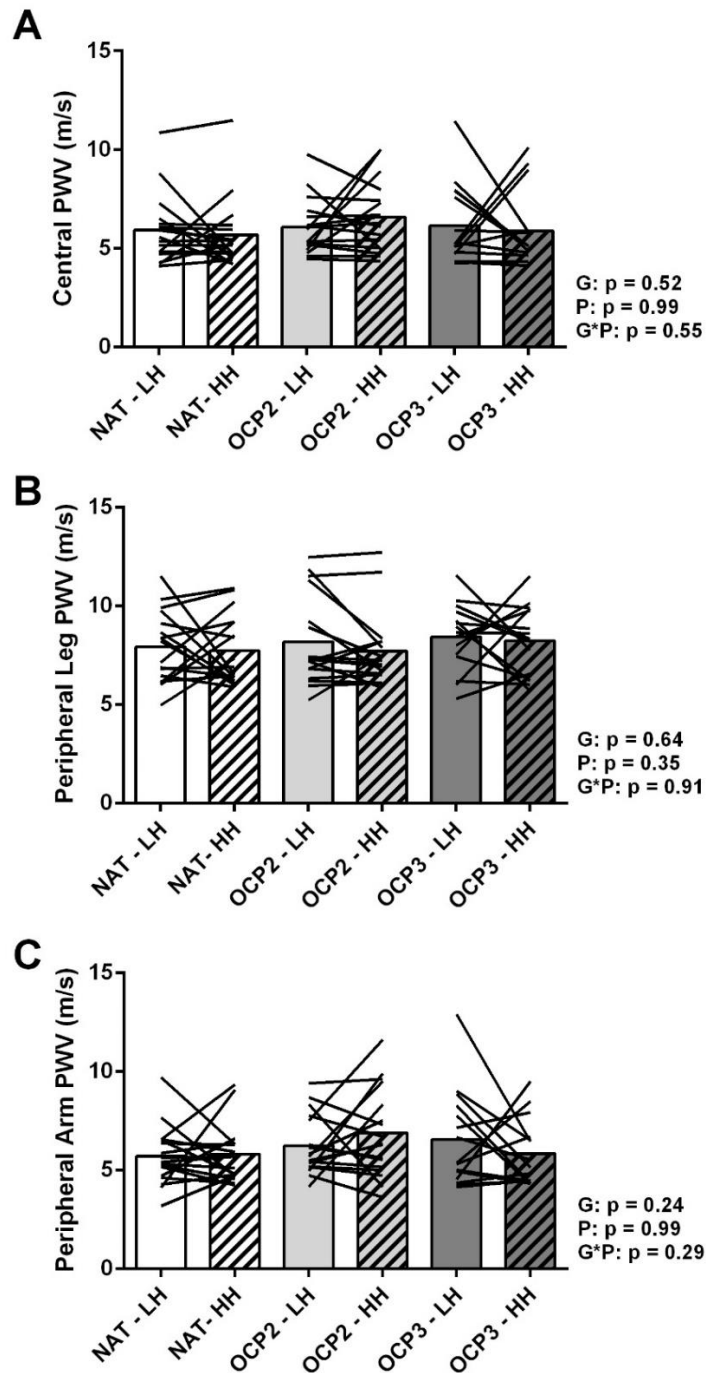
Figure 2. Central and Peripheral Arterial Stiffness. Measures of central PWV, peripheral leg PWV and peripheral arm PWV are detailed in panels A, B, and C respectively. Bars represent the mean, with connected lines representing individual responses across phases. NAT= naturally cycling; OCP2 = oral contraceptive pill 2nd generation users; OCP3 = oral contraceptive pill 3rd generation users; LH = low hormone; HH = high hormone; PWV = pulse wave velocity; G = group; P = phase.
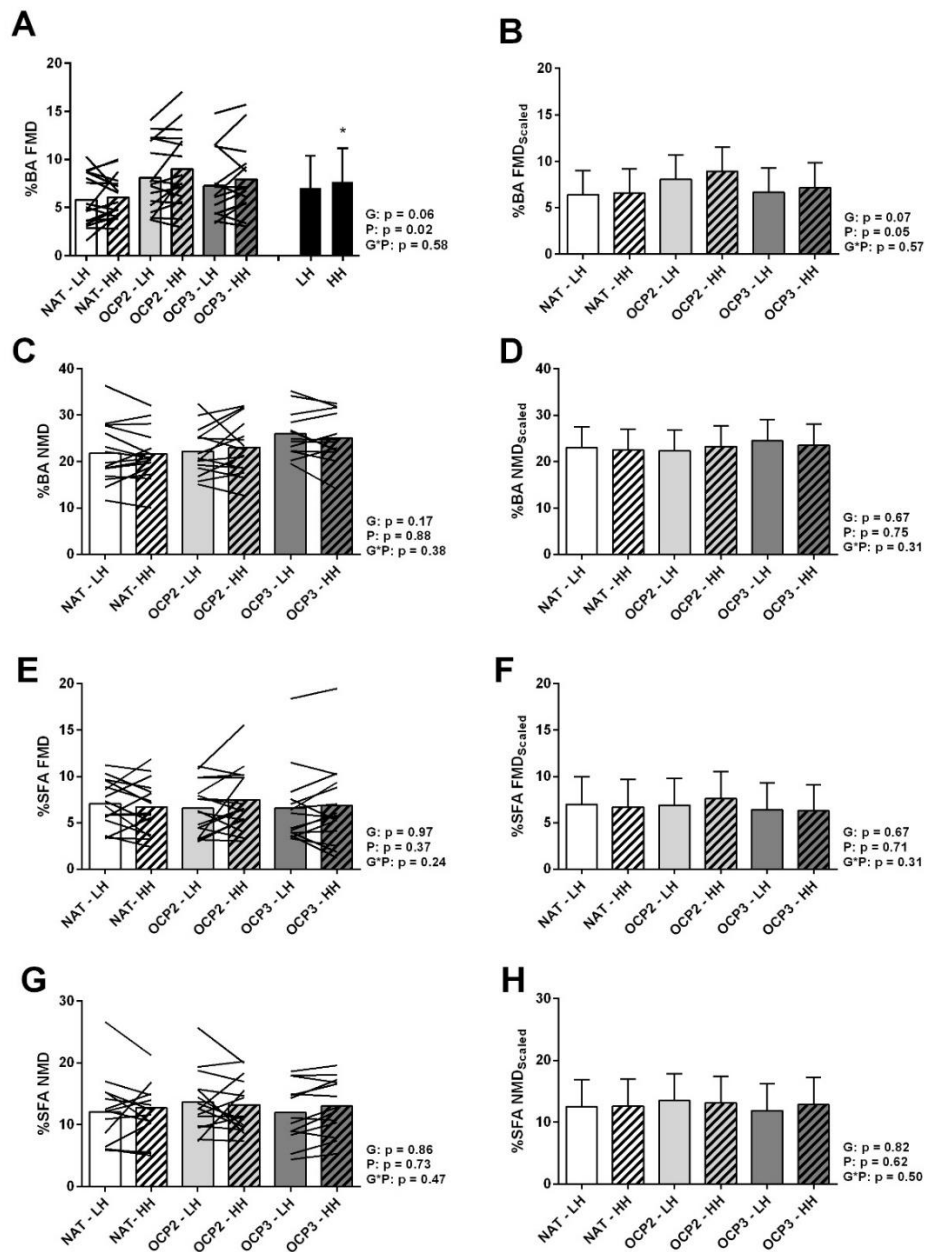
Figure 3. Endothelial Function & Smooth Muscle Function in the Brachial and Superficial Femoral Arteries. Brachial artery endothelial function (measured via a flow-mediated dilation test) is represented in panels A (unscaled) and B (allometrically scaled), while smooth muscle function (measured via a nitroglycerine-mediated vasodilation test) is represented in panels C (unscaled) and D (scaled). Superficial femoral artery endothelial function and smooth muscle function is similarly represented in panels E-H. Bars represent the mean, with connected lines representing individual responses across phases. NAT= naturally cycling; OCP2 = oral contraceptive pill 2nd generation users; OCP3 = oral contraceptive pill 3rd generation users; LH = low hormone; HH = high hormone; G = group; P = phase. *Significant elevation in %BA FMD in HH versus LH phase.
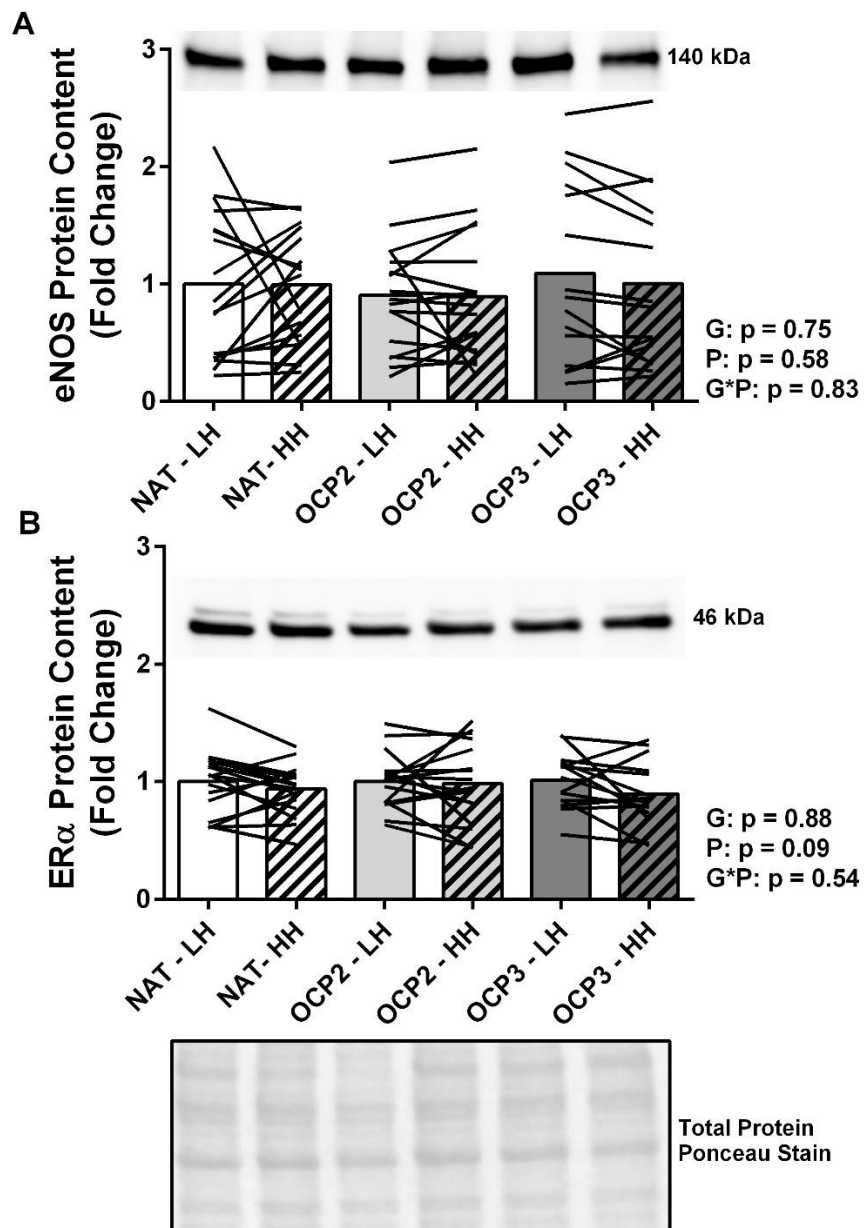
Figure 4. Serum Exposure Cellular Regulation. Endothelial nitric oxide synthase (eNOS) and estrogen receptor alpha (ERα) protein content was assessed using Western blotting following a 24h serum exposure period on female human umbilical vein endothelial cells. Fold change was used to compare changes in protein content with the NAT-LH phase as reference. Bars represent the mean, with connected lines representing individual responses across phases. NAT = naturally cycling; OCP2 = oral contraceptive pill 2nd generation users; OCP3 = oral contraceptive pill 3rd generation users; LH = low hormone; HH = high hormone; G = group; P = phase.

Discussion

This was the first study to comprehensively examine the influence of NAT and two generations of OCPs on a suite of cardiovascular outcomes and associated cellular regulation. NAT and OCP cycles have no influence on carotid or peripheral arterial stiffness, blood pressure, or smooth muscle function in both the brachial and femoral arteries, or endothelial function in the femoral artery. Further, we observed that serum exposure in female HUVECs had no impact on eNOS or ERα protein content. However, there was a small influence of the HH phase (elevated endogenous or exogenous estrogen and progesterone) on brachial artery endothelial function, which was no longer significant when %FMD was allometrically scaled. This increase in unscaled %FMD during the HH phase may be likely a product of alterations in vascular tone (i.e., constricted baseline artery diameter) with potential changes in autonomic nervous system activity (e.g., small but consistent elevation in HR). This study has implications on reconciling existing research and providing guidance for future investigations involving female participants.

Impact of Hormonal Cycling on Endothelial Function

In examining the influence of NAT and OCP cycles on brachial artery relative %FMD, we found a small (d=~0.2) effect of the HH phase versus LH phase when endogenous and exogenous estradiol and progesterones were elevated (increase of 0.7% in FMD: LH: 7.0±3.3%, HH: 7.7±3.5%); however, this effect was absent when scaled for differences in baseline artery diameter. These observations are in line with some previous literature on NAT[47, 48] although findings are mixed, reporting either varying degrees of magnitude of improvement or null effects.[7, 16, 49, 50] Early research

reported very large elevations in brachial artery relative %FMD during the late follicular and mid-luteal phases as compared to the early follicular phase in NAT.[48] For example, early work by Hashimoto *et al* (1995) reported a ~6% increase in relative %FMD from the early follicular to mid-luteal phases of the NAT cycle[48]; similarly, Kawano *et al* (2001) and (1996) reported a ~4% elevation.[47, 51] Further work by Harris *et al* (2012) also reported a ~3% elevation in relative %FMD during the mid-luteal phase.[52] The findings from these early studies have driven debate as to the relevance of considering NAT and other hormonal cycles when testing vascular function.[53] However, the majority of more recent literature did not observe the same elevation in relative %FMD[7]; for example, work by Shenouda *et al* (2018) reported a non-significant ~0.4% elevation in relative %FMD ,[16] while Rakobowchuck *et al* (2013) reported a non-significant ~1.5% decrease in relative %FMD.[54] To reconcile this conflicting literature, a recent meta-analysis by our group observed a small, non-significant increase endothelial function in the NAT mid-luteal phase compared to early follicular phase (n=12 studies, SMD: 0.37), although there was considerable heterogeneity of the findings.[7] In contrast to early findings, this meta-analysis found a more subtle influence of NAT on relative %FMD, potentially due to differences in FMD analysis methodologies (i.e., discrete vs. continuous analysis), in line with the small magnitude of %FMD elevation in the HH phase in the current study.[7]

Early cross-sectional research reported no influence of OCP cycle phases on relative %FMD when OCP types were grouped together, which may have masked the reported differences between generations of OCPs.[55] In contrast, there is some literature showing decreased relative %FMD during the active phase in 2nd generation

very low dose users (20 mcg ethinyl estradiol/100 mcg levonorgestrel), with no difference across phases in low dose users (30 mcg ethinyl estradiol/150 mcg levonorgestrel).[19] Similarly, research on 3rd generation OCPs have observed an increase in FMD with low dose (30 mcg ethinyl estradiol/150 mcg desogestrel) but no change at a very low dose composition (20 mcg ethinyl estradiol/150 mcg desogestrel).[11, 15] Participants in the current study were on OCP dosages similar to the previous study (very low dose OCP2 and low dose OCP3 as detailed above) and we found an overall elevation in relative %FMD in the HH phase regardless of group. There may be differences in study population or testing parameters, such as familiarization and random order of testing which could explain these subtle differences in results between studies.

One initially proposed explanation for the elevated relative %FMD observed in the BA during the HH phase of the current study is the elevation of nitric oxide via the ERα-eNOS signalling pathway.[9] However, serum exposure experiments do not support this hypothesis as there was no alignment between *in vitro* results and *in vivo* FMD findings, and further no relationship between these associated cellular proteins and functional vascular outcomes. An alternative hypothesis may be that the vasculature is experiencing alterations in autonomic nervous system activity, evidenced by an small increase in HR (~2 bpm) and vasoconstriction of the brachial and femoral arteries at baseline during the HH phase. Specifically, constriction of the brachial artery in the HH phase may have artificially increased relative %FMD (unscaled), which, when scaled for differences in baseline artery diameter, resulted in no main effect of phase. Briefly, there is an existing body of literature that has observed elevated HR and either increased

sympathetic activity or increased vagal withdrawal (parasympathetic activity) when progesterone is elevated.[56, 57] Exploring additional alternative hypotheses, some previous literature has identified a small increase in HR (~3-5%) across cycle phases associated with a concurrent increase in basal body temperature during supine rest.[58, 59] It is plausible that mimicry of exogenous progesterones may act similarly to endogenous progesterone in elevating HR through autonomic nervous system alterations and/or through elevations in body temperature.

We did not observe group-level differences in brachial artery endothelial function across NAT and OCP groups. This finding is not aligned with the majority of previous literature which has observed impaired relative %FMD in OCP2 compared to NAT and OCP3.[18, 60, 61] In contrast, evidence in Figure 3A&B suggest that there may be a non-significant trend towards elevated relative %FMD in OCP2 (main effect of group p = 0.06; NAT vs OCP2 p = 0.06). Previous work by Heidarzadeh *et al* (2014) reported lower FMD in OCP2 compared to NAT.[60] The same finding was reported in several other cross-sectional and interventional trials.[18, 61] Research by Shenouda *et al* (2018) did not observe group-level differences between NAT and OCP; however, this previous study did not have the sample size to disaggregate OCP users by generation type.[16] What may be unique about the present study, and in contrast to previous mixed literature, is matching between groups. We were able to match for body composition and cardiorespiratory fitness (Table 1), which are not commonly assessed in contraceptive research studies and may confound some previous vascular outcome reporting.

We did not observed an effect of NAT or OCP cycle groups or phases on femoral artery endothelial function apart from a slight constriction of the vessel during the HH phase – similar to the brachial artery during the FMD test. This finding has not been previously reported in the literature. The discordance between the brachial and femoral arteries is likely a product of structural differences and/or the sensitivity to neural stimuli between the artery beds.[62-64] The femoral artery is more resistant to changes in functional interventions, such as through exercise or other blood flow perturbations,[64, 65] unless in a cohort already predisposed to atherosclerosis development (i.e., smoking).[66] In a young, healthy cohort, this may be a result of greater use of the lower limbs in activities of daily living, providing protection against any perturbations in function, as suggested previously by King & Pyke (2019).[65]

Impact of Hormonal Cycling on Smooth Muscle Function

This was the first study to examine both upper and lower limb smooth muscle function responses to NAT and OCP cycles and phases. We observed no effect of hormonal cycling on smooth muscle function. While literature is sparse, there is some evidence that these findings align with the majority of previous work investigating smooth muscle function.[16, 47, 48, 51, 67] For example, similar to brachial endothelial function research, early work by Hashimoto *et al* (1995) identified an increase in brachial artery smooth muscle function in the late follicular and mid-luteal phases.[48] However, subsequent research by Sorensen *et al* (2006) did not identify phasic differences in relative %NMD.[67] Similarly, work by Shenouda *et al* (2018) also did not find any phasic differences in relative %NMD in NAT or OCP users.[16] In line with this body of literature, a previous meta-analysis by our group observed no differences

across the NAT cycle early follicular to mid-luteal phase on smooth muscle function (n=7 studies, SMD: 0.19) – though the studies available only examined the brachial artery.[7] However, Shenouda *et al* (2018) did note a significantly lower scaled NMD in NAT compared to OCP users.[16] This finding contrasts the lack of group-based findings from this study, but may point to differences in NAT versus OCP groups in the prior study, as discussed previously. There is a paucity of research comparing NAT versus OCP groups, and the present study is one of the first studies to confirm that there were no group-level differences in arterial smooth muscle function. Considering OCP cycles, all previous literature examining smooth muscle function in OCP2 and OCP3 observed no influence of OCP phase on smooth muscle function.[11, 15, 16, 19] Given the findings described in the majority of previous literature, and concurrent with the present study, we can posit that hormonal cycles do not influence upper or lower limb vascular smooth muscle function.

Impact of Hormonal Cycling on Arterial Stiffness

We observed no effect of NAT or OCP groups or phases on measures of carotid artery stiffness, including distensibility, compliance, and β-stiffness, or structural measures of IMT. There is a paucity of literature to compare to, however, early work by Franceschini *et al* (2013) and Lizarelli *et al* (2009) reported no impact of OCP2 on β-stiffness.[18, 61] These findings have been corroborated by our group previously,[17] and in the present study. Considering IMT, there is some prior cross-sectional research that reported elevated thickness in OCP2 compared to NAT; however, the effects were moderate (increase of ~0.09mm) and found in a population using OCPs for longer duration (~3 years versus 4.5 years).[60] While increases in carotid IMT among OCP2

users has been corroborated by another interventional study after 6 months of OCP2

use, there was no difference cross-sectionally comparing OCP2 with NAT.[18] Finally,

previous work by our group has not observed differences between groups or phases for

IMT.[17] While we have been unable to corroborate prior literature finding elevated IMT in

OCP2, further research is needed to confirm these findings.

Similarly, we observed no influence of NAT or OCP groups or phases on

measures of central or peripheral arterial stiffness, specifically central, peripheral arm or

peripheral leg PWV. This is consistent with recent research by Priest *et al* (2018) that

did not observe alterations in central or peripheral PWV across NAT or combined

generation OCP phases.[17] There was only one previous study that reported a small

increase in central PWV with OCP versus NAT; however, the small increase in PWV

was not clinically relevant and also did not disaggregate by OCP generation, rendering

it unable to be compared to the present study.[68] PWV, when measured by digital pulse

waveform acquisition, did not indicate phasic differences in OCP2 or OCP3, or across

groups[15], and this research has been since confirmed another study.[69] In summary,

based on previous literature and the present study, OCP and NAT cycles do not appear

to influence measures of central or peripheral PWV.

Duration of OCP Use on Vascular Outcomes

We observed no effect of the duration of contraceptive use on brachial artery

endothelial function in either OCP2 or OCP3 groups, and also no effect on central PWV.

The former was not in concordance with previous literature reported by Shenouda *et al*

(2018) who found a moderate negative relationship between duration of OCP2 use and

brachial artery FMD, but not combined 3rd and 4th generation OCP users, even after

controlling for age.[16] Both studies had comparable averages and spreads of use (OCP2: ~29 months Shenouda, ~40 months Williams; OCP3: ~57 months Shenouda, ~29 months Williams), although it is plausible that the shorter duration of use of OCP2 in the paper by Shenouda *et al* (2018) may point to earlier remodeling changes.[16] In contrast, it is more likely that, due to small sample sizes, selection of OCP2 participants with a more dramatic decline in FMD may have resulted in this spurious finding. Longer duration of contraceptive use studies, and studies involving longitudinal study designs, are necessary to draw any conclusions on the long-term influence of OCP use.

Limitations

While there were several strengths to this study, including the quantity and quality of vascular measures, inclusion of both *in vivo* outcomes and *in vitro* cellular regulation experimentation, and methodological rigour regarding hormonal cycle considerations, there are some limitations to recognize. First, this study focused on only two generations of OCPs rather than including 1st and 4th generation OCPs, or non-oral hormonal contraceptive options. As this study utilized convenience sampling, the availability of participants using OCPs was taken into consideration, with 2nd and 3rd generation OCPs as the most commonly prescribed OCPs in Canada. Second, this study focused primarily on young, generally healthy, premenopausal females, and was therefore limited in its generalizability to older populations who have other conditions (e..g, hypertension, diabetes) and we were additionally limited in examining duration of use of OCPs. Third, we only examined two cellular proteins known previously to be connected to hormone-related changes in endothelial function; further research is needed to examine the influence of other cellular or organ-level regulation pathways

(e.g., progesterone receptor activation, smooth muscle proliferation, interactions with lipids/lipoproteins, glucose regulation) and central and peripheral nervous system output on vascular outcomes. Fourth, while we were able to quantify endogenous 17β-estradiol and progesterone levels, and synthetic progestin levonorgestrel (found in OCP2), we were unable to quantify synthetic ethinyl estradiol or progestins norgestimate or desogestrel (found in OCP3). This limitation is due to the lack of accessible in-house or clinical assays to test these hormones; further development of synthetic hormone assays would greatly enhance the impact of future research involving contraceptive use. Another limitation to this study was the range of hours since participants have taken their pill and the vascular data collection sessions, recognizing that exogenous hormone levels fluctuate since the time ingested; however, a strength of this study is in reporting the duration of time for context which is uncommon in research studies. Finally, the cardiovascular outcomes were examined during supine rest, rather than during dynamic challenges such as stress tasks, small limb exercise tests, or metabolic challenges (e.g., oral glucose tolerance test, high fat meal challenge); dynamic challenges may be better able to manifest any subtle influence of the hormonal cycles on vascular outcomes and thus requires further study.[52, 70]

Conclusions & Future Directions

The natural menstrual cycle and 2nd and 3rd generation OCP cycles appear to minimally influence a comprehensive suite of cardiovascular outcomes, including blood pressure, carotid and peripheral arterial stiffness, brachial and femoral artery smooth muscle function, femoral artery endothelial function, and underlying cellular regulation (eNOS, ERα protein content). However, there may be a small effect of the HH (mid-

luteal/active) phase on brachial artery endothelial function, which may be partially explained by constricted resting vascular tone and autonomic nervous system activation. This study provides novel and timely insights into the role of the natural menstrual cycle and OCP cycles on peripheral vascular physiology, given the calls for increased inclusion of female participants in human research.[21, 71, 72] This study also challenges the notion that female hormonal cycles must be controlled for in cardiovascular physiology research; in contrast, researchers are encouraged to *consider* the hormonal cycles alongside other experimental controls depending on the study design. Further research is needed to explore the potential influence of the hormonal cycles on nervous system activity and its regulation on vascular tone. Finally, future examination of other forms of hormonal contraceptives, especially intrauterine devices, and considering older premenopausal populations who may have been exposed to contraceptives for a longer duration is needed.

References

1. United Nations Department of Economic and Social Affairs. Contraceptive Use by Method 2019: Data Booklet. *United Nations*. 2019.

2. Kaminski P, Szpotanska-Sikorska M and Wielgos M. Cardiovascular risk and the use of oral contraceptives. *Neuro endocrinology letters*. 2013;34:587-9.

3. Tanis BC, van den Bosch MA, Kemmeren JM, Cats VM, Helmerhorst FM, Algra A, van der Graaf Y and Rosendaal FR. Oral contraceptives and the risk of myocardial infarction. *The New England journal of medicine*. 2001;345:1787-93.

4. Green DJ, Jones H, Thijssen D, Cable NT and Atkinson G. Flow-mediated dilation and cardiovascular event prediction: does nitric oxide matter? *Hypertension*. 2011;57:363-9.

5. Mitchell GF, Hwang S-J, Vasan RS, Larson MG, Pencina MJ, Hamburg NM, Vita JA, Levy D and Benjamin EJ. Arterial stiffness and cardiovascular events: the Framingham Heart Study. *Circulation*. 2010;121:505-511.

6. Yeboah J, Folsom AR, Burke GL, Johnson C, Polak JF, Post W, Lima JA, Crouse JR and Herrington DM. Predictive value of brachial flow-mediated dilation for incident cardiovascular events in a population-based study: the multi-ethnic study of atherosclerosis. *Circulation*. 2009;120:502-9.

7. Williams JS, Dunford EC and MacDonald MJ. Impact of the menstrual cycle on peripheral vascular function in premenopausal women: systematic review & meta-analysis. *Submitted to American Journal of Physiology - Heart and Circulatory Physiology*. 2020.

8. Williams JS and MacDonald MJ. Influence of hormonal contraceptives on peripheral vascular function and structure in premenopausal females: a review. *American Journal of Physiology-Heart and Circulatory Physiology*. 2021;320:H77-H89.

9. Gavin KM, Seals DR, Silver AE and Moreau KL. Vascular endothelial estrogen receptor alpha is modulated by estrogen status and related to endothelial function and endothelial nitric oxide synthase in healthy women. *J Clin Endocrinol Metab*. 2009;94:3513-20.

10. Meendering JR, Torgrimson BN, Miller NP, Kaplan PF and Minson CT. Estrogen, medroxyprogesterone acetate, endothelial function, and biomarkers of cardiovascular risk in young women. *American Journal of Physiology-Heart and Circulatory Physiology*. 2008;294:H1630-H1637.

11. Meendering JR, Torgrimson BN, Miller NP, Kaplan PF and Minson CT. Ethinyl estradiol-to-desogestrel ratio impacts endothelial function in young women. *Contraception*. 2009;79:41-49.

12. Meendering JR, Torgrimson BN, Miller NP, Kaplan PF and Minson CT. A combined oral contraceptive containing 30 mcg ethinyl estradiol and 3.0 mg drospirenone does not impair endothelium-dependent vasodilation. *Contraception*. 2010;82:366-72.

13. Torgrimson BN, Meendering JR, Kaplan PF and Minson CT. Endothelial function across an oral contraceptive cycle in women using levonorgestrel and ethinyl estradiol. *American Journal of Physiology-Heart and Circulatory Physiology*. 2007;292:H2874-H2880.

14. Torgrimson BN, Meendering JR, Miller NP, Kaplan PF and Minson CT. Endothelial function, endothelin-1, and fibrinogen in young women using the vaginal contraceptive ring. *Fertility and Sterility*. 2009;92:441-447.

15. Thompson AK, Przemska A, Vasilopoulou D, Newens KJ and Williams CM. Combined oral contraceptive pills containing desogestrel or drospirenone enhance large vessel and microvasculature vasodilation in healthy premenopausal women. *Microcirculation*. 2011;18:339-46.

16. Shenouda N, Priest SE, Rizzuto VI and MacDonald MJ. Brachial artery endothelial function is stable across a menstrual and oral contraceptive pill cycle but lower in premenopausal women than in age-matched men. *Am J Physiol Heart Circ Physiol*. 2018;315:H366-H374.

17. Priest SE, Shenouda N and MacDonald MJ. Effect of sex, menstrual cycle phase, and monophasic oral contraceptive pill use on local and central arterial stiffness in young adults. *Am J Physiol Heart Circ Physiol*. 2018;315:H357-h365.

18. Franceschini SA, Vieira CS, Martins WP, Franca JB and Ferriani RA. Effects of combined oral contraceptives containing levonorgestrel or chlormadinone on the endothelium. *Contraception*. 2013;87:766-772.

19. Torgrimson BN, Meendering JR, Kaplan PF and Minson CT. Endothelial function across an oral contraceptive cycle in women using levonorgestrel and ethinyl estradiol. *American Journal of Physiology - Heart and Circulatory Physiology*. 2007;292:H2874-H2880.

20. Heart and Stroke Foundation. Ms. Understood: Women's hearts are victims of a system that is ill-equipped to diagnose, treat, and support them. *Heart and Stroke Foundation: Annual Heart Report*. 2018.

21. Sims ST and Heather AK. Myths and Methodologies: Reducing scientific design ambiguity in studies comparing sexes and/or menstrual cycle phases. *Exp Physiol*. 2018;103:1309-1317.

22. Lakens D and Caldwell AR. Simulation-Based Power-Analysis for Factorial ANOVA Designs. 2019.

23. Cheng JL, Williams JS, Hoekstra SP and MacDonald MJ. Improvements in vascular function in response to acute lower limb heating in young healthy males and females. *J Appl Physiol (1985)*. 2021;131:277-289.

24. Torgrimson BN, Meendering JR, Miller NP, Kaplan PF and Minson CT. Endothelial function, endothelin-1, and fibrinogen in young women using the vaginal contraceptive ring. *Fertility & Sterility*. 2009;92:441-7.

25. Williams JS, Stone JC, Masood Z, Bostad W, Gibala MJ and MacDonald MJ. The impact of natural menstrual cycle and oral contraceptive pill phase on substrate oxidation during rest and acute submaximal aerobic exercise. *Journal of Applied Physiology*. 2023.

26. von Elm E, Altman DG, Egger M, Pocock SJ, Gøtzsche PC and Vandenbroucke JP. The Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) statement: guidelines for reporting observational studies. *The Lancet*. 2007;370:1453-1457.

27. Ringqvist A, Caidahl K, Petersson AS and Wennmalm A. Diurnal variation of flow-mediated vasodilation in healthy premenopausal women. *Am J Physiol Heart Circ Physiol*. 2000;279:H2720-5.

28. Thijssen DHJ, Bruno RM, van Mil A, Holder SM, Faita F, Greyling A, Zock PL, Taddei S, Deanfield JE, Luscher T, Green DJ and Ghiadoni L. Expert consensus and evidence-based recommendations for the assessment of flow-mediated dilation in humans. *Eur Heart J*. 2019;40:2534-2547.

29. Barton M, Turner AT, Newens KJ, Williams CM and Thompson AK. Minimum Recovery Time Between Reactive Hyperemia Stimulus in the Repeated Measurement of Brachial Flow-Mediated Dilatation. *Ultrasound Med Biol*. 2011;37:879-883.

30. Myers J, Arena R, Franklin B, Pina I, Kraus WE, McInnis K and Balady GJ. Recommendations for clinical exercise laboratories: a scientific statement from the american heart association. *Circulation*. 2009;119:3144-61.

31. Gillen JB, Percival ME, Skelly LE, Martin BJ, Tan RB, Tarnopolsky MA and Gibala MJ. Three minutes of all-out intermittent exercise per week increases skeletal muscle oxidative capacity and improves cardiometabolic health. *PLoS One*. 2014;9:e111489.

32. Tanaka H, Monahan KD and Seals DR. Age-predicted maximal heart rate revisited. *J Am Coll Cardiol*. 2001;37:153-6.

33. Howley ET, Bassett DR, Jr. and Welch HG. Criteria for maximal oxygen uptake: review and commentary. *Med Sci Sports Exerc*. 1995;27:1292-301.

34. Bostad W, McCarthy DG, Richards DL, Helal L and Gibala MJ. Peak Cardiac Output Determined Using Inert Gas Rebreathing: A Comparison of Two Exercise Protocols. *Med Sci Sports Exerc*. 2023;55:1014-1022.

35. Bostad W, Valentino SE, McCarthy DG, Richards DL, MacInnis MJ, MacDonald MJ and Gibala MJ. Twelve weeks of sprint interval training increases peak cardiac output in previously untrained individuals. *European Journal of Applied Physiology*. 2021;121:2449-2458.

36. Van Bortel LM, Laurent S, Boutouyrie P, Chowienczyk P, Cruickshank JK, De Backer T, Filipovsky J, Huybrechts S, Mattace-Raso FU, Protogerou AD, Schillaci G, Segers P, Vermeersch S and Weber T. Expert consensus document on the measurement of aortic stiffness in daily practice using carotid-femoral pulse wave velocity. *J Hypertens*. 2012;30:445-8.

37. Adkisson EJ, Casey DP, Beck DT, Gurovich AN, Martin JS and Braith RW. Central, peripheral and resistance arterial reactivity: fluctuates during the phases of the menstrual cycle. *Exp Biol Med (Maywood)*. 2010;235:111-8.

38. Williams JS, Stimpson TV, Tremblay JC, Fenuta AM and Pyke KE. No impact of acute hyperglycaemia on arterial stiffness in the early and late follicular phases of the menstrual cycle in young females. *Exp Physiol*. 2020;105:174-183.

39. Wendelhag I, Liang Q, Gustavsson T and Wikstrand J. A new automated computerized analyzing system simplifies readings and reduces the variability in ultrasound measurement of intima-media thickness. *Stroke*. 1997;28:2195-200.

40. Ghiadoni L, Faita F, Salvetti M, Cordiano C, Biggi A, Puato M, Di Monaco A, De Siati L, Volpe M, Ambrosio G, Gemignani V, Muiesan ML, Taddei S, Lanza GA and Cosentino F. Assessment of flow-mediated dilation reproducibility: a nationwide multicenter study. *J Hypertens*. 2012;30:1399-405.

41. Greyling A, van Mil AC, Zock PL, Green DJ, Ghiadoni L and Thijssen DH. Adherence to guidelines strongly improves reproducibility of brachial artery flow-mediated dilation. *Atherosclerosis*. 2016;248:196-202.

42. Pyke KE, Hartnett JA and Tschakovsky ME. Are the dynamic response characteristics of brachial artery flow-mediated dilation sensitive to the magnitude of increase in shear stimulus? *J Appl Physiol (1985)*. 2008;105:282-92.

43. Shenouda N, Gillen JB, Gibala MJ and MacDonald MJ. Changes in brachial artery endothelial function and resting diameter with moderate-intensity continuous but not sprint interval training in sedentary men. *Journal of applied physiology (Bethesda, Md : 1985)*. 2017;123:773-780.

44. Ghariq M, Thijs RD, Bek LM, van Zwet EW, Benditt DG and van Dijk JG. A higher proportion of men than of women fainted in the phase without nitroglycerin in tilt-induced vasovagal syncope. *Clinical Autonomic Research*. 2020;30:441-447.

45. Berthois Y, Katzenellenbogen JA and Katzenellenbogen BS. Phenol red in tissue culture media is a weak estrogen: implications concerning the study of estrogen-responsive cells in culture. *Proceedings of the National Academy of Sciences of the United States of America*. 1986;83:2496-2500.

46. Brunt VE, Weidenfeld-Needham KM, Comrada LN, Francisco MA, Eymann TM and Minson CT. Serum from young, sedentary adults who underwent passive heat therapy improves endothelial cell angiogenesis via improved nitric oxide bioavailability. *Temperature (Austin, Tex)*. 2019;6:169-178.

47. Kawano H, Motoyama T, Kugiyama K, Hirashima O, Ohgushi M, Yoshimura M, Ogawa H, Okumura K and Yasue H. Menstrual cyclic variation of endothelium-dependent vasodilation of the brachial artery: possible role of estrogen and nitric oxide. *Proc Assoc Am Physicians*. 1996;108:473-80.

48. Hashimoto M, Akishita M, Eto M, Ishikawa M, Kozaki K, Toba K, Sagara Y, Taketani Y, Orimo H and Ouchi Y. Modulation of endothelium-dependent flow-mediated dilatation of the brachial artery by sex and menstrual cycle. *Circulation*. 1995;92:3431-5.

49. D'Urzo KA, King TJ, Williams JS, Silvester MD and Pyke KE. The impact of menstrual phase on brachial artery flow-mediated dilatation during handgrip exercise in healthy premenopausal women. *Exp Physiol*. 2018;103:291-302.

50. Williams JS, Stimpson TV, Tremblay JC, Fenuta AM and Pyke KE. The influence of acute hyperglycaemia on brachial artery flow-mediated dilatation in the early and late follicular phases of the menstrual cycle. *Experimental Physiology*. 2019;104:957-966.

51. Kawano H, Motoyama T, Ohgushi M, Kugiyama K, Ogawa H and Yasue H. Menstrual cyclic variation of myocardial ischemia in premenopausal women with variant angina. *Annals of Internal Medicine*. 2001;135:977-981.

52. Harris RA, Tedjasaputra V, Zhao J and Richardson RS. Premenopausal women exhibit an inherent protection of endothelial function following a high-fat meal. *Reprod Sci*. 2012;19:221-8.

53. Giersch GEW, Charkoudian N, Pereira T, Edgell H, Freeberg KA, Craighead DH, Neill M, Allison EY, Zapcic AK, Smith KJ, Bock JM, Casey DP, Shenouda N, Ranadive SM, Tremblay JC, Williams AM, Simpson LL, Meah VL, Ruediger SL, Bailey TG, Pereira HM, Lei TH, Perry B, Mündel T, Freemas JA, Worley ML, Baranauskas MN, Carter SJ, Johnson BD, Schlader ZJ, Bates LC, Stoner L, Zieff G, Poles J, Adams N, Meyer ML, Hanson ED, Greenlund IM, Bigalke JA, Carter JR, Kerr ZY, Stanford K, Pomeroy A, Boggess K, de Souza HLR, Meireles A, Arriel RA, Leite LHR, Marocolo M, Chapman CL, Atencio JK, Kaiser BW, Comrada LN, Halliwill JR, Minson CT, Williams JS, Dunford EC, MacDonald MJ, Santisteban KJ, Larson EA, Reed E, Needham KW, Gibson BM, Gillen J, Barbosa TC, Cardoso LLY, Gliemann L, Tamariz-Ellemann A, Hellsten Y, DuBos LE, Babcock MC, Moreau KL, Wickham KA, Vagula M, Moir ME, Klassen SA and Rodrigues A. Commentaries on Point:Counterpoint: Investigators should/should not control for menstrual cycle phase when performing studies of vascular control. *J Appl Physiol (1985)*. 2020;129:1122-1135.

54. Rakobowchuk M, Parsloe ER, Gibbins SE, Harris E and Birch KM. Prolonged low flow reduces reactive hyperemia and augments low flow mediated constriction in the brachial artery independent of the menstrual cycle. *PLoS One*. 2013;8:e55385.

55. Valtonen P, Punnonen K, Saarelainen H, Heiskanen N, Raitakari OT, Juonala M, Viikari JS, Alfthan G, Kahonen M, Laaksonen R, Lyyra-Laitinen T, Laitinen T and Heinonen S. ADMA concentration changes across the menstrual cycle and during oral contraceptive use: the Cardiovascular Risk in Young Finns Study. *European Journal of Endocrinology*. 2010;162:259-65.

56. Barba-Moreno L, Cupeiro R, Romero-Parra N, Janse de Jonge XAK and Peinado AB. Cardiorespiratory Responses to Endurance Exercise Over the Menstrual Cycle and With Oral Contraceptive Use. *J Strength Cond Res*. 2022;36:392-399.

57. Schmalenberger KM, Eisenlohr-Moul TA, Würth L, Schneider E, Thayer JF, Ditzen B and Jarczok MN. A Systematic Review and Meta-Analysis of Within-Person Changes in Cardiac Vagal Activity across the Menstrual Cycle: Implications for Female Health and Future Studies. *Journal of Clinical Medicine*. 2019;8:1946.

58. Baker FC, Siboza F and Fuller A. Temperature regulation in women: Effects of the menstrual cycle. *Temperature*. 2020;7:226-262.

59. Schoene RB, Robertson HT, Pierson DJ and Peterson AP. Respiratory drives and exercise in menstrual cycles of athletic and nonathletic women. *Journal of Applied Physiology*. 1981;50:1300-1305.

60. Heidarzadeh Z, Asadi B, Saadatnia M, Ghorbani A and Fatehi F. The effect of low-dose combined oral contraceptive pills on brachial artery endothelial function and common carotid artery intima-media thickness. *Journal of stroke and cerebrovascular diseases : the official journal of National Stroke Association*. 2014;23:675-80.

61. Lizarelli PM, Martins WP, Vieira CS, Soares GM, Franceschini SA, Ferriani RA and Patta MC. Both a combined oral contraceptive and depot medroxyprogesterone acetate impair endothelial function in young women. *Contraception*. 2009;79:35-40.

62. Thijssen DH, Dawson EA, Black MA, Hopman MT, Cable NT and Green DJ. Heterogeneity in conduit artery function in humans: impact of arterial size. *Am J Physiol Heart Circ Physiol*. 2008;295:H1927-34.

63. Rakobowchuk M, Tanguay S, Burgomaster KA, Howarth KR, Gibala MJ and MacDonald MJ. Sprint interval and traditional endurance training induce similar improvements in peripheral arterial stiffness and flow-mediated dilation in healthy humans. *Am J Physiol Regul Integr Comp Physiol*. 2008;295:R236-42.

64. Totosy de Zepetnek JO, Jermey TL and MacDonald MJ. Superficial Femoral Artery Endothelial Responses to a Short-Term Altered Shear Rate Intervention in Healthy Men. *PLOS ONE*. 2014;9:e113407.

65. King TJ and Pyke KE. Evidence of a limb- and shear stress stimulus profile-dependent impact of high-intensity cycling training on flow-mediated dilation. *Applied Physiology, Nutrition, and Metabolism*. 2019;45:135-145.

66. Gaenzer H, Neumayr G, Marschang P, Sturm W, Kirchmair R and Patsch Josef R. Flow-mediated vasodilation of the femoral and brachial artery induced by exercise in healthy nonsmoking and smoking men. *Journal of the American College of Cardiology*. 2001;38:1313-1319.

67. Sorensen MB, Franks S, Robertson C, Pennell DJ and Collins P. Severe endothelial dysfunction in young women with polycystic ovary syndrome is only partially explained by known cardiovascular risk factors. *Clinical Endocrinology*. 2006;65:655-9.

68. Hickson SS, Miles KL, McDonnell BJ, Yasmin, Cockcroft JR, Wilkinson IB, McEniery CM and ENIGMAStudyInvestigators. Use of the oral contraceptive pill is associated with increased large artery stiffness in young women: The ENIGMA Study. *J Hypertens*. 2011;29:1155-1159.

69. Yu A, Giannone T, Scheffler P, Doonan RJ, Egiziano G, Gomez YH, Papaioannou TG and Daskalopoulou SS. The effect of oral contraceptive pills and the natural menstrual cYCLe on arterial stiffness and hemodynamICs (CYCLIC). *J Hypertens*. 2014;32:100-7.

70. Luca MC, Liuni A, Harvey P, Mak S and Parker JD. Effects of estradiol on measurements of conduit artery endothelial function after ischemia and reperfusion in premenopausal women. *Can J Physiol Pharmacol*. 2016;94:1304-1308.

71. Elliott-Sale KJ, Minahan CL, de Jonge X, Ackerman KE, Sipilä S, Constantini NW, Lebrun CM and Hackney AC. Methodological Considerations for Studies in Sport and Exercise Science with Women as Participants: A Working Guide for Standards of Practice for Research on Women. *Sports Med*. 2021;51:843-861.

72. Lew LA, Williams JS, Stone JC, Au AKW, Pyke KE and MacDonald MJ. Examination of Sex-Specific Participant Inclusion in Exercise Physiology Endothelial Function Research: A Systematic Review. *Frontiers in sports and active living*. 2022;4:860356.

CHAPTER 6: The impact of natural menstrual cycle and oral contraceptive pill phase on substrate oxidation during rest and acute submaximal aerobic exercise.

Jennifer S. Williams, Jenna C. Stone, Zaryan Masood, William Bostad, Martin J. Gibala, Maureen J. MacDonald

american physiological society®

JOURNAL OF APPLIED PHYSIOLOGY.

RESEARCH ARTICLE

Sex Differences in the Response to Exercise Training

The impact of natural menstrual cycle and oral contraceptive pill phase on substrate oxidation during rest and acute submaximal aerobic exercise

Jennifer S. Williams,[1] Jenna C. Stone,[1] Zaryan Masood,[1] William Bostad,[2] Martin J. Gibala,[2] and Maureen J. MacDonald[1]

[1]Vascular Dynamics Lab, Department of Kinesiology, McMaster University, Hamilton, Ontario, Canada and [2]Human Performance Lab, Department of Kinesiology, McMaster University, Hamilton, Ontario, Canada

Abstract

Previous research has identified sex differences in substrate oxidation during submaximal aerobic exercise including a lower respiratory exchange ratio (RER) in females compared with males. These differences may be related to differences in sex hormones. Our purpose was to examine the impact of the natural menstrual cycle (NAT) and second- and third-generation oral contraceptive pill (OCP2 and OCP3) cycle phases on substrate oxidation during rest and submaximal aerobic exercise. Fifty female participants (18 NAT, 17 OCP2, and 15 OCP3) performed two experimental trials that coincided with the low (i.e., nonactive pill/early follicular) and the high hormone (i.e., active pill/midluteal) phase of their cycle. RER and carbohydrate and lipid oxidation rates were determined from gas exchange measurements performed during 10 min of supine rest, 5 min of seated rest, and two 8-min bouts of submaximal cycling exercise at ~40% and ~65% of peak oxygen uptake ($\dot{V}o_{2peak}$). For all groups, there were no differences in RER between the low and high hormone phases during supine rest (0.73 ± 0.05 vs. 0.74 ± 0.05), seated rest (0.72 ± 0.04 vs. 0.72 ± 0.04), exercise at 40% (0.77 ± 0.04 vs. 0.78 ± 0.04), and 65% $\dot{V}o_{2peak}$ (0.85 ± 0.04 vs. 0.86 ± 0.03; $P > 0.19$ for all). Similarly, carbohydrate and lipid oxidation rates remained largely unchanged across phases during both rest and exercise, apart from higher carbohydrate oxidation in NAT vs. OCP2 at 40% $\dot{V}o_{2peak}$ ($P = 0.019$) and 65% $\dot{V}o_{2peak}$ ($P = 0.001$). NAT and OCPs do not appear to largely influence substrate oxidation at rest and during acute submaximal aerobic exercise.

NEW & NOTEWORTHY This study was the first to examine the influence of NAT and two generations of OCPs on substrate oxidation during rest and acute submaximal aerobic exercise. We reported no differences across cycle phases or groups on RER, and minimal impact on carbohydrate or lipid oxidation apart from an increase in carbohydrate oxidation in NAT compared with OCP2 during exercise. Based on these findings, NAT/OCP phase controls may not be necessary in studies investigating substrate oxidation.

carbohydrate oxidation; estrogen; fuel utilization; hormonal contraceptive; lipid oxidation

INTRODUCTION

Humans primarily oxidize carbohydrates and lipids for energy provision during submaximal aerobic exercise. The respiratory exchange ratio (RER) is a valid, noninvasive, and cost-efficient method to estimate whole body substrate oxidation (1). Potential sex differences in substrate oxidation during submaximal aerobic exercise have been summarized comprehensively in several recent reviews (2–4). Females may have a lower rate of carbohydrate oxidation and a higher rate of lipid oxidation as compared with males during submaximal aerobic exercise and at rest (5–8). These differences are not seen in childhood, suggesting that fluctuations in hormone levels that occur with puberty, specifically the sex hormones 17β-estradiol and progesterone, play a role in the observed sex differences (9, 10). Previous research of oral 17β-estradiol supplementation in males showed a decrease in RER and carbohydrate oxidation and corresponding increase in lipid mobilization and oxidation via upregulation of fatty acid transport and β-oxidation enzymes, suggesting that 17β-estradiol may play a role in the regulation of fuel utilization and substrate oxidation (4, 6, 11). Although research on progesterone supplementation is limited, there is some evidence from animal and human research that progesterone (with or without 17β-estradiol) supplementation also increases lipid oxidation via upregulation of metabolic enzymes (12), but may restore carbohydrate oxidation compared with the decrease observed with 17β-estradiol supplementation alone (13).

There is renewed interest in the effects of fluctuations in endogenous and exogenous estrogen and progesterone levels in women across both the natural menstrual cycle and oral contraceptive pill cycle on exercise metabolism and performance (14). Some research has found that women had lower whole body carbohydrate oxidation and higher lipid oxidation rates in the luteal phase (when 17β-estradiol and progesterone are high) as compared with the follicular phase (when 17β-estradiol and progesterone are low) during submaximal cycling or running aerobic exercise (15–17). Similarly, muscle glycogen use during exercise has also been reported to be ~20%–25% lower in the luteal phase compared with the follicular phase, as lipids are preferentially selected for fuel utilization (15, 18–20). However, these findings are not universal (21, 22). One possible explanation for the discordant results may be a balance between the levels of 17β-estradiol and progesterone (17β-estradiol/progesterone ratio) with a larger magnitude of change in the ratio between phases needed to shift lipid oxidation rates (23). Finally, some research has indicated an intensity-dependent relationship between substrate utilization and menstrual cycle phase, such that differences between menstrual cycle phases in substrate oxidation may be present at low- and medium-intensity aerobic exercise [i.e., 35%–65% peak oxygen uptake ($\dot{V}O_{2peak}$)] but not at higher intensity aerobic exercise (i.e., 75% $\dot{V}O_{2peak}$) (24). This latter observation may also help explain some of the disagreement in the literature.

Combined oral contraceptive pills (OCPs) constitute one of the most common drug prescriptions for women in Canada (aged 15–49 yr) (25) and are likewise used by over 150 million women worldwide (26). OCPs differ in synthetic estrogen (i.e., ethinyl estradiol, dose: 0.01–0.05 mg) and progesterone (i.e., progestins levonorgestrel, desogestrel, norgestimate, norethindrone acetate, drospirenone; dose: 0.05–0.25 mg) content. OCPs are designed to mimic the natural menstrual cycle, often containing two "phases" of hormone levels: low hormone phase (nonactive/placebo pill phase) and high hormone phase (active pill phase) with one (monophasic), two (biphasic), or three (triphasic) levels of increasing progestin pill dosage alongside a single step increase in synthetic estrogen (27). Over the last 60 years, four generations of OCPs have been developed which differ in the type of synthetic progestin used. Variations in such hormones have in turn been linked to differences in carbohydrate and lipid oxidation rates at rest and during submaximal aerobic exercise (10, 16, 28–31); although other studies have observed no effects of OCP use or across phases (10, 21, 32). One reason for disagreement in the literature may be that prior research in this area often groups all OCP generations together or do not specify OCP type (10, 16, 28, 31), failing to consider that dissimilar hormonal profiles may differentially influence substrate oxidation during exercise and at rest, as suggested previously by Elliott-Sale et al. (33). Newer variants of progestins (i.e., third-generation progestins like desogestrel) have lower androgenicity or are anti-androgenic compared with older progestins (i.e., second-generation progestins like levonorgestrel) (34, 35), and lower androgenicity may have favorable impacts on lipid metabolism (36, 37), although research is limited. To highlight this gap, research by Isacco et al. (32) observed no differences in RER and substrate oxidation rates across OCP phases; however, this study grouped second-, third-, and fourth-generation OCP users together. These methodological considerations may be, in part, responsible for the disagreement in the literature.

The present study sought to address knowledge gaps in understanding the impact of oral contraceptives and natural menstrual cycle phase on fuel utilization in premenopausal females. Our purpose was to determine the effects of the natural menstrual cycle and oral contraceptive cycle of the two most prescribed generations of OCPs in Canada [second and third generations (25)] on substrate oxidation at rest and during submaximal aerobic exercise. We hypothesized that at rest and during 40% and 65% $\dot{V}O_{2peak}$ exercise, lipid oxidation would be increased in the high hormone (HH; active/midluteal) phase of both the natural menstrual cycle and oral contraceptive cycle, compared with the low hormone (LH; nonactive/early follicular) phase, with no differences between groups. This study contributes to current knowledge gaps that exist in understanding the impact of oral contraceptives and natural menstrual cycle phase on fuel utilization in premenopausal females. Determining the effect of oral contraceptives and natural menstrual cycle phase on substrate oxidation permits a deeper understanding of the potential impact of contraceptives on substrate metabolism and the role of both endogenous and exogenous sex hormones in fuel utilization during aerobic exercise.

METHODS

Participant Recruitment and Ethics

This study was part of a larger research project that recruited premenopausal females between 18–45 yr of age from McMaster University and surrounding areas in Hamilton, Ontario. All participants who consented to the larger research project also consented to participate in this project. Participants were recruited and stratified to the following groups, based on existing OCP use: *1*) females taking OPC2; *2*) females taking OCP3; and *3*) natural menstrual cycle (NAT) females. Although an a priori sample size calculation was not conducted, as this project was a part of a larger research project, a sample size calculation was determined using G*Power (α ≤ 0.05, power = 80%). Based on a similar study in NAT females with acute aerobic exercise, a sample size of approximately $n = 15$ per group would be needed to detect a change in RER of approximately 0.03 (0.87 vs. 0.84) across phases with a SD of 0.05 (17). Females were excluded from the study if they had a history of cardiovascular, metabolic, or musculoskeletal disease, were currently pregnant or pregnant within the last year, or were smokers. NAT participants were eumenorrheic, and excluded if their menstrual cycles were irregular (1 missed cycle in the last 6 mo) or if the cycle was outside of a "normal range" (21–35 day cycle length), as this may be indicative of menstrual cycle irregularity or other reproductive conditions (38). NAT participants were also excluded if they used oral or nonoral hormonal contraceptives within 6 mo of participating. OCP participants were excluded if they were not taking monophasic or triphasic OCP2 or OCP3 formulations, were not on the OCP for at least 3 mo before starting the study, or were not taking the OCP as prescribed (i.e., skipping the

nonactive/placebo pill phase). The study was approved by the Hamilton Integrated Research Ethics Board (HIREB; #7827).

Study Protocol

Screening and $\dot{V}O_{2peak}$ test visit.
Participants attended to the Vascular Dynamics Laboratory or participated in a virtual visit to be fully informed of the protocol and provide written informed consent (Fig. 1B). Following the initial visit, participants completed a medical information form to assess the inclusion criteria for the study as well as to provide the investigators with the information on their oral contraceptive pill use and cycle details (i.e., cycle duration, type of OCP used, and duration of OCP use). Participants then tracked their menstrual cycle for two or more months before participating in the study by providing researchers with the starting date of each consecutive period to calculate average menstrual cycle length. In the menstrual or oral contraceptive pill cycle (no specific phase) before the data collection visits, participants came to the laboratory in-person at any time of day for an assessment of basic anthropometric

measures such as age, height, and weight and completed a baseline $\dot{V}O_{2peak}$.

The $\dot{V}O_{2peak}$ test was completed with participants seated upright on a stationary cycle ergometer (Lode Excalibur Sport V 2.0, Groningen, Netherlands or Kettler Ergo Race, Kettler, Virginia Beach VA, same ergometer within each participant). Expired oxygen and carbon dioxide concentrations were determined using a daily calibrated metabolic cart with a mixing chamber (Quark CPET Metabolic Cart, COSMED, Italy). Heart rate (HR) was monitored throughout the exercise visits using a HR monitor (Polar A3, Lake Success, New York). Data were collected every 10 s for expired oxygen and carbon dioxide and every 1 s for HR. The $\dot{V}O_{2peak}$ test started with a 3-min warm-up at 50 W of resistance. The resistance was lowered if indicated by the participant as too intense for the warm-up within the first 30 s. Following the warm-up, the exercise phase involved an increase in resistance of 1 W every 2 s ($n = 37$) or 5 W every 10 s ($n = 13$), depending on the cycle ergometer used. Incremental increases in resistance continued until volitional exhaustion, evidenced by a failure to maintain a cadence of at least 60 rpm on the cycle
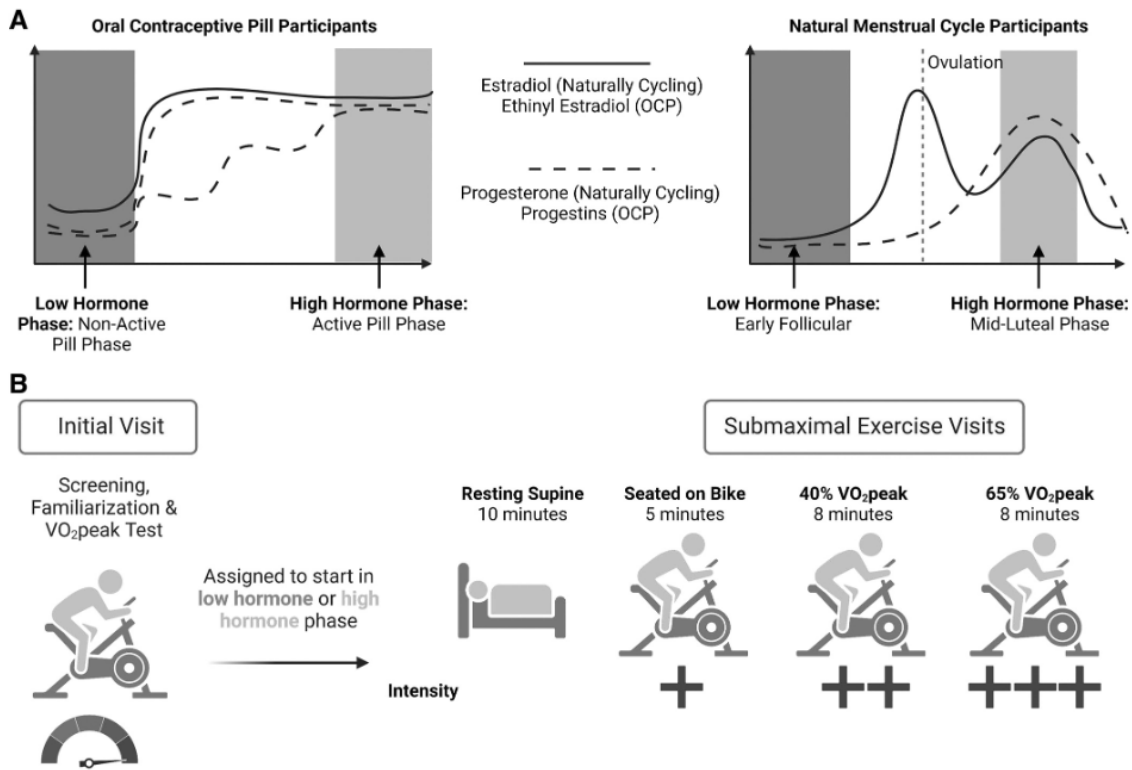
Figure 1. *A*: oral contraceptive pill (OCP) and natural menstrual (NAT) cycles. This schematic depicts a standard OCP cycle (*left*) and NAT cycle (*right*). The solid line represents fluctuating estradiol levels in NAT participants or ethinyl estradiol levels in OCP participants. The dotted line represents fluctuating levels of progesterone in NAT participants or progestins in OCP participants, with the single increase in progestin levels representing monophasic OCPs and three increasing levels of progestin representing triphasic OCPs. *B*: data collection protocol. Following an initial screening visit, participants took part in two identical submaximal exercise testing visits, assigned in a counterbalanced order, in the low hormone phase of their menstrual or oral contraceptive pill cycle (early follicular/nonactive pill) and in the high hormone phase of their cycle (midluteal/active pill). During the exercise visits, participants took part in 10 min of supine rest, followed by 5 min of seated rest on a cycle ergometer, followed by 8 min of cycling exercise at a power that corresponded to each of 40% and 65% peak oxygen uptake ($\dot{V}O_{2peak}$). Figure created in BioRender.

ergometer. Following exhaustion, the participant was instructed to cool down for 2 min at their warm-up intensity (50 W or lower). The $\dot{V}O_{2peak}$ test was considered successful if a minimum of two of the following criteria were met: *1*) the peak HR was within 10 beats/min of 208−0.7*age (39), *2*) the RER > 1.1, *3*) a clear plateau in the volume of oxygen consumed ($\dot{V}O_2$), and *4*) perceived exertion ≥17 (Borg scale 6–20). All participants achieved $\dot{V}O_{2peak}$. To determine $\dot{V}O_{2peak}$, the three highest 10-s averages of $\dot{V}O_2$ (typically achieved at the end of the test) were averaged to determine the maximum value (40, 41) and to subsequently calculate 40% and 65% of $\dot{V}O_{2peak}$.

Data collection visits.

Participants returned to the laboratory for two data collection visits during the subsequent menstrual or OCP cycle phase. With the onset of menses as *day 1*, eumenorrheic NAT participants were returned during the LH phase (early follicular phase: *days 3–7*) and the HH phase (midluteal phase: ∼7 days after ovulation). Ovulation was determined using standard at-home ovulation kits (BFP Ovulation Test Strips, Fairhaven Health, Bellingham, WA). Females using oral contraceptives were asked to return 3–7 days into the LH phase (nonactive pill/placebo phase) and 2–7 days in the third week of the HH phase (active pill phase; Fig. 1A). The order of visits was assigned and counterbalanced within a group (NAT, OCP2, and OCP3) during participant recruitment (i.e., first participant in a group began in the HH phase, while second participant in a group began in the LH phase), such that approximately half of the participants within each group had their first data collection visit during their LH phase ($n = 23$), while the other half had their first visit in the HH phase ($n = 27$). Odd number of participants in two of the groups and scheduling difficulties with one participant resulted in unequal starting phase numbers. Two OCP2 visits and one OCP3 visit required rescheduling to the next cycle due to technical difficulties ($n = 2$) or illness ($n = 1$).

Participants were asked to refrain from vigorous physical activity >24 h, caffeine or alcohol consumption >8 h, food consumption >6 h, and nonprescription medication use (i.e., anti-inflammatory and pain medications) >12 h before testing on each day. Data collection sessions were completed at the same time of day within a participant, and during the morning to midday hours. During each data collection visit, participants were asked to complete a four-stage rest and submaximal aerobic exercise test on the same cycle ergometer, with gas exchange variables collected using the same metabolic cart used during the $\dot{V}O_{2peak}$ test. Finally, participants took part in a whole body dual-energy X-ray absorptiometry (DXA) scan during their low/no hormone phase visit.

During each data collection visit, participants completed 10 min of supine rest with the lights in the room turned off. This was followed by 5 min of seated rest on the exercise bike followed by 8 min of cycling exercise at each of 40% and 65% of $\dot{V}O_{2peak}$, with the lights in the room turned on. All phases (rest and submaximal exercise) were completed sequentially (Fig. 1B), aligned with other sequential exercise protocols (17, 24). The only delay in timing of the testing was in the transition from supine to seated; participants were given a 2-min window to transition from the supine rest to seated position on the cycle ergometer, after which the 5-min seated rest

phase began. The exercise workloads were determined during the first submaximal exercise visit by adjusting the resistance on the bike to achieve the target $\dot{V}O_2$, while maintaining a pedal cadence of ∼70 rpm. During the second submaximal exercise visit, the workloads were set to match the first visit. The respective workloads were calculated from the $\dot{V}O_{2peak}$ test results using the following equation (42):

$$\text{Estimated Power(Watts)} = [\text{Target}\dot{V}O_2(\text{L/min}) - 0.435]/0.01141 \quad (1)$$

For the submaximal tests, data were averaged every 10 s for HR, gas exchange, and RER during last 2 min of supine and seated rest and during 40% and 65% of $\dot{V}O_{2peak}$ exercise bouts. Gas exchange variables included breathing frequency (Rf, breaths/min), tidal volume (VT, L/min), minute ventilation of gas exhaled ($\dot{V}E$, L/min), $\dot{V}O_2$ (L/min), and volume of carbon dioxide expired per minute ($\dot{V}CO_2$, L/min). Oxidation rates (g/min) were calculated using *Eqs. 2* and *3* as shown below (43) where any negative values were assumed as zero oxidation for that substrate:

Carbohydrate Oxidation(g/min)
$$= (4.858 \times \dot{V}CO_2(\text{L/min})) - (3.2255 \times \dot{V}O_2(\text{L/min})) \quad (2)$$

Lipid Oxidation(g/min)
$$= (1.6946 \times \dot{V}O_2(\text{L/min})) - (1.7012 \times \dot{V}CO_2(\text{L/min})) \quad (3)$$

COVID-19 Pandemic-Related Missing Data

Initial participants were recruited and tested in Winter 2020 before the coronavirus disease of 2019 (COVID-19) pandemic shutdown of research laboratories, and the protocol was altered slightly when testing proceeded in Fall 2021. As a result of equipment limitations in initial testing in 2020, gas exchange variables are missing for the supine rest condition for $n = 14/50$ participants ($n = 6$ NAT, $n = 6$ OCP2, and $n = 2$ OCP3). Further, an additional refinement between 2020 and 2021 included the addition of recording heart rate (HR) and rating of perceived exertion (RPE) during each interval; as a result, this data is missing during all intervals for $n = 14/50$ participants (same groups as above). Finally, one dual-energy X-ray absorptiometry (DXA) scan is missing in a NAT participant, due to COVID-19 pandemic-related research laboratory closures.

Statistical Analysis

All statistical analyses were performed using SPSS, version 22 (IBM, Chicago, IL). All data, unless otherwise noted, are reported as the mean ± SD, and statistical significance was set as $P < 0.05$. Figures 2 and 3 represent the data in box-and-whisker plots, where the middle line represents the median, the interquartile range (50% of the data) is represented by the box, and the whiskers represent the minimum and maximum points in the data. All participant characteristics were analyzed for differences by using a one-way analysis of variance (ANOVA) across the three groups. A two-way repeated measures linear mixed model (compound symmetry) ANOVA was conducted with three levels of group (OCP2, OCP3, and NAT) and two levels of phase (LH phase and HH phase) for each of the gas exchange, substrate oxidation, HR, and RPE outcomes during seated and supine rest and during

145

Figure 2. Heart rate (HR) during supine rest, seated rest, and exercise at 40% and 65% peak oxygen uptake ($\dot{V}O_{2peak}$). HR during supine rest (A); HR during resting on bike (B); HR at 40% of $\dot{V}O_{2peak}$ during submaximal aerobic exercise (C); and HR at 65% of $\dot{V}O_{2peak}$ during submaximal aerobic exercise (D). A two-way repeated measures linear mixed model (compound symmetry) ANOVA was conducted with three levels of group (OCP2, OCP3, and NAT) and two levels of phase (low hormone and high hormone). Data are represented as box-and-whisker plots, with the middle line representing the median and the whiskers representing the 5 and 95 percentiles. *HR was higher in the HH phase compared with LH phase during supine rest (main effect of phase: $P = 0.006$). G, group; G*P, group by phase interaction; HH, high hormone; LH, low hormone; NAT HH, naturally menstruating and in high hormone phase (midluteal phase); NAT LH, naturally menstruating and in low hormone phase (early follicular phase); OCP2 HH, second-generation oral contraceptive pill and in high hormone phase (active phase); OCP2 LH, second-generation oral contraceptive pill and in low hormone phase (nonactive pill phase); OCP3 HH, third-general oral contraceptive pill and in the high hormone phase (active phase); OCP3 LH, third-generation oral contraceptive pill and in the low hormone phase (nonactive pill phase); P, phase.
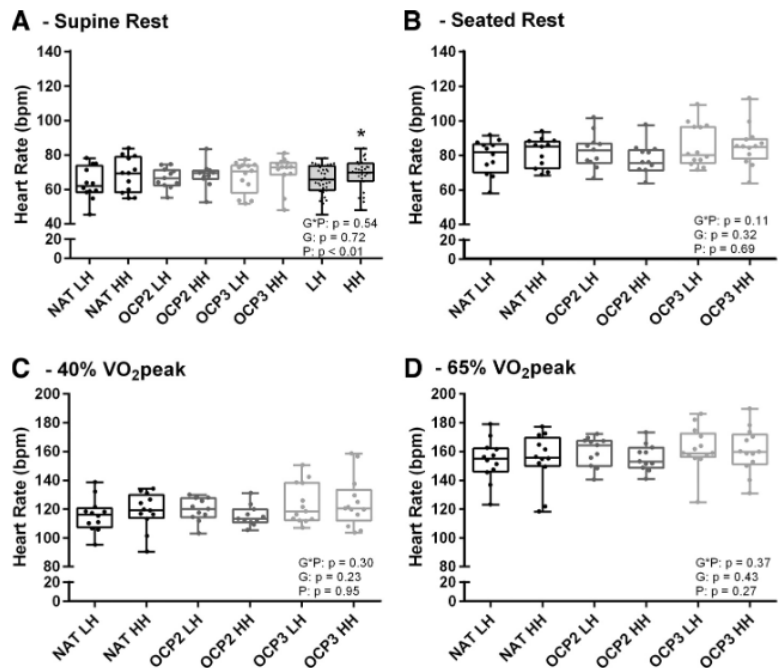
40% $\dot{V}O_{2peak}$ and 65% $\dot{V}O_{2peak}$ stages of exercise. Post hoc analysis was performed on significant main effects and interactions within group, using the Bonferroni-adjusted post hoc test for multiple comparisons. Significant effect sizes were quantified for the main analysis using a Cohen's d calculation, where a small effect is $d = 0.2$, medium effect is $d = 0.5$, and large effect is $d > 0.8$ (44). Although not determined as an a priori analysis, an exploratory three-way repeated

Figure 3. Respiratory exchange ratio (RER) during supine rest, seated rest, and exercise at 40% and 65% peak oxygen uptake ($\dot{V}O_{2peak}$). RER during supine rest (A); RER during resting on bike (B); RER at 40% of $\dot{V}O_{2peak}$ during submaximal aerobic exercise (C); and RER at 65% of $\dot{V}O_{2peak}$ during submaximal aerobic exercise (D). ANOVA was conducted with three levels of group (OCP2, OCP3, NAT) and two levels of phase (low hormone, high hormone). Data are represented as box-and-whisker plots, with the middle line representing the median and the whiskers representing the 5 and 95 percentiles. G, group; G*P, group by phase interaction; NAT HH, naturally menstruating and in high hormone phase (midluteal phase); NAT LH, naturally menstruating and in low hormone phase (early follicular phase); OCP2 HH, second-generation oral contraceptive pill and in high hormone phase (active phase); OCP2 LH, second-generation oral contraceptive pill and in low hormone phase (nonactive pill phase); OCP3 HH, third-generation oral contraceptive pill and in the high hormone phase (active phase); OCP3 LH, third-generation oral contraceptive pill and in the low hormone phase (nonactive pill phase); P, phase.
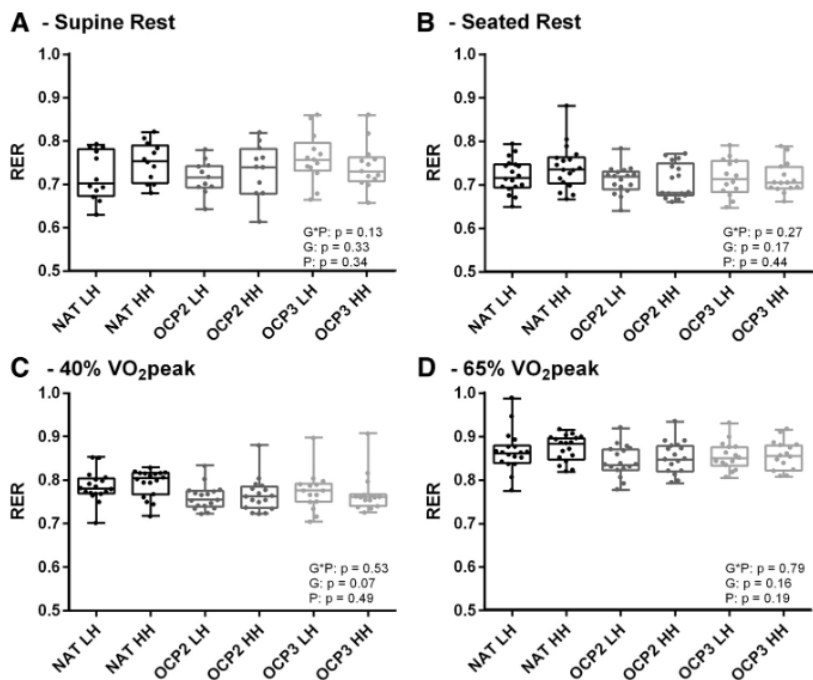
146

Table 1. *Participant characteristics*

	NAT (*n* = 18)	OCP2 (*n* = 17)	OCP3 (*n* = 15)	P Value
Age, yr	21±2	21±2	21±3	0.90
Height, cm	164.2±8.1	163.7±7.0	164.4±5.8	0.96
Weight, kg	66.0±14.4	59.2±9.0	61.8±7.6	0.19
BMI, kg/m^2	24.5±5.0	22.1±3.1	22.8±2.2	0.16
Hip-waist circumference ratio	0.77±0.05	0.78±0.05	0.76±0.04	0.41
DXA lean body mass, kg	41.2±4.7	38.8±4.1	39.5±3.7	0.25
DXA body fat, kg	23.1±11.5	18.0±6.0	20.2±6.5	0.22
DXA % body fat	33.2±9.1	29.9±5.7	32.1±7.3	0.43
Relative $\dot{V}O_{2peak}$, mL/min/kg	37.9±8.7	40.1±6.9	38.5±7.0	0.68

Means of participant characteristics across groups are given along with standard deviations and respective *P* values from the one-way ANOVA test. BMI, body mass index; DXA, dual-energy X-ray absorptiometry; NAT, naturally menstruating; OCP2, second-generation oral contraceptive pill; OCP3, third-generation oral contraceptive pill; $\dot{V}O_{2peak}$, peak volume of oxygen.

measures linear mixed model (compound symmetry) ANOVA was conducted with three levels of group (OCP2, OCP3, and NAT), two levels of phase (LH phase and HH phase), and four levels of time (supine rest, seated rest, 40% $\dot{V}O_{2peak}$, and 65% $\dot{V}O_{2peak}$ stages) with Bonferroni-adjusted post hoc tests to capture differences across rest and exercise intensities. Further, exploratory analysis to examine a Pearson correlation between RER responses in the LH versus HH phase within each level of rest and exercise phases was conducted to examine the consistency of response across the cycle.

RESULTS

Participant Characteristics and Menstrual/Oral Contraceptive Pill Cycles

Participant characteristics are detailed in Table 1. There were no differences between groups for any participant characteristics. Menstrual and oral contraceptive pill cycle information is detailed in Table 2. All OCP2 participants used monophasic contraceptives (*n* = 5 Alesse/*n* = 12 Alysena) containing daily active pills of 0.02 mg ethinyl estradiol and 0.1 mg levonorgestrel. OCP3 participants were either using monophasic contraceptives (*n* = 7, Marvelon/Mirvala/Freya, daily 0.03 mg ethinyl estradiol, 0.15 mg desogestrel) or one of three triphasic contraceptives (*n* = 8) with testing occurring during the highest active dose week: Linessa (*n* = 3, 0.025 mg ethinyl estradiol, 0.15 mg desogestrel), Tricira Lo (*n* = 3, 0.025 mg ethinyl estradiol, 0.25 mg norgestimate), or Tri-Jordyna (*n* = 2, 0.035 mg ethinyl estradiol, 0.25 mg norgestimate).

Supine Rest

During supine rest, there were no differences between groups or across phases for most gas exchange outcomes,

including Rf, VT, $\dot{V}E$, $\dot{V}O_2$, and $\dot{V}CO_2$ (Table 3, *top*). Similarly, there were no effects of group or phase on RPE (Table 3, *top*). However, there was a main effect of phase [*d* = 0.39 (small-medium effect), *P* < 0.01] for HR, such that HR was ~3 beats/min higher in the HH phase compared with the LH phase (Fig. 2*A*). There was no difference between groups or across phases for RER (Fig. 3*A*), or carbohydrate oxidation (Table 3, *top*). However, there was an interaction between group and phase (*P* = 0.04; Table 3, *top*) for lipid oxidation, resulting in lower lipid oxidation in the HH phase compared with the LH phase in NAT [*d* = 1.02 (large effect), *P* = 0.02] but no differences between phases in OCP2 (*P* = 0.50) or OCP3 (*P* = 0.20).

Seated Rest

During seated rest, there were no differences between groups or across phases for gas exchange variables Rf, $\dot{V}E$, $\dot{V}O_2$, and $\dot{V}CO_2$ (Table 3, *top*), HR (Fig. 2*B*), or RPE (Table 3, *top*). However, VT was higher in the HH phase compared with the LH phase [*d* = 0.25 (small effect), *P* < 0.01; Table 3, *top*]. Finally, there were no differences between groups or across phases for RER (Fig. 3*B*) or lipid and carbohydrate oxidation (Table 3, *top*).

Exercise at 40% $\dot{V}O_{2peak}$

During submaximal exercise at 40% $\dot{V}O_{2peak}$, there were no differences between groups or across phases for gas exchange variables Rf, $\dot{V}E$, $\dot{V}O_2$, and $\dot{V}CO_2$ (Table 3, *bottom*). There were also no differences between groups or phases for RPE (Table 3, *bottom*) or HR (Fig. 2*C*). However, VT was higher in the HH phase compared with LH phase (group-*phase interaction: *P* = 0.05) in the OCP2 [*d* = 0.27 (small effect), *P* = 0.02] and OCP3 [*d* = 0.30 (small-medium effect), *P* = 0.04] groups but not in the NAT group (*P* = 0.39; Table 3, *bottom*). Examining substrate oxidation, there was no

Table 2. *Menstrual and oral contraceptive pill cycle information*

	NAT (*n* = 18)	OCP2 (*n* = 17)	OCP3 (*n* = 15)
LH testing date (early follicular/nonactive pill phase)	5±1 days	5±1 days	5±1 days
HH testing date (midluteal/active pill phase)	23±3 days (7±1 days postovulation)	26±2 days	26±1 days
Average menstrual cycle length	29±2 days		
Average ovulation date	16±3 days		
Length of time on contraceptive		40±28 mo (3.3±2.3 yr)	29±23 mo (2.4±1.9 yr)

Counting of the dates of testing in the low hormone (LH) and high hormone (HH) phases, average menstrual cycle length, and ovulation date used the onset of menses to represent *day 0*. NAT, naturally menstruating; OCP2, second-generation oral contraceptive pill; OCP3, third-generation oral contraceptive pill.

significant main effect of group ($P = 0.07$) for RER (Fig. 3C), but a main effect of group ($P = 0.03$; Table 3, bottom) for carbohydrate oxidation, with higher oxidation in NAT compared with OCP2 [$d = 0.96$ (large effect), $P = 0.03$], but not OCP3 ($P = 0.38$). There were no differences across groups or phases for lipid oxidation (Table 3, bottom).

Exercise at 65% $\dot{V}O_{2peak}$

During submaximal exercise at 65% $\dot{V}O_{2peak}$, there were no differences between groups or across phases for gas exchange variables Rf, $\dot{V}E$, V_T, $\dot{V}O_2$, and $\dot{V}CO_2$ or RPE (Table 3, bottom) and HR (Fig. 2D). There were also no differences across groups or phases for RER (Fig. 3D) and carbohydrate or lipid oxidation (Table 3, bottom).

Exploratory Analysis

As expected, for all outcomes (HR, RPE, Rf, V_T, $\dot{V}E$, $\dot{V}O_2$, $\dot{V}CO_2$, RER, carbohydrate oxidation, and lipid oxidation), there was a main effect of time ($P < 0.001$). Measures of HR, Rf, $\dot{V}O_2$, RER, and lipid oxidation all increased from supine rest to seated rest to 40% $\dot{V}O_{2peak}$ to 65% $\dot{V}O_{2peak}$, with significant increases in all measures over exercise stages ($P < 0.001$). In contrast, measures of RPE, V_T, $\dot{V}E$, $\dot{V}CO_2$, and carbohydrate oxidation did not increase from supine rest to seated rest, but then increased from seated rest 40% $\dot{V}O_{2peak}$ and again from 40% $\dot{V}O_{2peak}$ to 65% $\dot{V}O_{2peak}$ exercise phases ($P < 0.001$).

In addition, for RER, there was an interaction between group and phase ($P = 0.031$), with RER higher in the HH phase compared with LH phase in NAT (LH: 0.78 ± 0.07, HH: 0.79 ± 0.07, $P = 0.005$), and no difference in OCP2 or OCP3 groups. Similarly, with carbohydrate oxidation, but not lipid oxidation, there was an interaction between group and time ($P = 0.001$), such that carbohydrate oxidation was higher in NAT versus OCP2 at 40% $\dot{V}O_{2peak}$ (NAT: 0.61 ± 0.20 g/min, OCP2: 0.44 ± 0.15 g/min, $P = 0.019$) and 65% $\dot{V}O_{2peak}$ (NAT: 1.57 ± 0.44 g/min, OCP2: 1.35 ± 0.32 g/min, $P = 0.001$).

Finally, RER was significantly positively correlated between LH and HH phases during seated rest ($r = 0.350$, $P = 0.014$), 40% $\dot{V}O_{2peak}$ ($r = 0.652$, $P < 0.001$), and 65% $\dot{V}O_{2peak}$ ($r = 0.662$, $P < 0.001$) but not during supine rest ($r = 0.265$, $P = 0.118$).

DISCUSSION

This is the first study to examine the influence of the NAT and OCP cycles on substrate oxidation during rest and acute submaximal aerobic exercise, stratifying based on the natural menstrual cycle and generation of OCP used. We found no differences across phases in NAT, OCP2, or OCP3 users on RER at rest or during submaximal aerobic exercise. Similarly, there was minimal impact of NAT or OCP phase or contraceptive group on carbohydrate or lipid oxidation during rest or submaximal aerobic exercise, apart from elevated carbohydrate oxidation in NAT compared with OCP2, but not OCP3, during 40% and 65% $\dot{V}O_{2peak}$. This study provides evidence that additional NAT or OCP phase controls, commonly cited as a barrier to the inclusion of females in exercise physiology research (45, 46), may not be required for studies examining fuel provision during acute aerobic exercise.

Influence of NAT and OCPs on Substrate Oxidation at Rest

During both supine and seated rest, there were no group or phase differences observed in most gas exchange variables, such Rf, $\dot{V}E$, $\dot{V}O_2$, $\dot{V}CO_2$, along with RER and carbohydrate oxidation. These findings are consistent with other studies examining RER and NAT or OCP cycles at rest (10, 15, 17, 22, 30, 31, 47). For example, research by Horton et al. (47) identified that RER and glucose kinetics were similar across the early follicular, midfollicular, and midluteal phases at rest in eumenorrheic NAT females. Few studies have examined differences in NAT versus OCP users, although there is some evidence of decreased RER and preference for lipid oxidation in OCP versus NAT at rest (29, 48). However, one study in monophasic OCP users while actively taking a contraceptive containing the same level of ethinyl estradiol but either low or high levels of norethisterone (first-generation progestin) identified no difference in RER between OCP types, in line with current study findings (31).

Despite the lack of differences in RER in the current study, there was a lower lipid oxidation rate during supine rest in the midluteal phase compared with the early follicular phase in the NAT group only. The observation that RER was unaltered across rest is consistent with previous literature (15, 21); however, the latter finding exclusively in the NAT group runs contrary to the reported sparing effect of muscle glycogen in the midluteal phase, when progesterone levels are elevated (18, 20). Early research has identified higher muscle glycogen stores in the midluteal compared with the midfollicular phase at rest, and less glycogen depletion postexercise to exhaustion (20) or during a submaximal cycling exercise test (15), pointing to a higher proportion of lipid oxidation during the midluteal phase (18). Further research is needed to explore this conflicting finding, considering the use of specific metabolic tracers (i.e., glycerol and palmitate) to examine a potential menstrual cycle-dependent preference for lipid utilization.

In addition to substrate oxidation findings, HR was ~3 beats/min higher in the HH phase compared with the LH phase during supine rest. During seated rest, while the elevated HR in the HH phase was not observed, there was an increase in V_T in the HH compared with the LH phase; there was also no difference in V_T between groups. This finding is in agreement with previous studies that have observed elevated ventilation and HR at rest and during exercise in the HH phase (49–51). Underlying these cardiorespiratory effects may be increases in body temperature: the midluteal phase was associated with higher basal body temperature by ~0.3°C–0.7°C and a ~3%–5% increase in HR, most apparent during supine rest, as reviewed recently (52). V_T was elevated in the HH phase for the OCP2 and OCP3, but not NAT group at 40% $\dot{V}O_{2peak}$; however, there were no difference at 65% $\dot{V}O_{2peak}$. It is possible that the cardiorespiratory effect the HH phase may only be apparent at lower levels of cardiorespiratory activation.

Influence of NAT and OCPs on Substrate Oxidation during Submaximal Aerobic Exercise

During submaximal exercise, there were minimal differences across NAT and OCP phases for gas exchange variables, including RER, and carbohydrate or lipid oxidation rates at

ORAL CONTRACEPTIVES AND SUBSTRATE OXIDATION

Table 3. Gas exchange and substrate oxidation measures at supine rest, seated rest, 40% $\dot{V}O_{2peak}$, and 65% $\dot{V}O_{2peak}$

	NAT		OCP2		OCP3		P Values	
	LH	HH	LH	HH	LH	HH		
				Supine rest				
RPE	6±0	6±0	6±1	6±1	6±0	6±0	G*P G P	P = 0.57 P = 0.10 P = 0.44
Rf, breaths/min	14.4±5.5	14.0±5.4	15.3±5.0	15.6±5.5	16.2±4.1	16.5±4.7	G*P G P	P = 0.89 P = 0.53 P = 0.89
VT, L/min	0.56±0.20	0.63±0.25	0.48±0.16	0.50±0.23	0.47±0.07	0.48±0.09	G*P G P	P = 0.50 P = 0.15 P = 0.13
$\dot{V}E$, L/min	6.7±1.2	6.8±1.6	6.4±1.6	6.8±1.5	7.3±1.2	7.5±1.4	G*P G P	P = 0.84 P = 0.29 P = 0.36
$\dot{V}O_2$, L/min	0.22±0.03	0.21±0.04	0.20±0.04	0.20±0.03	0.22±0.02	0.22±0.03	G*P G P	P = 0.21 P = 0.12 P = 0.54
$\dot{V}CO_2$, L/min	0.16±0.03	0.16±0.04	0.14±0.04	0.15±0.03	0.17±0.02	0.17±0.02	G*P G P	P = 0.63 P = 0.13 P = 0.94
Carbohydrate oxidation, g/min	0.07±0.06	0.09±0.06	0.05±0.04	0.08±0.06	0.10±0.06	0.08±0.05	G*P G P	P = 0.16 P = 0.34 P = 0.38
Lipid oxidation, g/min	0.12±0.02	0.10±0.02*	0.11±0.02	0.10±0.01	0.10±0.02	0.11±0.02	**G*P** G P	**P = 0.04** P = 0.45 P = 0.27
				Seated rest				
RPE	6±1	6±0	7±1	7±1	6±1	6±1	G*P G P	P = 0.81 P = 0.18 P = 0.36
Rf, breaths/min	17.4±4.1	19.0±6.2	19.8±6.1	18.6±4.9	18.2±3.7	17.7±5.5	G*P G P	P = 0.17 P = 0.71 P = 0.97
VT, L/min	0.54±0.18	0.59±0.23	0.47±0.20	0.53±0.22	0.53±0.10	0.57±0.14	G*P G **P**	P = 0.97 P = 0.54 **P < 0.017^**
$\dot{V}E$, L/min	8.0±1.1	9.0±1.6	8.2±1.3	8.4±1.5	8.9±1.3	8.9±1.2	G*P G P	P = 0.21 P = 0.30 P = 0.09
$\dot{V}O_2$, L/min	0.26±0.05	0.27±0.04	0.26±0.04	0.26±0.05	0.28±0.04	0.28±0.04	G*P G P	P = 0.48 P = 0.21 P = 0.62
$\dot{V}CO_2$, L/min	0.19±0.03	0.20±0.03	0.18±0.03	0.18±0.04	0.20±0.03	0.20±0.03	G*P G P	P = 0.15 P = 0.15 P = 0.49
Carbohydrate oxidation, g/min	0.07±0.05	0.10±0.06	0.06±0.04	0.05±0.05	0.07±0.05	0.07±0.04	G*P G P	P = 0.11 P = 0.12 P = 0.67
Lipid oxidation, g/min	0.14±0.03	0.14±0.03	0.14±0.02	0.15±0.04	0.16±0.03	0.16±0.03	G*P G P	P = 0.95 P = 0.28 P = 0.96
				40% $\dot{V}O_{2peak}$				
RPE	10±1	10±1	10±2	10±2	10±1	10±1	G*P G P	P = 0.84 P = 0.85 P = 0.90
Rf, breaths/min	23.5±4.9	24.7±5.2	26.8±5.1	24.7±5.0	26.0±6.5	24.8±5.9	G*P G P	P = 0.09 P = 0.59 P = 0.26
VT, L/min	1.14±0.31	1.12±0.30	0.96±0.23	1.04±0.33**	1.03±0.24	1.10±0.24**	**G*P** G **P**	**P = 0.05** P = 0.37 **P = 0.04**
$\dot{V}E$, L/min	25.7±5.1	26.4±6.4	24.4±4.4	24.2±4.7	25.2±4.6	26.1±5.6	G*P G P	P = 0.37 P = 0.58 P = 0.17
$\dot{V}O_2$, L/min	0.98±0.21	0.97±0.24	0.93±0.18	0.92±0.20	0.95±0.17	0.97±0.18	G*P G P	P = 0.42 P = 0.70 P = 0.72

Continued

149

Table 3.— Continued

	NAT		OCP2		OCP3			P Values
	LH	HH	LH	HH	LH	HH		
$\dot{V}CO_2$, L/min	0.77±0.17	0.78±0.19	0.71±0.13	0.70±0.15	0.74±0.15	0.75±0.14	G*P	P = 0.77
							G	P = 0.40
							P	P = 0.87
Carbohydrate oxidation, g/min	0.58±0.19	0.64±0.21	0.43±0.13	0.45±0.16	0.52±0.24	0.50±0.24	G*P	P = 0.31
							G	**P = 0.03#**
							P	P = 0.37
Lipid oxidation, g/min	0.43±0.11	0.40±0.10	0.45±0.11	0.43±0.13	0.43±0.10	0.45±0.11	G*P	P = 0.15
							G	P = 0.77
							P	P = 0.35
			65% $\dot{V}O_{2peak}$					
RPE	14±1	14±1	13±2	13±1	14±2	14±2	G*P	P = 0.55
							G	P = 0.54
							P	P = 0.49
Rf, breaths/min	29.7±6.2	31.0±5.9	31.3±5.4	30.8±5.1	29.1±7.1	29.9±5.4	G*P	P = 0.39
							G	P = 0.73
							P	P = 0.38
V_T, L/min	1.53±0.40	1.51±0.41	1.42±0.39	1.48±0.41	1.59±0.34	1.56±0.31	G*P	P = 0.15
							G	P = 0.67
							P	P = 0.92
$\dot{V}E$, L/min	44.1±10.7	45.7±12.1	43.2±7.1	44.1±8.4	44.6±8.1	44.9±8.0	G*P	P = 0.63
							G	P = 0.92
							P	P = 0.07
$\dot{V}O_2$, L/min	1.60±0.40	1.38±0.41	1.52±0.29	1.53±0.31	1.56±0.28	1.55±0.28	G*P	P = 0.57
							G	P = 0.86
							P	P = 0.88
$\dot{V}CO_2$, L/min	1.4±0.3	1.4±0.4	1.28±0.23	1.30±0.25	1.32±0.23	1.33±0.21	G*P	P = 0.64
							G	P = 0.65
							P	P = 0.46
Carbohydrate oxidation, g/min	1.54±0.45	1.59±0.44	1.31±0.31	1.38±0.32	1.42±0.31	1.43±0.24	G*P	P = 0.70
							G	P = 0.17
							P	P = 0.22
Lipid oxidation, g/min	0.50±0.18	0.47±0.16	0.53±0.16	0.51±0.16	0.51±0.14	0.51±0.16	G*P	P = 0.66
							G	P = 0.81
							P	P = 0.23

Means ± SD. Bold font indicates statistical significance. G, group; G*P, group by phase interaction; HH, high hormone; LH, low hormone; NAT, naturally menstruating; OCP2, second-generation oral contraceptive pills; OCP3, third-generation oral contraceptive pills; P, phase; Rf, breathing frequency; RPE, rating of perceived exertion; $\dot{V}CO_2$, volume of carbon dioxide; $\dot{V}E$, minute ventilation; $\dot{V}O_2$, volume of oxygen; V_T, tidal volume. *Lipid oxidation is lower in the HH phase compared with LH phase within NAT. **V_T is higher in HH phase compared with LH phase in OCP2 and OCP3. ^V_T is higher in HH phase compared with LH phase. #Carbohydrate oxidation is higher in NAT compared with OCP2.

40% or 65% $\dot{V}O_{2peak}$, in agreement with some previous research (10, 15, 22, 28–30, 32, 47, 53, 54). For example, research by Casazza et al. (10) identified no differences between phases in RER in eumenorrheic NAT across early follicular and midluteal phases in a 60-min cycling exercise intervention at either 45% or 65% $\dot{V}O_{2peak}$. This study further identified no differences between phases in RER after NAT participants were prescribed the same triphasic, third-generation OCP for 4 mo (10). Although this duration of contraceptive use may not be sufficient in duration to see impacts on metabolism, participants in the OCP3 group were using contraceptives for ~2.5 yr and we observed similar effects. Further work by Horton et al. (47) has also identified no differences between phases in RER in NAT in a 90-min cycling bout at 50% $\dot{V}O_{2peak}$ across the early follicular, midfollicular, and midluteal phases. Similarly, more recent research by Rael et al. (54) identified no differences between phases in NAT across the early follicular, late follicular, and midluteal phases during a 5-min cycling warm-up phase at 60% $\dot{V}O_{2peak}$ or throughout a high-intensity exercise intervention (8 bouts × 3 min at 85% $\dot{V}O_{2peak}$ with 90 s active recovery between each bout at 30% $\dot{V}O_{2peak}$).

Some previous studies have, however, identified differences between phases in RER or substrate oxidation rates apparent at moderate intensities (17, 19, 21, 24), in contrast with the findings of this current study. Early research by Hackney et al. (24) identified a lower RER in the midluteal versus midfollicular phase across 10-min treadmill exercise bouts at 35% and 65% $\dot{V}O_{2peak}$ but not at 75% $\dot{V}O_{2peak}$, pointing to higher lipid oxidation and lower carbohydrate oxidation at low- and moderate-intensity, short-duration aerobic exercise, but not high-intensity aerobic exercise. Curiously, Zderic et al. (17) observed no differences between phases in carbohydrate or lipid oxidation between the follicular and luteal phases in a 25-min cycling exercise bout at 70% of $\dot{V}O_2$ at lactate threshold (~40% $\dot{V}O_{2peak}$). However, higher lipid oxidation and lower carbohydrate oxidation was observed during a similar cycling exercise bout of 25 min at 90% of $\dot{V}O_2$ at lactate threshold (~52% $\dot{V}O_{2peak}$) (17).

Although it is unclear why there are notable discrepancies in the literature, and in contrast to the current findings, this lack of agreement may be a product of the varying fitness status of participants or individual variability in the responses. Participants in the previously mentioned studies

150

that observed differences between phases had a higher average cardiorespiratory fitness level than the present study ($\sim$48 mL/kg/min vs. $\sim$37–40 mL/kg/min depending on the group in the current study). Further, the present study included a wider range of activity levels in participants (i.e., sedentary to highly elite athletes) in contrast to previous studies in recreational athletes (17) or participants who were "well trained" (24). Further, specifically in the NAT group, while there were no significant differences in body composition and cardiorespiratory fitness across groups (Table 1), the NAT group was bordering overweight (>25 kg/m^2) on average and included more spread of body mass indexes (BMIs) and cardiorespiratory fitness than the other groups. These participant characteristics may have introduced between-subject variability beyond any minor within-subject fluctuations in RER across NAT and OCP phases, particularly in the NAT group. Further, it is well established that there are responders and nonresponders to exercise-induced responses, in addition to variability in hormone fluctuations, especially in circulating levels of sex hormones in NAT, which may have additionally contributed to the lack of differences between phases observed in this study (15, 55). Other sources of variability could have been in participant experience with cycling-based exercise, fitness status, age (both biological and gynecological), or in the reproductive history of participants; all of which should be considered in future research examining fuel use during exercise.

In addition, it is possible that while whole body substrate oxidation assessments did not detect differences between phases, metabolic substrates, and local utilization at the skeletal muscle identified via isotope tracers may be more sensitive to small changes in oxidation across the NAT or OCP cycle (17). Similarly, it is possible that differences between groups and across phases may become more apparent only with longer-duration exercise, in contrast to the short-duration intervention used in this study. Finally, it is possible that we were underpowered to detect a difference in RER across phases within each group (NAT $n = 18$, OCP2 $n = 17$, and OCP3 $n = 15$). However, post hoc sample size calculations performed across phases (LH-HH) using G*Power ($\alpha \leq 0.05$, 80% power) indicated that when groups were pooled, a sample size of $n = 411$ ($d = 0.39$, supine rest), $n = 488$ ($d = 0.14$, seated rest), $n = 882$ ($d = 0.09$, 40% $\dot{V}O_{2peak}$), and $n = 211$ ($d = 0.17$, 65% $\dot{V}O_{2peak}$) would be needed. Similarly, examining differences between phases within each group during exercise, the OCP2 group would require a sample size of $n = 276$ ($d = 0.17$) at 40% $\dot{V}O_{2peak}$ or $n = 73$ ($d = 0.25$) at 65% $\dot{V}O_{2peak}$; the OCP3 group would require a sample size of $n = 698$ ($d = 0.09$) at 40% $\dot{V}O_{2peak}$ or $n = 2,202$ ($d = 0.05$) at 65% $\dot{V}O_{2peak}$; and the NAT group would require a sample size of $n = 106$ ($d = 0.24$) at 40% $\dot{V}O_{2peak}$ or $n = 139$ ($d = 0.19$) at 65% $\dot{V}O_{2peak}$. While it is plausible that a much larger sample size could have resulted in significant differences across phases or groups, this post hoc analysis and associated effect sizes provide further support that RER is largely unchanged by NAT or OCP phases.

In addition to the absence of differences between phases in substrate oxidation, we observed few differences between groups (i.e., NAT, OCP2, and OCP3), despite similar participant characteristics. There was some evidence of differences across groups, with significantly elevated carbohydrate oxidation in the NAT group compared with OCP2 at 40% $\dot{V}O_{2peak}$ and at 65% $\dot{V}O_{2peak}$ in agreement with previous comparisons between NAT and OCP groups (10, 22, 28, 29, 32). For example, research by Suh et al. (22) identified higher glucose appearance (Ra) and disappearance rate (Rd) and metabolic clearance rate during 60-min cycling exercise at both 45% and 65% $\dot{V}O_{2peak}$ in NAT compared with OCP users. Further work by Casazza et al. (10) also identified lower glycerol Ra and Rd during 60-min cycling exercise at both 45% and 65% $\dot{V}O_{2peak}$ in NAT versus OCP users and was confirmed in shorter duration work by Bonen et al. (29) identifying decreased availability of free fatty acids during 30-min treadmill exercise at both 40% $\dot{V}O_{2peak}$ and 85% $\dot{V}O_{2peak}$ in NAT versus OCP users. However, in contrast, one study by Isacco et al. (48) identified that while there was increased lipid oxidation at rest in OCP versus NAT, this difference was not apparent during a 45-min exercise bout at 65% $\dot{V}O_{2peak}$. Given that most studies identifying differences in lipid oxidation in NAT versus OCP used metabolic tracers, it is possible that whole body assessments do not have sufficient precision to detect substrate oxidation differences between groups. Further, there were no differences in outcomes at rest or during exercise between OCP2 and OCP3, despite known androgenicity differences in progestins in OCPs which may influence metabolism (37). While this may point to a null effect of differing progestins on metabolism, it is possible that there are subtle differences present at the skeletal-muscle level that are not apparent in whole body measurements. As discussed in a recent review by Boisseau and Isacco (3), more studies are needed to understand how (and if) OCP use alters whole body lipid oxidation and skeletal muscle lipolytic activity.

Limitations

Although this study includes strengths in its study design, including using multiple stages of rest and exercise to mimic daily movement transitions and its relatively large sample size, in addition to the diverse fitness and body compositions of the population recruited, there are several limitations to discuss. All NAT participants were instructed to use ovulation kits to determine the correct midluteal days for testing. Blood samples were not analyzed to confirm NAT or OCP phases in this study. Traditionally, phase verification is most accurately measured through a three-step verification method. These methods include menstrual cycle tracking, followed by ovulation testing and blood testing (56). However, in a similar study, Mattu et al. (57) have attested that ovulation kits are an accurate and reliable measure for phase determination, thus mitigating the need for more invasive blood draws. Similarly, recent research by Lew et al. (58) found that standard ovulation tests (used in the present study) were as effective as advanced ovulation tests for detecting ovulation and scheduling visits. However, without measurement of serum hormone levels, this study is limited in its ability to make conclusions about fluctuations in specific sex hormones and to be able to confirm that the positive ovulation resulted in elevated midluteal hormone levels. Another limitation of this study was initial challenges with resting data acquisition in the first group of

participants ($n = 14$). Equipment limitations excluded supine resting gas exchange data for initial participants as noted in the study methods. Further, another limitation of this study was the inclusion of participants in OCP2 using only monophasic OCPs, while participants in OCP3 were using both monophasic and triphasic formulations, due to limitations in participant recruitment for the OCP3 group. However, we tested triphasic participants during their highest period of active pill hormones, in the latter half of the highest week, to attempt to examine the acute influence of the hormones. Finally, a limitation of this study was the lack of direct assessments of substrate utilization at the level of the exercising skeletal muscle. It is possible that muscle-level differences in substrate utilization exist at a molecular level, which may not be present at a whole body substrate oxidation level (11). However, as the first study to examine NAT and different generations of OCPs across phases, it was important to first determine whole body metabolic differences in phase comparisons before embarking on an invasive study design.

Conclusions and Future Directions

The present study found that there was largely no influence of NAT or OCP cycles on substrate utilization at rest or during submaximal aerobic exercise; although there may be higher carbohydrate oxidation in NAT participants compared with OCP2 users during aerobic exercise. This study contributes to understanding the influence of NAT or OCP cycles on substrate oxidation and provides a better understanding of female metabolic physiology. Current guidelines in physiology (56, 59) and specifically sport and exercise science (38) detail the importance of controlling for NAT or OCP cycles (often by testing in the LH phase) in research study design. The findings from this study challenge if NAT or OCP cycle controls are necessary when examining whole body substrate oxidation outcomes and builds on previous research noting the lack of NAT or OCP cycles influence in exercise physiology research. Altogether, this study may positively influence the inclusion of females in future study design (46). Future studies should examine how different hormonal contraceptives (oral and nonoral) impact substrate oxidation, with a focus on longer duration exercise interventions with more invasive tracer methodology to assess potential NAT or OCP cycle influences.

DISCLOSURES

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

AUTHOR CONTRIBUTIONS

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

REFERENCES

1. **Jeukendrup AE, Wallis GA.** Measurement of substrate oxidation during exercise by means of gas exchange measurements. *Int J Sports Med* 26, Suppl 1: S28–S37, 2005. doi:10.1055/s-2004-830512.

2. **Beaudry KM, Devries MC.** Sex-based differences in hepatic and skeletal muscle triglyceride storage and metabolism. *Appl Physiol Nutr Metab* 44: 805–813, 2019. doi:10.1139/apnm-2018-0635.

3. **Boisseau N, Isacco L.** Substrate metabolism during exercise: sexual dimorphism and women's specificities. *Eur J Sport Sci* 22: 672–683, 2022. doi:10.1080/17461391.2021.1943713.

4. **Tarnopolsky MA.** Sex differences in exercise metabolism and the role of 17-beta estradiol. *Med Sci Sports Exerc* 40: 648–654, 2008. doi:10.1249/MSS.0b013e31816212ff.

5. **Friedlander AL, Casazza GA, Horning MA, Huie MJ, Piacentini MF, Trimmer JK, Brooks GA.** Training-induced alterations of carbohydrate metabolism in women: women respond differently from men. *J Appl Physiol (1985)* 85: 1175–1186, 1998. doi:10.1152/jappl.1998.85.3.1175.

6. **Hamadeh MJ, Devries MC, Tarnopolsky MA.** Estrogen supplementation reduces whole body leucine and carbohydrate oxidation and increases lipid oxidation in men during endurance exercise. *J Clin Endocrinol Metab* 90: 3592–3599, 2005. doi:10.1210/jc.2004-1743.

7. **Phillips SM, Atkinson SA, Tarnopolsky MA, Macdougall JD.** Gender differences in leucine kinetics and nitrogen-balance in endurance athletes. *J Appl Physiol (1985)* 75: 2134–2141, 1993. doi:10.1152/jappl.1993.75.5.2134.

8. **Tarnopolsky LJ, MacDougall JD, Atkinson SA, Tarnopolsky MA, Sutton JR.** Gender differences in substrate for endurance exercise. *J Appl Physiol (1985)* 68: 302–308, 1990. doi:10.1152/jappl.1990.68.1.302.

9. **Aucouturier J, Baker JS, Duché P.** Fat and carbohydrate metabolism during submaximal exercise in children. *Sports Med* 38: 213–238, 2008. doi:10.2165/00007256-200838030-00003.

10. **Casazza GA, Jacobs KA, Suh SH, Miller BF, Horning MA, Brooks GA.** Menstrual cycle phase and oral contraceptive effects on triglyceride mobilization during exercise. *J Appl Physiol (1985)* 97: 302–309, 2004. doi:10.1152/japplphysiol.00050.2004.

11. **Fu MH, Maher AC, Hamadeh MJ, Ye C, Tarnopolsky MA.** Exercise, sex, menstrual cycle phase, and 17β-estradiol influence metabolism-related genes in human skeletal muscle. *Physiol Genomics* 40: 34–47, 2009. doi:10.1152/physiolgenomics.00115.2009.

12. **Campbell SE, Febbraio MA.** Effect of ovarian hormones on mitochondrial enzyme activity in the fat oxidation pathway of skeletal muscle. *Am J Physiol Endocrinol Physiol* 281: E803–E808, 2001. doi:10.1152/ajpendo.2001.281.4.E803.

13. **D'Eon T, Braun B.** The roles of estrogen and progesterone in regulating carbohydrate and fat utilization at rest and during exercise. *J Womens Health Gend Based Med* 11: 225–237, 2002. doi:10.1089/152460902753668439.

14. **McNulty KL, Elliott-Sale KJ, Dolan E, Swinton PA, Ansdell P, Goodall S, Thomas K, Hicks KM.** The effects of menstrual cycle phase on exercise performance in eumenorrheic women: a systematic review and meta-analysis. *Sports Med* 50: 1813–1827, 2020. doi:10.1007/s40279-020-01319-3.

15. **Devries MC, Hamadeh MJ, Phillips SM, Tarnopolsky MA.** Menstrual cycle phase and sex influence muscle glycogen utilization and glucose turnover during moderate-intensity endurance exercise. *Am J Physiol Regul Integr Comp Physiol* 291: R1120–R1128, 2006. doi:10.1152/ajpregu.00700.2005.

16. **Mattu AT, Iannetta D, MacInnis MJ, Doyle-Baker PK, Murias JM.** Menstrual and oral contraceptive cycle phases do not affect submaximal and maximal exercise responses. *Scand J Med Sci Sports* 30: 472–484, 2020. doi:10.1111/sms.13590.

17. **Zderic TW, Coggan AR, Ruby BC.** Glucose kinetics and substrate oxidation during exercise in the follicular and luteal phases. *J Appl Physiol (1985)* 90: 447–453, 2001. doi:10.1152/jappl.2001.90.2.447.

18. **Hackney AC.** Effects of the menstrual cycle on resting muscle glycogen content. *Horm Metab Res* 22: 647–647, 1990. doi:10.1055/s-2007-1004994.

19. **Hackney AC, Muoio D, Meyer WR.** The effect of sex steroid hormones on substrate oxidation during prolonged submaximal exercise in women. *Jpn J Physiol* 50: 489–494, 2000. doi:10.2170/jjphysiol.50.489.

20. **Nicklas BJ, Hackney AC, Sharp RL.** The menstrual cycle and exercise: performance, muscle glycogen, and substrate responses. *Int J Sports Med* 10: 264–269, 1989. doi:10.1055/s-2007-1024913.

21. **Ortega-Santos CP, Barba-Moreno L, Cupeiro R, Peinado Lozano AB.** Substrate oxidation in female adults during endurance exercise throughout menstrual cycle phases: IronFEMME pilot study. *J Hum Sport Exerc* 13: 553–565, 2018. doi:10.14198/jhse.2018.133.07.

22. **Suh SH, Casazza GA, Horning MA, Miller BF, Brooks GA.** Effects of oral contraceptives on glucose flux and substrate oxidation rates during rest and exercise. *J Appl Physiol (1985)* 94: 285–294, 2003. doi:10.1152/japplphysiol.00693.2002.

23. **Hackney AC, Koltun KJ, Williett HN.** Menstrual cycle hormonal changes: estradiol-β-17 and progesterone interactions on exercise fat oxidation. *Endocrine* 76: 240–242, 2022. doi:10.1007/s12020-022-02998-w.

24. **Hackney AC, McCracken-Compton MA, Ainsworth B.** Substrate responses to submaximal exercise in the midfollicular and midluteal phases of the menstrual cycle. *Int J Sport Nutr* 4: 299–308, 1994. doi:10.1123/ijsn.4.3.299.

25. **Rotermann M, Dunn S, Black A.** Oral contraceptive use among women aged 15 to 49: results from the Canadian Health Measures Survey. *Health Rep* 26: 21–28, 2015.

26. **Park H, Kim K.** Trends and factors associated with oral contraceptive use among Korean women. *Healthcare (Basel, Switzerland)* 9: 1386, 2021. doi:10.3390/healthcare9101386.

27. **Burrows M, Peters CE.** The influence of oral contraceptives on athletic performance in female athletes. *Sports Med* 37: 557–574, 2007. doi:10.2165/00007256-200737070-00001.

28. **Bemben DA, Boileau RA, Bahr JM, Nelson RA, Misner JE.** Effects of oral contraceptives on hormonal and metabolic responses during exercise. *Med Sci Sports Exerc* 24: 434–441, 1992.

29. **Bonen A, Haynes FW, Graham TE.** Substrate and hormonal responses to exercise in women using oral contraceptives. *J Appl Physiol (1985)* 70: 1917–1927, 1991. doi:10.1152/jappl.1991.70.5.1917.

30. **Jacobs KA, Casazza GA, Suh S-H, Horning MA, Brooks GA.** Fatty acid reesterification but not oxidation is increased by oral contraceptive use in women. *J Appl Physiol (1985)* 98: 1720–1731, 2005. doi:10.1152/japplphysiol.00685.2004.

31. **Redman LM, Scroop GC, Westlander G, Norman RJ.** Effect of a synthetic progestin on the exercise status of sedentary young women. *J Clin Endocrinol Metab* 90: 3830–3837, 2005. doi:10.1210/jc.2004-2401.

32. **Isacco L, Duché P, Boisseau N.** Influence of hormonal status on substrate utilization at rest and during exercise in the female population. *Sports Med* 42: 327–342, 2012. doi:10.2165/11598900-000000000-00000.

33. **Elliott-Sale KJ, Smith S, Bacon J, Clayton D, McPhilimey M, Goutianos G, Hampson J, Sale C.** Examining the role of oral contraceptive users as an experimental and/or control group in athletic performance studies. *Contraception* 88: 408–412, 2013. doi:10.1016/j.contraception.2012.11.023.

34. **Darney PD.** The androgenicity of progestins. *Am J Med* 98, Suppl 1: S104–S110, 1995. doi:10.1016/s0002-9343(99)80067-9.

35. **Williams JS, MacDonald MJ.** Influence of hormonal contraceptives on peripheral vascular function and structure in premenopausal females: a review. *Am J Physiol Heart Circ Physiol* 320: H77–H89, 2021. doi:10.1152/ajpheart.00614.2020.

36. **Linn ES.** Clinical significance of the androgenicity of progestins in hormonal therapy in women. *Clin Ther* 12: 447–455, 1990.

37. **Silva-Bermudez LS, Toloza FJK, Perez-Matos MC, de Souza RJ, Banfield L, Vargas-Villanueva A, Mendivil CO.** Effects of oral contraceptives on metabolic parameters in adult premenopausal women: a meta-analysis. *Endocr Connect* 9: 978–998, 2020. doi:10.1530/EC-20-0423.

38. **Elliott-Sale KJ, Minahan CL, de Jonge X, Ackerman KE, Sipilä S, Constantini NW, Lebrun CM, Hackney AC.** Methodological considerations for studies in sport and exercise science with women as participants: a working guide for standards of practice for research on women. *Sports Med* 51: 843–861, 2021. doi:10.1007/s40279-021-01435-8.

39. **Tanaka H, Monahan KD, Seals DR.** Age-predicted maximal heart rate revisited. *J Am Coll Cardiol* 37: 153–156, 2001. doi:10.1016/s0735-1097(00)01054-8.

40. **Bostad W, McCarthy DG, Richards DL, Helal L, Gibala MJ.** Peak cardiac output determined using inert gas rebreathing: a comparison of two exercise protocols. *Med Sci Sports Exerc* 55: 1014–1022, 2023. doi:10.1249/MSS.0000000000003125.

41. **Bostad W, Valentino SE, McCarthy DG, Richards DL, MacInnis MJ, MacDonald MJ, Gibala MJ.** Twelve weeks of sprint interval training increases peak cardiac output in previously untrained individuals. *Eur J Appl Physiol* 121: 2449–2458, 2021. doi:10.1007/s00421-021-04714-4.

42. **Hawley JA, Noakes TD.** Peak power output predicts maximal oxygen uptake and performance time in trained cyclists. *Eur J Appl Physiol Occup Physiol* 65: 79–83, 1992. doi:10.1007/BF01466278.

43. **Péronnet F, Massicotte D.** Table of nonprotein respiratory quotient: an update. *Can J Sport Sci* 16: 23–29, 1991.

44. **Cohen J.** *Statistical Power Analysis for the Behavioral Sciences.* New York: Routledge, 1988.

45. **Cowley ES, Olenick AA, McNulty KL, Ross EZ.** "Invisible sportswomen": the sex data gap in sport and exercise science research. *Women Sport Phys Act J* 29: 146–151, 2021. doi:10.1123/wspaj.2021-0028.

46. **Lew LA, Williams JS, Stone JC, Au AKW, Pyke KE, MacDonald MJ.** Examination of sex-specific participant inclusion in exercise physiology endothelial function research: a systematic review. *Front Sports Act Living* 4: 860356, 2022. doi:10.3389/fspor.2022.860356.

47. **Horton TJ, Miller EK, Glueck D, Tench K.** No effect of menstrual cycle phase on glucose kinetics and fuel oxidation during moderate-intensity exercise. *Am J Physiol Endocrinol Physiol* 282: E752–E762, 2002. doi:10.1152/ajpendo.00238.2001.

48. **Isacco L, Thivel D, Pelle AM, Zouhal H, Duclos M, Duche P, Boisseau N.** Oral contraception and energy intake in women: impact on substrate oxidation during exercise. *Appl Physiol Nutr Metab* 37: 646–656, 2012. doi:10.1139/h2012-031.

49. **Barba-Moreno L, Cupeiro R, Romero-Parra N, Janse de Jonge XAK, Peinado AB.** Cardiorespiratory responses to c cycle and with oral contraceptive use. *J Strength Cond Res* 36: 392–399, 2022. doi:10.1519/JSC.0000000000003447.

50. **Schoene RB, Robertson HT, Pierson DJ, Peterson AP.** Respiratory drives and exercise in menstrual cycles of athletic and nonathletic

women. *J Appl Physiol Respir Environ Exerc Physiol* 50: 1300–1305, 1981. doi:10.1152/jappl.1981.50.6.1300.

51. **Williams TJ, Krahenbuhl GS.** Menstrual cycle phase and running economy. *Med Sci Sports Exerc* 29: 1609–1618, 1997. doi:10.1097/00005768-199712000-00010.

52. **Baker FC, Siboza F, Fuller A.** Temperature regulation in women: effects of the menstrual cycle. *Temperature (Austin)* 7: 226–262, 2020. doi:10.1080/23328940.2020.1735927.

53. **Horton TJ, Grunwald GK, Lavely J, Donahoo WT.** Glucose kinetics differ between women and men, during and after exercise. *J Appl Physiol (1985)* 100: 1883–1894, 2006. doi:10.1152/japplphysiol.01431.2005.

54. **Rael B, Alfaro-Magallanes VM, Romero-Parra N, Castro EA, Cupeiro R, Janse de Jonge XAK, Wehrwein EA, Peinado AB; IronFEMME Study Group.** Menstrual cycle phases influence on cardiorespiratory response to exercise in endurance-trained females. *Int J Environ Res Public Health* 18: 860, 2021. doi:10.3390/ijerph18030860.

55. **Bull JR, Rowland SP, Scherwitzl EB, Scherwitzl R, Danielsson KG, Harper J.** Real-world menstrual cycle characteristics of more than 600,000 menstrual cycles. *NPJ Digit Med* 2: 83, 2019. doi:10.1038/s41746-019-0152-7.

56. **Sims ST, Heather AK.** Myths and methodologies: reducing scientific design ambiguity in studies comparing sexes and/or menstrual cycle phases. *Exp Physiol* 103: 1309–1317, 2018. doi:10.1113/EP086797.

57. **Mattu AT, MacInnis MJ, Doyle-Baker PK, Murias JM.** Effects of the menstrual and oral contraceptive cycle phases on microvascular reperfusion. *Exp Physiol* 105: 184–191, 2020. doi:10.1113/EP088135.

58. **Lew LL, Liu KR, Pyke KE.** Comparison of two ovulation tests to improve predicted timing of the late follicular phase for menstrual cycle research. *Proceedings of the Canadian Society for Exercise Physiology Annual General Meeting—Health and Performance for the Future/Santé et performance pour l'avenir*. Fredericton, Canada, Nov 2–5, 2022, vol. 47, p. S78.

59. **Wenner MM, Stachenfeld NS.** Point: investigators should control for menstrual cycle phase when performing studies of vascular control that include women. *J Appl Physiol (1985)* 129: 1114–1116, 2020. doi:10.1152/japplphysiol.00443.2020.

CHAPTER 7: Differences in cardiovascular risk factors associated with biological sex, but not gender expression, in young, healthy adults.

Jennifer S. Williams, Elise Wiley, Jem L. Cheng, Jenna C. Stone, William Bostad, Joshua M. Cherubini, Martin J. Gibala, Ada Tang, Maureen J. MacDonald

Abstract

Background: Sex-differences exist in risk factors for cardiovascular disease including elevated blood pressure and arterial stiffness, and decreased endothelial function in males compared to females. Feminine gender expression may be associated with elevated risk of acute coronary syndrome. However, no study has investigated the associations between sex, gender identity, and gender expression and risk factors for cardiovascular disease in young adults.

Methods: One hundred and thirty participants (22±3y) underwent assessments of hemodynamics, arterial stiffness (pulse wave velocity; PWV), and brachial artery endothelial function (flow mediated dilation; %FMD). Participants completed a questionnaire capturing biological sex (50 male/80 female), gender identity (49 men/79 women/2 non-binary) and gender expression assessed by the Bem Sex Role Inventory-30 (39 androgynous/33 feminine/29 masculine/29 undifferentiated). Sex and gender identity groups were compared using unpaired t-tests and gender expression groups compared using one-way ANOVAs.

Results: Resting systolic and mean arterial pressure (p<0.01) were elevated in males *vs.* females. Central PWV was elevated in males *vs.* females (median [interquartile range]: 6.4[1.8] *vs.* 5.8[2.2] m/s, p=0.02); however, leg and arm PWV were not different between sexes. %FMD was elevated in males *vs.* females, after accounting for a larger baseline artery diameter in males (8.8±3.3% *vs.* 7.2±3.1%, p=0.02). While the same results were found in gender identity (men *vs.* women), there were no differences across gender expression groups for any outcome (p>0.05).

Conclusions: Biological sex and gender identity, but not gender expression, influence cardiovascular risk factors. Elevated systolic blood pressure and central arterial stiffness, but greater brachial artery endothelial function, was observed in males versus females and men versus women.

Keywords: cardiovascular health, sex-differences, sex- and gender-based analysis, flow mediated dilation, pulse wave velocity.

Plain English Summary

Biological sex (i.e., male, female, intersex) is defined as sex determined at birth through reproductive anatomy, and gender identity is defined as a human's preferred gender (i.e., man, woman, non-binary, transgender). In addition, gender expression (i.e., feminine, masculine, androgynous, undifferentiated) is defined as the way in which a human expresses their gender identity often through appearance, behaviours and gendered traits. There is evidence that biological sex influences risk for having cardiovascular disease; however, the impact of gender identity and expression on risk factors for cardiovascular disease are not well studied. The purpose of this study was to investigate how sex, gender identity, and gender expression impacted risk factors for cardiovascular disease. One hundred and thirty young, healthy adults were tested for known early risk factors for cardiovascular disease, including blood pressure, artery stiffness, and endothelial function (function of cells that line the arteries). We found that males and men had higher blood pressure and artery stiffness compared to females and women, in line with previous research. We also found that endothelial function was higher in males and men compared to females and women, when we took into account that males and men have greater artery size. Lastly, we did not find differences in any cardiovascular outcome for gender expression groups. Therefore, biological sex and gender identity, but not gender expression, appears to impact cardiovascular disease risk in young, healthy adults. Further research in middle-aged and older adults, along with gender diverse groups (i.e., non-binary, transgender) is needed.

Highlights

- Sex-differences in cardiovascular outcomes has been well studied across the lifespan, with elevated blood pressure and arterial stiffness, but lower endothelial function, in males compared to females; however, other physiological factors such as cardiorespiratory fitness and resting hemodynamics have not been previously considered in these associations. Furthermore, the associations between gender identity and expression and cardiovascular outcomes are not well understood.

- This study found that systolic blood pressure and central arterial stiffness were elevated in young males and men compared to females and women; similarly

endothelial function (scaled for differences in baseline artery diameter) was also elevated in males and men. There were no differences across gender expression groups for any outcome.

- Biological sex (aligned with gender identity in 99% of participants), but not gender expression, influenced cardiovascular risk factors. Further research examining sex- and gender-differences in middle-aged and older adults, and gender diverse participants (i.e., non-binary, transgender) is needed.

Introduction

Cardiovascular disease (CVD) is the leading cause of death worldwide (1-3). There are known sex-differences in its pathophysiology and associated traditional and novel risk factors (4-6). For example, CVD progression and mortality is accelerated in males compared to females, and the first myocardial infarction occurs ~9 years earlier in males (7). Approximately 80% of this age-related difference is attributed to a deleterious cardiovascular risk profile in males earlier in life owing to factors such as smoking, hypertension, and diabetes (7). However, females have a higher burden of disease and disability after the fifth decade of life; this burden is partly associated with the menopause transition (4, 6) and loss of the cardioprotective effects of the sex hormone estrogen (8, 9).

Early risk factors for cardiovascular disease can predict future CVD risk (10) and follow similar age- and sex-related patterns of decline (9, 11, 12). Two important variables are endothelial function, measured by brachial artery flow mediated dilation (FMD) and arterial stiffness, measured by pulse wave velocity (PWV). For example, a recent large multi-site trial identifying age- and sex-differences in brachial artery FMD, found that while females have a higher relative FMD early in life compared to males, females experience a more rapid decline in FMD across the aging lifespan. This may be attributed to changes in hormone levels across the menopause transition, namely the influence of 17β-estradiol (11). This study also found that males have a larger brachial artery diameter, regardless of age, which has been suggested to mask the capacity for the artery to dilate, thereby exacerbating the rapid decline observed in females (11). In contrast, research from our lab in young, healthy participants (age: 22±3 years)

identified sex-differences in brachial artery FMD, such that FMD was elevated in males as compared to females when the influence of larger artery diameter in males was accounted for using allometric scaling (13). Accounting for sex-differences in resting brachial artery diameter may be contribute to the discrepant study results. However, other factors, such as differences in resting blood pressure or cardiorespiratory fitness may also influence purposed sex-differences in FMD (14, 15).

Arterial stiffness appears to be higher in males compared to females early in life, until middle age when arterial stiffness equalizes across sex. The effect is potentially associated with the menopause transition and the role of 17β-estradiol (16) and/or differences in anatomical growth patterns (16, 17), where stiffness rapidly increases in females (18). Our laboratory has found that local stiffness measured as carotid artery compliance was elevated in young males compared to females (19). While this study did not observe sex-differences in central and peripheral arterial stiffness, measured via PWV (19), other studies have observed elevated central and peripheral arterial stiffness levels in males compared to females (20, 21). However, prior research examining the impact of biological sex on arterial stiffness and endothelial function did not consider other factors that may be underlying these sex-differences. These include cardiorespiratory fitness, resting hemodynamics such as systolic blood pressure, and other intersections of identity, such as gender identity and expression.

We recently found that less than 1% of cardiovascular exercise physiology research has considered gender as a factor or variable that may differentially impact responses, and that published studies only examined gender identity and not other constructs such as gender expression or roles (22). In contrast to biological sex, which

mainly examines sex assigned at birth (i.e., male, female, intersex) and sex-related factors such as anatomy, chromosomes, and sex hormones, gender considers the socially constructed identity, expression, roles, and institutional structures and policies (23, 24) that act independently and/or synergistically to influence health (25, 26). Gender identity is commonly used in biomedical and clinical research, characterizing men, women, and gender-diverse individuals (24, 25). Gender expression refers to how an individual portrays gendered personality traits, namely masculine and feminine gender traits (27). The Bem Sex Role Inventory was developed and validated in young and middle-aged adults to assess how individuals perceive their expression of gendered traits, to create a composite score of the balance between masculinity and femininity (27, 28). Gender expression, along with gender roles, may contribute to stress-related impacts on the cardiovascular system, such as the stress associated with caregiving, psychosocial stressors, and role strain/balance between work and home life (29-32). For example, one study found that anger expression and control were associated with impaired cardiovascular health indices (i.e., blood pressure, blood lipids) in men, but not in women (33). Another study found that feminine gender roles and expression may play a role in the recurrence of acute coronary syndrome in young individuals, suggesting that examining gender expression and roles alongside biological sex and gender identity is of clinical importance (34). Further, a recent review by our group focused on the intersections of aging and sex/gender influences on cardiovascular indices also noted the lack of research examining gender (35). Similarly, a recent narrative review by Seeland and colleagues (2021) and the VascAgeNet Gender Expert Group points to the importance of examining both sex- and gender-related factors in

vascular research and aging (36). We are not aware of any study to date that has examined the influence of gender identity and expression on early risk factors for CVD, including endothelial function or arterial stiffness, in young adults.

Given the sex- and gender-related gaps that exist, the primary objective of this study was to determine the association of biological sex, gender identity, and gender expression with endothelial function (measured using brachial artery FMD) and arterial stiffness (measured using PWV) in young, healthy adults. We hypothesized that males and men would have elevated FMD when baseline arterial diameter is considered, and this sex- and gender-difference may be attributed to higher cardiorespiratory fitness levels in males and men. Similarly, we hypothesized that PWV would be higher in males and men (37). We also hypothesize that there would be a blunted cardiovascular profile (i.e., lower FMD and higher PWV) in individuals classified as feminine, aligned with prior clinical research on acute coronary syndrome in middle-aged adults (34). The secondary objective of this study was to investigate the relationships between feminine and masculine gender expression scores, cardiorespiratory fitness, central PWV, and FMD. We also hypothesized that cardiorespiratory fitness would be associated with increased FMD and decreased central PWV (14, 15), and that that higher feminine scores would be associated with decreased FMD and elevated central PWV. As the first study to investigate the effects of sex and gender on early risk indicators for cardiovascular disease in a young adult population, we hoped to clarify the impact of these factors on future risk for CVD, aligned with recent calls for sex- and gender-based analysis in research (4, 23, 24, 38, 39).

Methods

Participant Recruitment & Ethics

One hundred and thirty male and female participants between the ages of 18 and 45 were recruited from the McMaster University and Hamilton communities through posters, online advertisements, and word of mouth. A sample size calculation estimated that 50 participants (25 male, 25 female) would be needed to find sex-differences in scaled %FMD, based on previous work by our lab (13) (%FMD: Male: 9.0±2.6%, Female: 6.5±2.1%, α ≤ 0.05, power: 95%; independent t-test in G*Power). However, given that no study had previously examined gender expression in a young, healthy cohort to base power calculations on, and given that a 1% change in %FMD is clinically relevant (10) and the robust change in clinical risk observed previously (34), we considered a 2% scaled FMD difference between gender expression groups (masculine, feminine, androgynous, undifferentiated; Δ2% change between feminine and masculine groups) to be meaningful. We determined that 120 participants were required to detect a significant difference assuming a SD of 2%, an alpha level of 0.05, and power of 90% based on a one-way ANOVA in G*Power. This study was approved by the Hamilton Integrated Research Ethics Board (#14884) and conforms to the standards set by the *Declaration of Helsinki*, apart from registration of this study in a database. Participants completed a medical screening form to determine eligibility and provided written informed consent before participating in the study. Participants were excluded if they reported cardiovascular or metabolic diseases, were pregnant or within the last year, were taking vasoactive medication (e.g., beta-blockers, ACE inhibitors, diuretics), or were smokers. Participants who had previously participated in a study in

our laboratory within the last year and agreed to be contacted for future studies were recruited (n=149), to which n=130 agreed to participate. Potential participants who had been recruited using existing trials were re-consented to have their data included for this specific research question. Most participants (n=114) were recruited prospectively through five existing studies that had embedded the demographic questionnaire into their study design and used lab-wide standardized methods for the relevant outcomes. A small number of participants (n=16) were recruited retrospectively after completion of a study and asked to complete the questionnaire through a virtual medium (Zoom).

Study Protocol

All study visits took place in the Vascular Dynamics Laboratory at McMaster University in a temperature (23.3°C) and humidity (15%) controlled, quiet room. Prior to commencing the study, participants attended a familiarization visit to become familiar with the lab environment, assess eligibility, and complete consent forms and medical screening forms. Following recruitment, anthropometric information, including age and height and weight to assess body mass index (kg/m^2) was collected during the familiarization visit. Participants were also familiarized to the FMD test as described below.

Following the familiarization visit, participants participated in a vascular assessment session and a cardiorespiratory fitness exercise test. The cardiorespiratory fitness test was completed in close temporal proximity to the vascular assessment session, either in the week prior to the vascular assessment visit during the familiarization session (n=64), immediately after on the same day (n=46), or within 3 days following the vascular assessment visit (n=20). Prior to the vascular assessment

session, participants completed at least a 6h overnight fast, 12h without alcohol or caffeine, 24h without moderate to vigorous physical activity, and 12h without the use of prescription or non-prescription medications (i.e., anti-inflammatory and pain medications). Participants were tested during the morning hours to control for diurnal variation in endothelial function (40).

Demographic Questionnaire: Participants were asked to complete a demographic questionnaire asking for information about their sex assigned at birth (i.e., biological sex), gender identity and expression, ethnicity, and race. Ethnicity and race were collected using questionnaires developed by the Governments of Canada (Canadian Census) (41), and Ontario (Ontario data standards for collection of race) (42), respectively. A two-step sex and gender question asked about biological sex at birth (options: female, male, intersex, prefer not to answer) and then about gender identity (woman, man, gender diverse/gender fluid, two-spirit, non-binary, prefer to self describe, prefer not to answer), as a widely used question in clinical research settings (43, 44). Finally, gender expression was assessed using the Bem Sex Role Inventory 30-item questionnaire (BSRI-30) that has been used previously in a university population (28, 45, 46). This version of the BSRI asks participants to identify with 30 traits from the BSRI according to a 7-point Likert-type scale from "1 - Never or almost never true" to "7 - Almost Always true" (Supplementary Table 1). Each trait was previously categorized as "masculine", "feminine", or "neutral", with ten traits in each category. A mean score for masculine (BSRI-masculine) and feminine (BSRI-feminine) was calculated based on the average of the ten traits in each category. The median BSRI-masculine (4.40) and BSRI-feminine (5.20) scores was determined from the

overall study population (n=130), and each participant was categorized according to the following criteria:

Feminine: High BSRI-Feminine (≥5.20), Low BSRI-Masculine (<4.40)

Masculine: High BSRI-Masculine (≥4.40), Low BSRI-Feminine (<5.20)

Androgynous: High BSRI-Masculine (≥ 4.40) and BSRI-Feminine (≥5.20)

Undifferentiated: Low BSRI-Masculine (<4.40) and BSRI-Feminine (<5.20)

Use of internal population-defined medians was chosen over the use of a 4.0 split or using Bem's original reported medians to reflect the gender expression unique to this population, such as generational or geographical differences in gender expression, supported by previous work (47, 48). The questionnaire was completed correctly in 92% of cases, with only n=11 participants requiring follow-up if the questionnaire was incomplete (i.e., not responding to 1 trait question/30 traits on the BSRI-30).

Resting Hemodynamics (Heart Rate, Blood Pressure): Resting heart rate (HR) and blood pressure (BP) [including systolic blood pressure (SBP), diastolic blood pressure (DBP), and mean arterial pressure (MAP)] were assessed using the average of the last two of three measurements collected via an automated BP assessment device (GE Dinamap ProSeries, Batesville IN). If the second and third systolic BP measurements collected were not within 5 mmHg of one another, a fourth measurement was collected with the last two averaged.

Arterial Stiffness (Pulse Wave Velocity): Pulse wave velocity (PWV, m/s) is a measure of regional arterial stiffness that uses applanation tonometry to detect the pressure waveform from the skin surface of an artery using micromanometer-tipped pressure

probes (SPT-301, Millar Instruments), as previously reported by our lab (19). These

pressure waveforms are then band-pass filtered between 5-30Hz to determine the foot

of each pulse for the calculation of the pulse transit time using LabChart (AD

Instruments, Colorado Springs, CO). Distance between the measured locations was

assessed as the average of two measurements using a measuring tape over the

surface of the body. Tonometers were placed on the carotid and femoral arterial sites

for the determination of central PWV, and calculated using the following formula: central

PWV = (0.8 * carotid-femoral distance)/carotid-femoral pulse transit time (49).

Peripheral leg PWV was calculated using waveforms at the femoral and dorsalis pedis

arteries according to the following formula: leg PWV = (femoral-dorsalis pedis

distance)/femoral-dorsalis pedis transit time (50, 51). Similarly, peripheral arm PWV was

calculated using waveforms at the carotid and radial arteries according to the following

formula: arm PWV = (radial-suprasternal notch distance – (carotid-suprasternal notch

distance))/carotid-radial pulse transit time (50, 51). Central PWV was collected in all

participants (n=130), but leg PWV was collected in n=128 participants and arm PWV in

only n=82 participants, due to different collection protocols for the studies included in

this project. Measurements were reported as the mean of two sets of 10 continuous

heart cycles. If the PWV measurements between the two sets were not within 0.5 m/s of

one another, a third set of 10 heart cycles was collected and averaged. Given the

dependent of PWV on arterial blood pressure (52), measures of blood pressure were

included as a covariate in statistical analysis of PWV outcomes.

Endothelial Function & Blood Velocity: Participants completed a brachial artery reactive

hyperemia FMD test to assess macrovascular endothelial function. Tests conducted in

the left arm of n=82 participants and in the right arm of n=48 participants, based on the initial study in which participants were recruited from. Using a Doppler ultrasound machine (Vivid Q, GE Medical Systems, Horten, Norway) attached to a 12 MHz linear array probe in duplex mode with an insonation angle of 68° (53), brachial artery diameter and blood velocity was collected before cuff inflation (baseline) for 30s. In line with current guidelines (54), a pneumatic blood pressure cuff was placed around the forearm and was rapidly inflated to suprasystolic pressure (~200 mmHg) for five minutes to occlude blood flow to the distal artery. Arterial diameters and blood velocity were measured again using the same Doppler ultrasound machine following 4 minutes of cuff inflation (occlusion; 30s) and in the three minutes immediately following cuff deflation. During the test, heart rate was collected using single-lead electrocardiogram into the Doppler ultrasound. Images were stored in a Digital Imaging and Communications in Medicine (DICOM) format, and end-diastolic frames were extracted and compiled (Sante DICOM Editor, v. 3.1.20, Santesoft, Athens, Greece, or an internally created extraction program called Pancakes). DICOM files were then analyzed using a semi-automated edge tracking software (Artery Measurement System (AMS) II, version 1.141, Gothenburg, Sweden) (55). Baseline diameter was determined as an average of the arterial diameters during rest in the 30s prior to inflation, and peak diameter was determined as the largest 5-heart cycle average of diameters in the 3 minutes following deflation. FMD was reported as both an absolute change (AbsFMD) and percentage change (%FMD) in diameter: FMD = peak diameter – baseline diameter; %FMD = ((peak diameter – baseline diameter)/baseline diameter) x 100%. Mean blood velocity (MBV) measures taken at the same time as ultrasound assessments, extracted as AVI

files, and were analyzed using a pixel-based tracking software (Measurements from

Arterial Ultrasound Imaging; Hedgehog Medical, Waterloo, ON, Canada). MBV was

similarly averaged into 5-heart cycle average time bins and used to calculate shear rate

(SR = 8 x MBV/arterial diameter), as described previously (56). The time to peak

diameter and SR areas under the curve to the time of peak diameter are reported.

Cardiorespiratory Fitness Test ($\dot{V}O_2peak$): Participants completed an incremental

exercise test to exhaustion seated upright on a stationary cycle ergometer (Lode

Excalibur Sport V 2.0, Groningen, Netherlands or Kettler Ergo Race, Kettler, Virginia

Beach VA) to determine $\dot{V}O_2peak$, in accordance with current guidelines (57). A

metabolic cart with an online gas collection system (Quark CPET metabolic cart,

COSMED, Italy) was used to determine oxygen consumption and carbon dioxide

production. HR was monitored continuously with a HR monitor (Polar A3, Lake

Success, NY). The $\dot{V}O_2peak$ test began with a 3-minute warm-up at 50W (or lower if the

participant indicated this intensity was too intense for a warm-up), after which the power

was increased by five watts every ten seconds until volitional exhaustion or the point at

which pedal cadence fell below 60 rpm, as described previously (58). Following

reaching this point, participants continued to cycle to cool down for two minutes at 50W

or less. $\dot{V}O_2peak$ (mL/kg/min) was defined as the highest oxygen consumption achieved

over a 30s period. The $\dot{V}O_2peak$ test was considered successful if at least of two of the

following four criteria were met: (1) perceived exertion was >17 on a Borg scale of 6-20;

(2) HR was within 10 beats per minute of age-predicted maximal HR (208-0.7*age); (3)

their respiratory exchange ratio was >1.1; and (4) a plateau in $\dot{V}O_2$ was reached. If

V̇O$_2$peak was not achieved, the participant returned on another day to perform the test again.

Statistical Analysis

All statistical analyses were performed using SPSS, version 22 (IBM, Chicago, IL, USA), and R Statistical Software (v.4.1.0, R Core Team, 2023) for allometric scaling analysis. All data was reported using descriptive statistics, including means and standard deviations for normally distributed continuous variables, and medians and interquartile ranges for non-normally distributed continuous variables, and frequencies (percentages) for categorical variables (i.e., race, ethnicity, sex, gender identity, gender expression). Statistical significance was set as $p < 0.05$.

Primary Objective (Association of Sex, Gender Identity, and Gender Expression with FMD & PWV): Data were first inspected for normality using the Shapiro-Wilk test, histograms and Quantile-Quantile (Q-Q) plots. If normally distributed, data were first compared between sexes using an independent t-test with equal variances assumed if Levene's test for equality of variances was not significant ($p>0.05$). Independent t-tests that failed to meet the assumption for homogeneity of variances ($p<0.05$) used an independent t-test with unequal variances assumed. If not normally distributed, Wilcoxon Rank Sum tests were conducted. Similarly, independent means for outcome data were compared between gender identities using an independent t-test to compare men and women, with outcomes from non-binary participants detailed but not included in the analysis due to small sample size (n=2), using the same statistical analysis methods outlined for comparisons between sexes. Then, all data were compared across gender expression categories (i.e., feminine, masculine, androgynous, undifferentiated)

using a one-way analysis of variance and Bonferroni corrected post-hoc tests for multiple comparisons, if applicable. We inspected for homogeneity, as well as the distribution of standardized residuals. Data with high residuals were examined for potential removal. If assumptions were not met, a Kruskal-Wallis test was conducted. If differences between the four gender expressions groups were observed, a two-sample Wilcoxon Rank sum tests with Dunn-Bonferroni post hoc pairwise correction tests were subsequently applied. A one-way analysis of covariance with Bonferroni post hoc pairwise correction tests, if applicable, was used to examine the potential influences of *a priori* covariates on the main effects of sex, gender identity or gender expression: blood pressure (MAP) for central PWV, leg PWV, and arm PWV; cardiorespiratory fitness for central PWV, leg PWV, arm PWV, and %FMD; and SRAUC for %FMD. Allometric scaling of the FMD results was performed, as supported by recent guidelines (54), if criteria were met (59, 60). Using Rtery (Github) (61), the difference between the natural logarithm of baseline artery diameter and national logarithm of peak artery diameter was entered as a dependent variable into a linear mixed effects model that included group as an independent variable and the logarithm of baseline diameter as a covariate. The back-transformed estimated marginal means and standard deviations are reported for %FMD$_{scaled}$. Significant effect sizes were also quantified using a Cohen's d calculation, where a small effect is d=0.2, medium effect is d=0.5, and large effect is d>0.8 (62).

Secondary Objective (Relationship Between Feminine & Masculine Gender Expression Scores with Cardiorespiratory Fitness, Central PWV, FMD): Analysis of the relationship between %FMD and Central PWV (m/s) and BSRI-Feminine score, BSRI-Masculine score, and cardiorespiratory fitness ($\dot{V}O_2$peak, mL/kg/min) were analyzed using a

Pearson correlation. Further, an analysis of the relationship between baseline diameter and %FMD was conducted using a Pearson correlation. To assess or internal consistency of the BSRI within this population, a Cronbach's alpha calculation was performed to examine the relationship between the 10 questions within each BSRI subcategory (masculine, feminine, neutral), with a score of 1 indicating perfect correlation and 0 indicating no correlation between items; generally, a score between 0.7 and 0.95 as indicative of acceptable scores for internal consistency (63-65).

Results

Participant Characteristics

Of the 130 participants who were recruited, 50 participants identified as male (38%) while 80 participants identified as female (62%). Gender identity groups included 49 men (38%), 79 women (61%), and 2 non-binary participants (2%). The proportion of participants categorized into gender expression groups is outlined in Figure 1. Ethnic and race make-up of participants is detailed in full in Supplementary Table 2. Briefly, the ethnic make-up of the participants included in this study is as follows: African Origins (n=5; 4%), Asian Origins (n=54; 42%), European Origins (n=38; 29%), Mixed Origins (n=30; 23%), and North American Origins (n=3; 2%). The race make-up of participants included in this study is as follows: Asian (n=48; 37%), Black or African American (n=2; 2%), Middle Eastern or North African (n=10; 8%), Mixed (n=8; 6%) Prefer to Self Describe (n=2; 2%), and White or Caucasian (n=60; 46%).

While the average age of participants across sex and gender identity groups were not different, all other characteristics were elevated in males compared to females, and men compared to women, including height, weight, BMI, and $\dot{V}O_2$peak (all $p < 0.01$;

Table 1). There was no difference across gender expression groups for any participant characteristics (Table 2).

Bem Sex Role Inventory Scores

BSRI-Feminine scores were higher in females compared to males (Female: 5.4 ± 0.8, 95% CI: 5.2, 5.8; Male: 5.0 ± 0.9, 95% CI: 4.7, 5.2; $p < 0.01$), and also higher in women compared to men (Women: 5.4 ± 0.8, 95% CI: 5.2, 5.6; Men: 5.0 ± 0.9, 95% CI: 4.7, 5.2; $p = 0.01$; Non-Binary: 5.4 & 3.7). In contrast, there was no difference for the BSRI-Masculine scores across sex (Female: 4.4 ± 0.7, 95% CI: 4.2, 4.5; Male: 4.4 ± 0.7, 95% CI: 4.2, 4.5, $p = 0.85$) or gender identity groups (Women: 4.4 ± 0.7, 95% CI: 4.2, 4.5, Men: 4.4 ± 0.7, 95% CI: 4.2, 4.6, $p = 0.93$; Non-Binary: 4.4 & 3.8).

BSRI-Feminine scores were higher in Feminine (5.9 ± 0.5) and Androgynous (5.8 ± 0.5) gender expression groups, compared to Masculine (4.5 ± 0.6) and Undifferentiated (4.4 ± 0.5) gender expression groups ($p<0.01$ for all), with no difference between the Feminine and Androgynous ($p = 1.00$) and the Masculine and Undifferentiated ($p = 1.00$) gender expression groups (Figure 1). BSRI-Masculine scores were lower in the Feminine (3.8 ± 0.3) and Undifferentiated (3.8 ± 0.4) groups compared to the Masculine (5.1 ± 0.4) and Androgynous (4.8 ± 0.4) gender expression groups ($p< 0.01$ for all), with no difference between the Feminine and Undifferentiated groups ($p = 1.00$; Figure 1). However, BSRI-Masculine was also higher in the Masculine compared to the Androgynous gender expression group ($p = 0.03$; Figure 1).

To assess the internal consistency of each subscale (feminine, masculine, neutral) of the BSRI, Cronbach's alphas were computed: BSRI-Feminine $\alpha = 0.87$,

BSRI-Masculine α = 0.75, BSRI-Neutral α = 0.40; BSRI-Feminine and BSRI-Masculine

are within acceptable scores for internal consistency of the score.(63-65)

Association of Sex and Gender with Resting Hemodynamics

Resting SBP and MAP were higher in males compared to females (d = 1.2, d =

0.7 respectively; both p<0.01; Table 1) and higher in men compared to women (d = 1.3,

d = 0.7 respectively; both p<0.01; Table 1). There were no sex or gender identity

differences for DBP or HR (Table 1). There were no differences across gender

expression groups for any hemodynamic measure (SBP, DBP, MAP, HR; Table 2).

Primary Objective: Association of Sex and Gender with Arterial Stiffness (PWV)

Central PWV was higher in males compared to females (6.6 ± 1.5 *vs* 5.1 ± 1.5

m/s, d = 0.3; p = 0.02; Figure 2A), and higher in men compared to women (6.6 ± 1.5 *vs*

5.1 ± 1.5 m/s, d = 0.3; p = 0.02; Figure 2D). There were no differences in peripheral leg

PWV (Figure 2B, 2E) or peripheral arm PWV (Figure 2C, 2F) between sex or gender

identity groups. Similarly, there were no differences across gender expression groups

for any PWV measures (Figure 2G, H, I). None of these findings were altered when the

independent association of MAP or $\dot{V}O_2$peak were added as covariates.

Primary Objective: Association of Sex and Gender with Endothelial Function (FMD)

Baseline brachial artery diameter and peak brachial artery diameter were both

larger in males compared to females (d = 1.3, d = 1.4 respectively, p < 0.01; Table 1)

and men compared to women (d = 1.4, d = 1.4, respectively; p < 0.01; Table 1).

AbsFMD was not different between sex or gender identity groups (Table 1). %FMD was

initially higher in females compared to males (8.5 ± 3.7 *vs* 6.9 ± 3.5%; d = 0.4, p = 0.01;

Figure 3A) and in women compared to men (8.6 ± 3.7 *vs* 7.0 ± 3.5%; d = 0.4, p = 0.02; Figure 3C); this result remained significant when $\dot{V}O_2peak$ or SRAUC were added as covariates in the model (both p = 0.02). After allometric scaling to consider differences in artery size, %FMD$_{scaled}$ was higher in males compared to females (8.8 ± 3.3 *vs* 7.2 ± 3.1%, d = 0.4, p = 0.03; Figure 3B) and in men compared to women (8.9 ± 3.3 *vs* 7.2 ± 3.1, d = 0.4, p = 0.02; Figure 3D).

Baseline MBV was higher in males compared to females (d = 0.3, p = 0.01; Table 1) and men compared to women (d = 0.5, p = 0.02; Table 1). There were no sex or gender identity group differences for baseline SR, SRAUC, or time to peak diameter (Table 1). There was no difference in any endothelial function or blood velocity outcome across gender expression groups (Table 2; Figure 3E); this result remained non-significant when %FMD was allometrically scaled (p = 0.39; Figure 3F) or when $\dot{V}O_2peak$ or SRAUC was considered as a covariate.

Non-Binary Participant Outcomes

Due to a low sample size for gender-diverse participants, non-binary participants were qualitatively, instead of statistically, examined. Compared to men and women, non-binary participants may have higher BMI (35.9 & 28.8 kg/m^2) and lower cardiorespiratory fitness (27.5 & 32.4 mL/kg/min; Table 1). Non-binary participants also may have elevated blood pressure (SBP: 110 & 138, MAP: 78 & 97 mmHg; Table 1) and resting heart rate (66 & 92 bpm; Table 1) compared to women, impaired endothelial function (AbsFMD: 0.18 & 0.09 mm; %FMD: 4.69 & 2.16%, Table 1 & Figure 3C&D), including after differences in artery size were considered using allometric scaling (%FMD$_{scaled}$: 4.7 ± 2.8%). However, non-binary participants had lower central (4.2 & 5.3

m/s) and peripheral arm PWV (4.8 & 4.5) but elevated peripheral leg PWV (9.2 & 9.3 m/s).

Secondary Objective: Relationship Between Gender Expression, Cardiorespiratory Fitness and Endothelial Function, Central PWV

There was no significant relationship between gender expression scores (BSRI-Feminine, BSRI-Masculine) and endothelial function or central PWV (Supplementary Figure 1A-D). Similarly, there was no relationship between cardiorespiratory fitness and endothelial function (Supplementary Figure 1E) or central PWV (Supplementary Figure 1F). Finally, there was a negative relationship between baseline diameter and %FMD (r = -0.55, p < 0.001; Supplementary Figure 1G).

	Males (n=50)	Females (n=80)	Men (n=49)	Women (n=79)	Non-Binary (n=2)	p-values
Age (years)	22 [4]	21 [4]	22 [4]	21 [4]	20, 18	S: p = 0.31 G: p = 0.24
Height (cm)	178 ± 7 (176, 180)	164 ± 7* (163, 166)	178 ±7 (176, 180)	164 ± 7** (163, 166)	165, 180	**S: p < 0.01** **G: p < 0.01**
Weight (kg)	81.4 [18.9]	60.8 [13.1]*	80.9 [18.3]	60.7 [12.0]**	98, 93	**S: p < 0.01** **G: p < 0.01**
BMI (kg/m^2)	25.0 [5.9]	22.7 [4.1]*	25.0 [5.8]	22.4 [4.1]**	35.9, 28.8	**S: p < 0.01** **G: p < 0.01**
$\dot{V}O_2$peak (mL/kg/min)	44.2 ± 8.9 (41.6, 46.7)	39.1 ± 8.0* (37.4, 40.9)	44.4 ± 8.9 (41.9, 46.9)	39.3 ± 7.9** (37.5, 41.1)	27.5, 32.4	**S: p < 0.01** **G: p < 0.01**
SBP (mmHg)	117 ± 8 (115, 120)	106 ± 7* (104, 107)	117 ± 8 (115, 119)	106 ± 7** (104, 107)	110, 138	**S: p < 0.01** **G: p < 0.01**
DBP (mmHg)	63 [7]	63 [9]	62 [7]	63 [10]	57, 72	S: p = 0.54 G: p = 0.71
MAP (mmHg)	84 ± 6 (82, 86)	79 ± 6* (77, 80)	84 ± 6 (82, 86)	79 ± 6** (77, 81)	78, 97	**S: p < 0.01** **G: p < 0.01**
HR (bpm)	64 ± 10 (61, 67)	62 ± 9 (60, 64)	64 ± 9 (61, 66)	62 ± 9 (60, 64)	66, 92	S: p = 0.20 G: p = 0.32
Baseline Diameter (mm)	4.03 [0.63]	3.19 [0.68]*	4.04 [0.67]	3.19 [0.65]**	3.86, 3.94	**S: p < 0.01** **G: p < 0.01**
Peak Diameter (mm)	4.38 [0.61]	3.53 [0.61]*	4.38 [0.61]	3.53 [0.61]**	4.04, 4.02	**S: p < 0.01** **G: p < 0.01**
AbsFMD (mm)	0.27 ± 0.12 (0.24, 0.30)	0.27 ± 0.11 (0.25, 0.30)	0.27 ± 0.12 (0.24, 0.31)	0.27 ± 0.11 (0.25, 0.30)	0.18, 0.09	S: p = 0.84 G: p = 0.94

Baseline MBV (cm/s)	8.0 [5.9]	6.5 [3.0]*	8.0 [6.1]	6.5 [3.0]**	6.1, 7.1	**S: p = 0.01** **G: p = 0.02**
Baseline SR (s^{-1})	163.2 [138.7]	161.3 [70.9]	163.3 [139.1]	161.5 [72.4]	127.3, 144.0	S: p = 0.91 G: p = 0.92
SRAUC ($\times 10^3\ s^{-1}$)	1.68 [1.90]	2.03 [2.14]	1.64 [1.83]	2.10 [2.15]	7.1, 37.4	S: p = 0.53 G: p = 0.38
Time to Peak Diameter (s)	46 [21]	42 [19]	46 [20]	42 [19]	29, 57	S: p = 0.46 G: p = 0.61

Table 1. Participant Characteristics, Resting Hemodynamics, and Flow Mediated Dilation Test Outcomes Characterized by Sex and Gender (Identity). Independent t-tests were performed comparing between sexes (male/female) and gender identity groups (men/women). Non-binary participants were characterized detailing the outcome values from each participant (n=2), but not included in statistical analysis due to low sample size. S = sex; G = gender identity; BMI = body mass index; $\dot{V}O_2peak$ = volume of oxygen at peak exercise capacity; SBP = systolic blood pressure; DBP = diastolic blood pressure; MAP = mean arterial pressure; HR = heart rate; AbsFMD = absolute flow mediated dilation response; MBV = mean blood velocity; SR = shear rate; SRAUC = shear rate area under the curve to peak dilation. Significant main effects are bolded. Normally distributed outcomes are represented as mean ± standard deviation (95% confidence interval), while non-normally distributed outcomes are represented as median [interquartile range]. *Significantly higher in males compared to females. **Significantly higher in men compared to women.

	Masculine (n=29)	Feminine (n=33)	Androgynous (n=39)	Undifferentiated (n=29)	p-values
Age (years)	20 [4]	22 [4]	20 [3]	22 [6]	p = 0.12
Height (cm)	168 ± 8 (165, 171)	169 ± 12 (165, 174)	169 ±9 (166, 172)	173 ± 8 (170, 176)	p = 0.21
Weight (kg)	65.9 [17.8]	64.2 [24.2]	63.9 [21.4]	71.3 [25.5]	p = 0.54
BMI (kg/m^2)	24.6 [5.3]	23.2 [3.5]	23.5 [4.8]	23.7 [5.0]	p = 0.70
$\dot{V}O_2$peak (mL/kg/min)	41.3 ± 8.1 (38.2, 44.40	39.6 ± 8.0 (36.7, 42.5)	41.5 ± 8.6 (38.7, 44.3)	42.0 ± 10.1 (38.1, 45.8)	p = 0.72
SBP (mmHg)	112 ± 10 (109, 116)	109 ± 11 (105, 112)	109 ± 8 (107, 112)	111 ± 10 (107, 115)	p = 0.36
DBP (mmHg)	62 [9]	62 [9]	63 [8]	63 [7]	p = 0.66
MAP (mmHg)	81 ± 6 (79, 84)	81 ± 8 (78, 84)	80 ± 6 (78, 82)	82 ± 6 (79, 84)	p = 0.83
HR (bpm)	62 ± 9 (59, 65)	63 ± 7 (60, 65)	63 ± 9 (60, 66)	64 ± 12 (60, 69)	p = 0.81
Baseline Diameter (mm)	3.85 [1.26]	3.39 [0.84]	3.44 [0.90]	3.65 [0.67]	p = 0.37
Peak Diameter (mm)	4.09 [1.16]	3.61 [0.76]	3.77 [0.93]	3.91 [0.90]	p = 0.40
AbsFMD (mm)	0.27 ± 0.13 (0.22, 0.32)	0.30 ±0.11 (0.26, 0.34)	0.27 ± 0.10 (0.23, 0.30)	0.25 ± 0.11 (0.21, 0.29)	p = 0.36

Baseline MBV (cm/s)	7.4 [5.3]	6.8 [2.9]	7.0 [5.9]	6.6 [3.5]	p = 0.60
Baseline SR (s^{-1})	166.8 [134.1]	163.2 [70.6]	174.8 [146.6]	144.0 [78.4]	p = 0.24
SRAUC (x10^3 s^{-1})	2.24 [2.89]	2.10 [2.43]	1.82 [1.68]	1.73 [1.87]	p = 0.56
Time to Peak Diameter (s)	48 [22]	49 [19]	41 [18]	40 [21]	p = 0.17

Table 2. Participant Characteristics and Resting Hemodynamics Characterized by Gender Expression. One-way ANOVAs were performed comparing across gender expression groups. BMI = body mass index; $\dot{V}O_2$peak = volume of oxygen at peak exercise capacity; SBP = systolic blood pressure; DBP = diastolic blood pressure; MAP = mean arterial pressure; HR = heart rate; AbsFMD = absolute flow mediated dilation response; MBV = mean blood velocity; SR = shear rate; SRAUC = shear rate area under . Normally distributed outcomes are represented as mean ± standard deviation (95% confidence interval), while non-normally distributed outcomes are represented as median [interquartile range].

Masculine
High Masculine
Low Feminine

22% (n=29):
  22% Male (n=11)
  23% Female (n=18)

BSRI-F: 4.5 ± 0.6
BSRI-M: 5.1 ± 0.4

Androgynous
High Masculine
High Feminine

30% (n=39):
  26% Male (n=13)
  33% Female (n=26)

BSRI-F: 5.8 ± 0.5
BSRI-M: 4.8 ± 0.4

BSRI-M
BSRI-M Median:4.40

BSRI-F
BSRI-F Median:5.20

BSRI-F

Undifferentiated
Low Masculine
Low Feminine

22% (n=29):
  30% Male (n=15)
  18% Female (n=14)

BSRI-F: 4.4 ± 0.5
BSRI-M: 3.8 ± 0.4

Feminine
Low Masculine
High Feminine

25% (n=33):
  22% Male (n=11)
  28% Female (n=22)

BSRI-F: 5.9 ± 0.5
BSRI-M: 3.8 ± 0.3
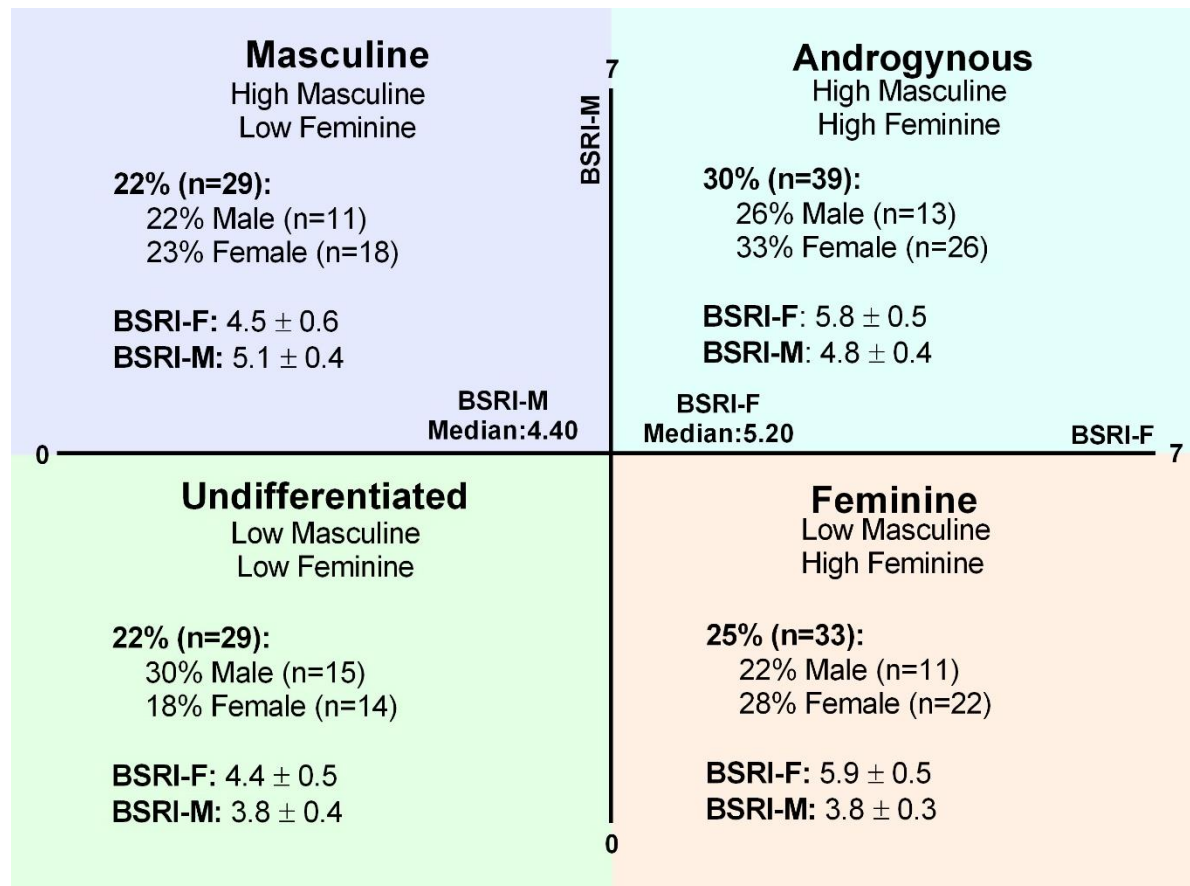
Figure 1. Gender Expression Groups. The Bem Sex Role Inventory (BSRI) creates gender scores for the feminine (BSRI-F) and masculine (BSRI-M) subscales that can be used to generate median-split quadrants for Masculine, Androgynous, Undifferentiated, and Feminine gender expression groups. The proportion of the overall sample in each category, and prevalence of biological males and females in each gender expression group are detailed on the figure. Mean scores for the BSRI-F and BSRI-M are represented (mean ± SD) in each gender expression category.
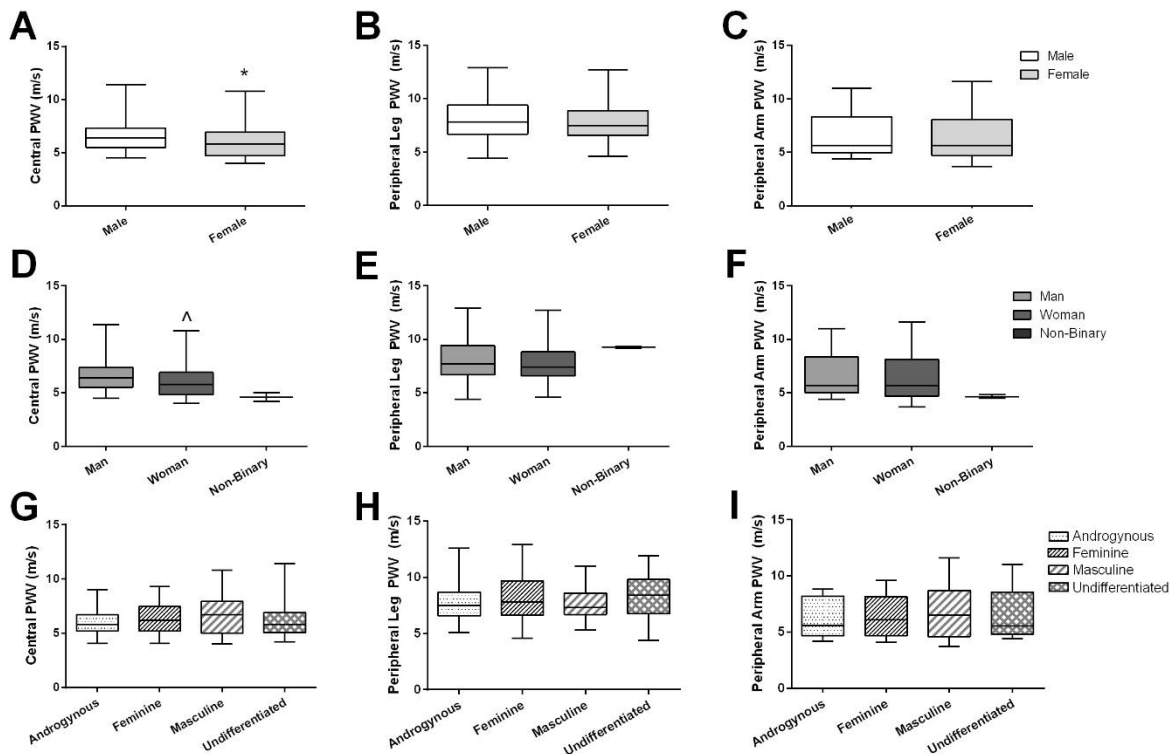
Figure 2. Arterial Stiffness (Central, Peripheral Leg, Peripheral Arm) across Sex, Gender Identity, and Gender Expression Groups. Data is illustrated in Panels A, B, C for sex, D, E, F, for gender identity groups (non-binary data is illustrated on the graphs, but not included in the statistical analysis), and G, H, I for gender expression groups. Box-and-whisker plots represent the median, the box represents the interquartile range, with the minimum and maximum points represented by the whiskers. Independent t-tests were performed between males and females and men and women. A one-way ANOVA was performed to examine %FMD across gender expression groups. *Females have lower Central PWV compared to males (p = 0.02). ^Women have lower Central PWV compared to men (p = 0.02). There were no differences between sexes or gender identity groups for peripheral leg PWV or peripheral Arm PWV, or across gender expression groups for any PWV outcome.
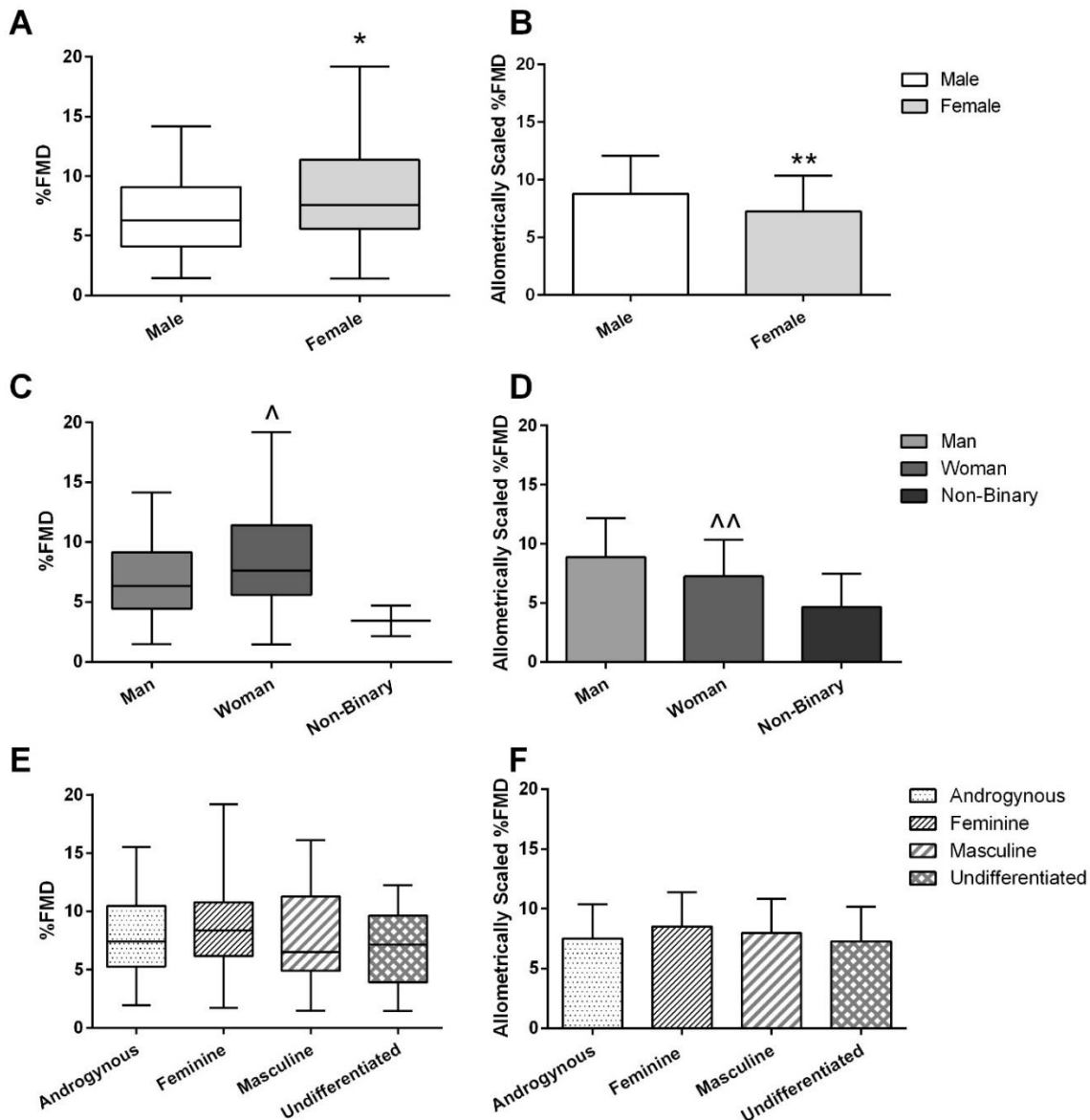
Figure 3. % Flow Mediated Dilation (FMD) across Sex (A, B), Gender Identity (C, D), and Gender Expression (E, F) groups. Left-side graphs are all %FMD, unscaled for baseline diameter (panels A, C, E), and right-side graphs are all %FMD allometrically scaled to baseline diameter (panels B, D, F). Graphs for %FMD (unscaled) show mean ± SD with individual data points, while %FMD (scaled) show no data points. Independent t-tests were performed between males and females and men and women. A one-way ANOVA was performed to examine %FMD across gender expression groups. *Females have higher %FMD (without controlling for baseline diameter differences) compared to males ($p = 0.01$). **Females have lower %FMD when controlling for baseline diameter using allometric scaling ($p = 0.03$). ^Women have higher %FMD (without controlling for baseline diameter differences compared to men ($p = 0.02$). ^^Women have lower %FMD when controlling for baseline diameter using allometric scaling ($p = 0.03$).

Discussion

This is the first study to investigate the effects of biological sex, gender identity, and gender expression on novel and traditional risk factors for CVD in a young, healthy population. We found that males had higher blood pressure (SBP, MAP) and central arterial stiffness (central PWV) compared to females in both unadjusted and adjusted models. However, males had a greater %FMD compared to females, when larger artery diameters in males were considered using allometric scaling. While the same finding was observed in men compared to women (gender identity), there were no differences across gender expression groups (i.e., Feminine, Masculine, Androgynous, Undifferentiated) for any outcome. Finally, there was no relationship between gender expression scores (BSRI-F and BSRI-M) or cardiorespiratory fitness and either central PWV or %FMD. These results suggest that in healthy, young adults, biological sex and gender identity influences several cardiovascular risk factors, but gender expression does not appear to influence these outcomes.

Association of Biological Sex with Hemodynamics and Arterial Stiffness

The present study found that males had elevated blood pressure (SBP, MAP) compared to females, with no difference in DBP or HR. These findings are in line with most previous research on sex-differences in hemodynamics, finding higher SBP, MAP and occasionally DBP in males compared to females (19, 20, 36, 66-70). Research by Harris and colleagues (2012) found that males had higher SBP compared to females (66). Similarly, research in our lab also found elevated SBP and MAP in males compared to two groups of premenopausal females: natural cycling and combined oral contraceptive pill users (19). Finally, a recent narrative review discussed that resting

blood pressure is elevated in males compared to females, and that this may be a critical factor in the increased risk for CVD in males earlier in life than females (36). The mechanisms underlying elevated BP in males compared to females include sex hormones which may involve testosterone increasing blood pressure and 17β-estradiol decreasing blood pressure (and the ratio of testosterone/estradiol) (71-74), and anatomical differences in height resulting in increased pulse wave propagation that must be accompanied by a heightened SBP in males (17). While sex hormones were not measured in this study, males were taller than females which could be in part responsible for these differences in blood pressure. Additionally, biological sex and gender factors are challenging to separate, gender-related lifestyle factors [i.e., smoking, alcohol consumption, sodium intake, sleep (75)] may also play a role influencing observed sex-differences in blood pressure. However, the finding that BP is higher in males compared to females is not always true; BP is reported to be the same or elevated in females compared to males in populations with elevated CVD risk factors, including elevated BMI and low fitness levels (21, 76). Therefore, it is plausible that the "female advantage" observed with blood pressure previously, may be outweighed by the influence of additional risk factors for CVD, such as obesity and low fitness.

In the present study, males had elevated central PWV, but not peripheral arm or leg PWV compared to females, which was not explained by controlling for blood pressure elevations in males. This is in line with some (19-21, 36, 67), but not all (69), studies that have observed sex-differences in local (carotid artery) and/or systemic arterial stiffness. For example, research by Baldo and colleagues (2018) found that central PWV was higher in males compared to females, across the aging lifespan (20).

The same was true in a population of patients with pre-hypertension to stage 1 hypertension, with males having higher central PWV than females, despite females having a higher SBP compared to males (21). Marlatt and colleagues (2013) identified that sex-differences in carotid artery compliance became present in early adulthood (~late 30s), but not in childhood (67). This study, alongside previous research, points to the role of sex hormones like 17β-estradiol to play a role in these apparent sex-differences and/or the influence of sex-differences in growth patterns between males and females (16).

Similarly, recent work found that β-Stiffness of the carotid artery was higher in males compared to females who were naturally cycling or used oral contraceptive pills (19). Despite finding a sex-difference in local arterial stiffness in the carotid artery, no differences were observed in central PWV, although it is possible that they were underpowered to detect a sex-difference in central PWV (19). Our study, with 130 participants found a significant sex-difference in central PWV between males and females (p = 0.02), but arguably the ~0.6 m/s difference may not be clinically significant. Prior research has determined that a 1 m/s increase in arterial stiffness is associated with a 15% (95% CI: 9-21%) increased risk for CVD mortality (77). While a 0.6 m/s difference in central PWV may still increase the risk for CVD marginally, it is unlikely to be solely responsible for differential rates in CVD in males and females.

Association of Biological Sex and Endothelial Function

We found that unscaled %FMD was elevated in female compared to male participants but %FMD may have been artificially inflated given that baseline brachial artery diameter was also smaller in females. After performing allometric scaling analysis

to account for differences in baseline artery diameter, we found that scaled %FMD was elevated in males compared to females. These findings are aligned with most prior %FMD (unscaled) research finding greater %FMD in females (11, 13, 66, 78, 79) and prior research from our lab finding %FMD (scaled) is greater in males (13).

The present study also aligns with a consistent finding that male arteries are, on average, larger than female arteries (11, 66, 78-81). Early research identified sex-differences in reductions in %FMD with aging, alongside sex-differences in %FMD and in artery diameter (82). Further, this study reported a negative relationship between %FMD and resting arterial diameter, which has been extensively replicated in healthy and clinical populations (11, 79, 82). Recent research by Holder and colleagues (2021) observed marked sex-differences in %FMD (higher in females), developing reference ranges in a large population of both healthy and clinical participants (11), following FMD guidelines (11, 54). We observed similar %FMD values compared to the reference intervals (11): present study data in the ~50th percentile of reported references: Male baseline diameter: 4.09 ± 0.49 mm; Male %FMD: 6.86 ± 3.50%; Female baseline diameter: 3.30 ± 0.46 mm, Female %FMD: 8.51 ± 3.70%.

Age- and sex-differences in %FMD relate to differences in baseline diameter and further allude to structural influences on artery function between sexes (11). One evident reason for sex-differences in artery diameter is a positive relationship between baseline diameter and height. Recent work by this same group also found that a "…10 cm increase in height is associated with a 0.16mm increase in baseline diameter and a 0.28% decrease in FMD…" and this finding was independent of sex (11). In our study, there was a 14±7cm difference in height between males and females, which would have

been attributed to a ~0.23mm increase in baseline diameter and ~0.39% decrease in

%FMD. While sex-differences in height do not fully explain the sex-differences in

baseline diameter and %FMD in the present study, artery size differences cannot be

ignored in examining sex-differences in endothelial function through allometric scaling of

%FMD to baseline diameter. Overall, while unscaled %FMD may initially suggest that

females have improved endothelial function compared to males, accounting for artery

size differences result in males having elevated %FMD (scaled) compared to females.

Researchers should consider using allometric scaling when comparing between sexes

to consider baseline differences in artery size and ensure valid interpretation of sex-

difference findings.

Association of Gender and CVD Risk Factors

While there is evidence of alterations in CVD and CVD risk factors associated

with gender and gender-related factors, the present study did not observe any variation

in novel and traditional CVD risk factors across gender expression groups. Prior

research has observed the effect of gender or gender-related factors on CVD; for

example, research by Pelletier and colleagues (2016) found that recurrent acute

coronary syndrome was associated with "femininity" as a composite score of gender

roles and expression (assessed by the BSRI) in middle-aged (aged ~48 years)

individuals, after adjusting for biological sex (34). Similarly, previous research has found

that gender-related roles including caregiver burden (29), role strain (e.g., workplace-

home life role stress), and other psychosocial stressors are predominant in women

compared to men and attributed to increased risk factors and development of CVD (30-

32). The present study did not see impaired cardiovascular health (i.e., blunted %FMD

or increased PWV) in young individuals (aged ~22 years) with higher femininity (gender expression) scores; however, it is possible that gender expression and other gender-related factors such as gender roles may not manifest until later in life. For example, caregiving burden may not be present until middle- and older-adulthood where parenting roles and care for aging relatives commonly occurs (83); therefore, any influence of these gender-related factors may not have yet progressed to the stage of negative remodeling in the vasculature. These factors may also intersect with known increases in CVD risk associated with age (9), though further research is necessary. It is also possible that gender roles have a stronger relationship with CVD health outcomes, and that gender expression is less associated; further research investigating these constructs is needed in young adults is needed. In addition, given the limitations of the BSRI in only examining gender expression, further examining of gender roles in professional and home life, along with exposure to gendered expression and roles in family and friend circles may have added further depth to this analysis. A newly developed questionnaire by Nielsen and colleagues (2021), examining seven gender-related variables across domains of gender norms, gender-related traits (gender expression), and gender relations in American undergraduate students and younger adults, may be useful questionnaire for extension of this research (84).

In examining gender identity groups, we observed the same biological sex-differences detailed above in gender identity groups of men and women. The finding that ~99% of participant biological sex aligned with their gender identity (male = man, female = woman) substantiates this overlap. Considering that sex and gender identity are highly interrelated, the same conclusions detailed for biological sex may also be

attributed to gender identity differences; and further, there may be gender identity influences on seemingly biological sex-differences. However, while sex-differences and gender-identity differences aligned in this study, these are two different identity constructs and should be represented separately, especially to allow for the representation of gender-diverse participants. In this study, we recruited a small number of gender-diverse individuals (n=2 non-binary; comprising ~1.5% of the total study population). While this is not representative of the diverse groups of gender queer participants that could have been recruited (i.e., transgender, genderqueer, two-spirit, etc.), this number of participants is proportionally representative of the number of gender-diverse individuals on average. For example, in Canada, 0.13% of the population reports being non-binary (85), though this may be an underestimation. When considering gender diverse individuals in analyzing for differences in cardiovascular outcomes across gender identity groups, we removed non-binary individuals from the analysis due to this low sample size and instead reported the data qualitatively in hopes of stimulating further needed research in this population. Qualitatively, it appears that the two non-binary participants in this study may have some elevated risk factors for CVD, including elevated blood pressure (above 90th percentile for SBP in the present study), impaired endothelial function (below 15th percentile for %FMD in the present study), and elevated leg PWV (above 75th percentile in the present study), though lower central and arm PWV. This could be in part due to higher BMI and lower cardiorespiratory fitness in these participants. While these findings are limited and must be explored further with a larger sample size, they do align with current literature indicating increased CVD risk factors in gender-diverse populations (86, 87), in part

attributed to gender minority stress (86, 88) and increased allostatic load, or the accumulation of stress and life events, in some gender and sexual minority groups (89).

Overall, while gender expression differences were not observed in this study, this does not mean that differences do not exist or should not be studied by researchers. On the contrary, given that this study was in a young, healthy population, we may have yet to observe CVD risk factor elevations or disease manifestation later in the time course of CVD development. Similarly, it is possible that cardiovascular risk may only be apparent during periods in which gender expression or roles are challenged. For example, research by Kramer and colleagues (2017) found that men presented with low masculinity feedback experienced an exaggerated vagal withdrawal response during a speech task compared to those who received higher feedback (90). Overall, it is critical for researchers to continue to examine gender identity, expression, and gender-related factors, alongside biological sex and sex-related factors, and how they influence novel and traditional CVD risk factors.

Limitations

While this study had several strengths, including its inclusion of several novel and traditional CVD risk factor outcomes, recruitment of participants with a wide range of cardiorespiratory fitness levels, compilation of outcomes collected using the same standardized methodologies, and a reasonably large sample size, there are some limitations to consider. First, the findings of this study are only generalizable to young, healthy adults who were primarily university students, and where the majority of which had alignment between their biological sex and gender identity. As a result, further research is necessary, particularly in middle-aged and older adults when gender

expression changes alongside critical gender milestones and other contextual factors (91-93). Similarly, though representative of the proportions of gender-diverse populations in Canada, the number of non-binary participants was too low to make conclusions; further research is needed in this population adequately powered to draw conclusions about CV health. Second, the gender assessment in this study was limited to gender identity and expression, measured by the BSRI. While other gender assessment tools exist (46, 84), their utility in a university population is challenged as many questions ask about gender-related roles in familial structures, financial status in a family or income, caregiver strain, workplace role and environment, among others, many of which are less applicable in a university student context. The BSRI, in contrast, was created using data from a university population and has applicability in this context (28), albeit a limited assessment of gender expression. However, the BSRI also may use some outdated gender stereotypical traits to explore gender expression, as it was constructed in the 1970s and gender norms have shifted since (27, 94-96); though at the time of the study's design, it was the only validated and widely-used gender scoring system available recommended by experts (24). Further research is necessary to create additional relevant gender assessment methods in young adults. Another limitation of the questionnaire was that a small number of participants (n=11) required follow-up prompting to complete 1 question on the BSRI-30; this is unlikely to alter the results of the study given the lower number of participants (~8%) requiring follow-up and the high internal consistency scores of the BSRI-30 questionnaire. Finally, the type of contraceptive or hormonal cycle phase were not controlled for in this study. Previous research has found conflicting results on the influence of contraceptives and the

hormonal cycle on endothelial function and arterial stiffness (13, 50, 66, 97-100).

However, recent work from our lab and others have observed no impact of

contraceptives, contraceptive cycle or menstrual cycle on arterial stiffness (19), and a

small influence of the menstrual cycle on endothelial function (101). Therefore, any

effects of contraceptive type or hormonal cycle phase is unlikely to have changed the

findings from the current study.

Perspectives and Significance

The present study found that biological sex and gender identity, but not gender

expression groups, influenced novel and traditional risk factors for CVD in a young,

healthy population. Specifically, blood pressure (SBP, MAP) and central PWV was

elevated in males compared to females and men compared to women, but %FMD, once

larger artery diameter in males and men was controlled for, was improved in males and

men compared to females and women respectively. While young, otherwise healthy

males and men appear to have elevated measures of central stiffness this may be

compensated by elevated vasodilatory capacity of a major conduit artery (brachial

artery). Given that this study is only generalizable to a young, healthy population of

primarily university students of Asian and European/Caucasian racial and ethnic origins,

further research is necessary to examine sex and gender considerations in other more

ethnically diverse and representative groups of young adults, older adults, and those

with CVD. Similarly, further research on populations of gender-diverse adults, including

non-binary and transgender populations is warranted. Finally, further research is also

needed examining participants in conditions where gender expression is challenged

(i.e., masculinity stressors), or in populations that may experience gender-related stress

(i.e., non-binary and transgender participants) and their influence on novel and

traditional risk factors for CVD.

Declarations

References

1. Vos T, Lim SS, Abbafati C, Abbas KM, Abbasi M, Abbasifard M, et al. Global burden of 369 diseases and injuries in 204 countries and territories, 1990–2019: a systematic analysis for the Global Burden of Disease Study 2019. The Lancet. 2020;396(10258):1204-22.

2. Mensah GA, Roth GA, Fuster V. The Global Burden of Cardiovascular Diseases and Risk Factors. Journal of the American College of Cardiology. 2019;74(20):2529-32.

3. Aggarwal NR, Patel HN, Mehta LS, Sanghani RM, Lundberg GP, Lewis SJ, et al. Sex Differences in Ischemic Heart Disease. Circulation: Cardiovascular Quality and Outcomes. 2018;11(2):e004437.

4. Norris CM, Yip CYY, Nerenberg KA, Clavel MA, Pacheco C, Foulds HJA, et al. State of the Science in Women's Cardiovascular Disease: A Canadian Perspective on the Influence of Sex and Gender. Journal of the American Heart Association. 2020;9(4):e015634.

5. Vogel B, Acevedo M, Appelman Y, Bairey Merz CN, Chieffo A, Figtree GA, et al. The Lancet women and cardiovascular disease Commission: reducing the global burden by 2030. The Lancet. 2021;397(10292):2385-438.

6. Pacheco C, Mullen K-A, Coutinho T, Jaffer S, Parry M, Van Spall HGC, et al. The Canadian Women's Heart Health Alliance Atlas on the Epidemiology, Diagnosis, and Management of Cardiovascular Disease in Women — Chapter 5: Sex- and Gender-Unique Manifestations of Cardiovascular Disease. CJC Open. 2022;4(3):243-62.

7. Anand SS, Islam S, Rosengren A, Franzosi MG, Steyn K, Yusufali AH, et al. Risk factors for myocardial infarction in women and men: insights from the INTERHEART study. European Heart Journal. 2008;29(7):932-40.

8. Moreau KL, Stauffer BL, Kohrt WM, Seals DR. Essential role of estrogen for improvements in vascular endothelial function with endurance exercise in postmenopausal women. J Clin Endocrinol Metab. 2013;98(11):4507-15.

9. Seals DR, Jablonski KL, Donato AJ. Aging and vascular endothelial function in humans. Clinical science (London, England : 1979). 2011;120(9):357-75.

10. Inaba Y, Chen JA, Bergmann SR. Prediction of future cardiovascular outcomes by flow-mediated vasodilatation of brachial artery: a meta-analysis. Int J Cardiovasc Imaging. 2010;26(6):631-40.

11. Holder SM, Bruno RM, Shkredova DA, Dawson EA, Jones H, Hopkins ND, et al. Reference Intervals for Brachial Artery Flow-Mediated Dilation and the Relation With Cardiovascular Risk Factors. Hypertension (0194911X). 2021;77(5):1469-80.

12. Skaug E-A, Aspenes ST, Oldervoll L, Mørkedal B, Vatten L, Wisløff U, et al. Age and gender differences of endothelial function in 4739 healthy adults: the HUNT3 Fitness Study. Eur J Prev Cardiol. 2013;20(4):531-40.

13. Shenouda N, Priest SE, Rizzuto VI, MacDonald MJ. Brachial artery endothelial function is stable across a menstrual and oral contraceptive pill cycle but lower in premenopausal women than in age-matched men. Am J Physiol Heart Circ Physiol. 2018;315(2):H366-H74.

14. Green DJ, Hopkins ND, Jones H, Thijssen DH, Eijsvogels TM, Yeap BB. Sex differences in vascular endothelial function and health in humans: impacts of exercise. Exp Physiol. 2016;101(2):230-42.

15. Maruhashi T, Soga J, Fujimura N, Idei N, Mikami S, Iwamoto Y, et al. Relationship between flow-mediated vasodilation and cardiovascular risk factors in a large community-based study. Heart. 2013;99(24):1837-42.

16. DuPont JJ, Kenney RM, Patel AR, Jaffe IZ. Sex differences in mechanisms of arterial stiffness. Br J Pharmacol. 2019;176(21):4208-25.

17. London GM, Guerin AP, Pannier B, Marchais SJ, Stimpel M. Influence of Sex on Arterial Hemodynamics and Blood Pressure. Hypertension. 1995;26(3):514-9.

18. Ogola BO, Zimmerman MA, Clark GL, Abshire CM, Gentry KM, Miller KS, et al. New insights into arterial stiffening: does sex matter? Am J Physiol Heart Circ Physiol. 2018;315(5):H1073-h87.

19. Priest SE, Shenouda N, MacDonald MJ. Effect of sex, menstrual cycle phase, and monophasic oral contraceptive pill use on local and central arterial stiffness in young adults. Am J Physiol Heart Circ Physiol. 2018;315(2):H357-h65.

20. Baldo MP, Cunha RS, Molina MdCB, Chór D, Griep RH, Duncan BB, et al. Carotid-femoral pulse wave velocity in a healthy adult sample: The ELSA-Brasil study. International Journal of Cardiology. 2018;251:90-5.

21. Collier SR, Frechette V, Sandberg K, Schafer P, Ji H, Smulyan H, et al. Sex differences in resting hemodynamics and arterial stiffness following 4 weeks of resistance versus aerobic exercise training in individuals with pre-hypertension to stage 1 hypertension. Biol Sex Differ. 2011;2(1):9.

22. Lew LA, Williams JS, Stone JC, Au AKW, Pyke KE, MacDonald MJ. Examination of Sex-Specific Participant Inclusion in Exercise Physiology Endothelial Function Research: A Systematic Review. Frontiers in sports and active living. 2022;4:860356.

23. Tannenbaum C, Ellis RP, Eyssel F, Zou J, Schiebinger L. Sex and gender analysis improves science and engineering. Nature. 2019;575(7781):137-46.

24. Tannenbaum C, Greaves L, Graham ID. Why sex and gender matter in implementation research. BMC Medical Research Methodology. 2016;16(1):145.

25. Krieger N. Genders, sexes, and health: what are the connections—and why does it matter? International Journal of Epidemiology. 2003;32(4):652-7.

26. Mauvais-Jarvis F, Bairey Merz N, Barnes PJ, Brinton RD, Carrero J-J, DeMeo DL, et al. Sex and gender: modifiers of health, disease, and medicine. The Lancet. 2020;396(10250):565-82.

27. Donnelly K, Twenge JM. Masculine and Feminine Traits on the Bem Sex-Role Inventory, 1993–2012: a Cross-Temporal Meta-Analysis. Sex Roles. 2017;76(9):556-65.

28. Bem SL. Bem sex role inventory. Journal of personality and social psychology. 1981.

29. Lee S, Colditz GA, Berkman LF, Kawachi I. Caregiving and risk of coronary heart disease in U.S. women: A prospective study. American Journal of Preventive Medicine. 2003;24(2):113-9.

30. von Känel R, Mausbach BT, Patterson TL, Dimsdale JE, Aschbacher K, Mills PJ, et al. Increased Framingham Coronary Heart Disease Risk Score in Dementia Caregivers Relative to Non-Caregiving Controls. Gerontology. 2008;54(3):131-7.

31. Parry M. Caregiver Burden and Cardiovascular Disease: Can We Afford to Keep the Health of Caregivers in Canada Invisible? Canadian Journal of Cardiology. 2019;35(10):1267-9.

32. Mortensen J, Dich N, Lange T, Ramlau-Hansen CH, Head J, Kivimäki M, et al. Weekly hours of informal caregiving and paid work, and the risk of cardiovascular disease. European Journal of Public Health. 2017;28(4):743-7.

33. Doster JA, Purdum MB, Martin LA, Goven AJ, Moorefield R. Gender Differences, Anger Expression, and Cardiovascular Risk. The Journal of Nervous and Mental Disease. 2009;197(7).

34. Pelletier R, Khan NA, Cox J, Daskalopoulou SS, Eisenberg MJ, Bacon SL, et al. Sex Versus Gender-Related Characteristics: Which Predicts Outcome After Acute Coronary Syndrome in the Young? Journal of the American College of Cardiology. 2016;67(2):127-35.

35. Williams JS, Dunford EC, Cheng JL, Moncion K, Valentino SE, Droog CA, et al. The impact of the 24-h movement spectrum on vascular remodeling in older men and women: a review. American Journal of Physiology-Heart and Circulatory Physiology. 2021;320(3):H1136-H55.

36. Seeland U, Nemcsik J, Lønnebakken MT, Kublickiene K, Schluchter H, Park C, et al. Sex and Gender Aspects in Vascular Ageing - Focus on Epidemiology, Pathophysiology, and Outcomes. Heart, lung & circulation. 2021;30(11):1637-46.

37. Diaz-Canestro C, Pentz B, Sehgal A, Montero D. Sex differences in cardiorespiratory fitness are explained by blood volume and oxygen carrying capacity. Cardiovascular Research. 2021;118(1):334-43.

38. Greaves L, Ritz SA. Sex, Gender and Health: Mapping the Landscape of Research and Policy. Int J Environ Res Public Health. 2022;19(5).

39. Lindsey ML, Carter JR, Ripplinger CM, Kassiri Z, Hansell Keehan K, Brunt KR, et al. Sex still matters in cardiovascular research. American Journal of Physiology-Heart and Circulatory Physiology. 2022;324(1):H79-H81.

40. Etsuda H, Takase B, Uehata A, Kusano H, Hamabe A, Kuhara R, et al. Morning attenuation of endothelium-dependent, flow-mediated dilation in healthy young men: possible connection to morning peak of cardiac events? Clin Cardiol. 1999;22(6):417-21.

41. Statistics Canada. Appendix 2.5 Ethnic or cultural origins disseminated in 2021, 2016, and 2011. Dictionary, Census of Population 2021. 2022.

42. Ontario Goverment Collection of Personal Information: Collection of Personal Information on Race. Data Standards for the Identification and Monitoring of Systemic Racism. 2021.

43. Lagos D, Compton D. Evaluating the Use of a Two-Step Gender Identity Measure in the 2018 General Social Survey. Demography. 2021;58(2):763-72.

44. Bauer GR, Braimoh J, Scheim AI, Dharma C. Transgender-inclusive measures of sex/gender for population surveys: Mixed-methods evaluation and recommendations. PLOS ONE. 2017;12(5):e0178043.

45. Schmitt BH, Millard RT. Construct validity of the bem sex role inventory (BSRI): Does the BSRI distinguish between gender-schematic and gender-aschematic individuals? Sex Roles. 1988;19(9):581-8.

46. Pelletier R, Ditto B, Pilote L. A composite measure of gender and its association with risk factors in patients with premature acute coronary syndrome. Psychosom Med. 2015;77(5):517-26.

47. Carver LF, Vafaei A, Guerra R, Freire A, Phillips SP. Gender Differences: Examination of the 12-Item Bem Sex Role Inventory (BSRI-12) in an Older Brazilian Population. PLOS ONE. 2013;8(10):e76356.

48. Spence JT, Helmreich R, Stapp J. Ratings of self and peers on sex role attributes and their relation to self-esteem and conceptions of masculinity and femininity. Journal of Personality and Social Psychology. 1975;32:29-39.

49. Van Bortel LM, Laurent S, Boutouyrie P, Chowienczyk P, Cruickshank JK, De Backer T, et al. Expert consensus document on the measurement of aortic stiffness in daily practice using carotid-femoral pulse wave velocity. J Hypertens. 2012;30(3):445-8.

50. Adkisson EJ, Casey DP, Beck DT, Gurovich AN, Martin JS, Braith RW. Central, peripheral and resistance arterial reactivity: fluctuates during the phases of the menstrual cycle. Exp Biol Med (Maywood). 2010;235(1):111-8.

51. Williams JS, Stimpson TV, Tremblay JC, Fenuta AM, Pyke KE. No impact of acute hyperglycaemia on arterial stiffness in the early and late follicular phases of the menstrual cycle in young females. Exp Physiol. 2020;105(1):174-83.

52. Koivistoinen T, Lyytikäinen L-P, Aatola H, Luukkaala T, Juonala M, Viikari J, et al. Pulse Wave Velocity Predicts the Progression of Blood Pressure and Development of Hypertension in Young Adults. Hypertension. 2018;71(3):451-6.

53. Pyke KE, Hartnett JA, Tschakovsky ME. Are the dynamic response characteristics of brachial artery flow-mediated dilation sensitive to the magnitude of increase in shear stimulus? J Appl Physiol (1985). 2008;105(1):282-92.

54. Thijssen DHJ, Bruno RM, van Mil A, Holder SM, Faita F, Greyling A, et al. Expert consensus and evidence-based recommendations for the assessment of flow-mediated dilation in humans. Eur Heart J. 2019;40(30):2534-47.

55. Wendelhag I, Liang Q, Gustavsson T, Wikstrand J. A new automated computerized analyzing system simplifies readings and reduces the variability in ultrasound measurement of intima-media thickness. Stroke. 1997;28(11):2195-200.

56. 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. Journal of applied physiology (Bethesda, Md : 1985). 2017;123(4):773-80.

57. Myers J, Arena R, Franklin B, Pina I, Kraus WE, McInnis K, et al. Recommendations for clinical exercise laboratories: a scientific statement from the american heart association. Circulation. 2009;119(24):3144-61.

58. Gillen JB, Percival ME, Skelly LE, Martin BJ, Tan RB, Tarnopolsky MA, et al. Three minutes of all-out intermittent exercise per week increases skeletal muscle oxidative capacity and improves cardiometabolic health. PLoS One. 2014;9(11):e111489.

59. Atkinson G, Batterham AM. The percentage flow-mediated dilation index: A large-sample investigation of its appropriateness, potential for bias and causal nexus in vascular medicine. Vascular Medicine. 2013;18(6):354-65.

60. Atkinson G, Batterham AM. Allometric scaling of diameter change in the original flow-mediated dilation protocol. Atherosclerosis. 2013;226(2):425-7.

61. Cherubini JM. Rtery: An R package for endothelial function analysis. GitHub. Retrieved Feburary 21, 2023.

62. Cohen J. Statistical Power Analysis for the Behavioral Sciences. . New York: Routledge. 1988.

63. Cronbach LJ. Coefficient alpha and the internal structure of tests. Psychometrika. 1951;16(3):297-334.

64. Tavakol M, Dennick R. Making sense of Cronbach's alpha. International journal of medical education. 2011;2:53-5.

65. Streiner DL. Starting at the Beginning: An Introduction to Coefficient Alpha and Internal Consistency. Journal of Personality Assessment. 2003;80(1):99-103.

66. Harris RA, Tedjasaputra V, Zhao J, Richardson RS. Premenopausal women exhibit an inherent protection of endothelial function following a high-fat meal. Reprod Sci. 2012;19(2):221-8.

67. Marlatt KL, Kelly AS, Steinberger J, Dengel DR. The influence of gender on carotid artery compliance and distensibility in children and adults. J Clin Ultrasound. 2013;41(6):340-6.

68. Vermeersch SJ, Rietzschel ER, De Buyzere ML, De Bacquer D, De Backer G, Van Bortel LM, et al. Age and gender related patterns in carotid-femoral PWV and carotid and femoral stiffness in a large healthy, middle-aged population. J Hypertens. 2008;26(7):1411-9.

69. Sun P, Chen X, Zeng Z, Li S, Wang J, Yu F, et al. Sex differences in lower-limb arterial stiffness following acute aerobic exercise. Science & Sports. 2020;35(2):e39-e48.

70. Holder SM, Brislane A, Dawson EA, Hopkins ND, Hopman MTE, Cable NT, et al. Relationship Between Endothelial Function and the Eliciting Shear Stress Stimulus in Women: Changes Across the Lifespan Differ to Men. Journal of the American Heart Association. 2019;8(4).

71. Ali Hamza M, Abdulhameed A, Ali Mansour A. Total Testosterone to Estradiol Ratio as a Predictor Marker of Metabolic Syndrome in Males. Archives of Razi Institute. 2022;77(1):351-7.

72. Khaw KT, Barrett-Connor E. Blood pressure and endogenous testosterone in men: an inverse relationship. J Hypertens. 1988;6(4):329-32.

73. Dubey RK, Oparil S, Imthurn B, Jackson EK. Sex hormones and hypertension. Cardiovascular Research. 2002;53(3):688-708.

74. Reckelhoff JF. Gender Differences in the Regulation of Blood Pressure. Hypertension. 2001;37(5):1199-208.

75. Choi JY, Yun EK, Yeun EJ, Jeong ES. Factors influencing blood pressure classification for adults: Gender differences. International Journal of Nursing Practice. 2019;25(3):e12706.

76. Black MA, Cable NT, Thijssen DHJ, Green DJ. Impact of age, sex, and exercise on brachial artery flow-mediated dilatation. American journal of physiology Heart and circulatory physiology. 2009;297(3):H1109-H16.

77. Vlachopoulos C, Aznaouridis K, Stefanadis C. Prediction of cardiovascular events and all-cause mortality with arterial stiffness: a systematic review and meta-analysis. J Am Coll Cardiol. 2010;55(13):1318-27.

78. Levenson J, Pessana F, Gariepy J, Armentano R, Simon A. Gender differences in wall shear-mediated brachial artery vasoconstriction and vasodilation. J Am Coll Cardiol. 2001;38(6):1668-74.

79. Mizia-Stec K, Gasior Z, Mizia M, Haberka M, Holecki M, Zwolińska W, et al. Flow-mediated dilation and gender in patients with coronary artery disease: arterial size influences gender differences in flow-mediated dilation. Echocardiography (Mount Kisco, NY). 2007;24(10):1051-7.

80. Black MA, Cable NT, Thijssen DH, Green DJ. Impact of age, sex, and exercise on brachial artery flow-mediated dilatation. Am J Physiol Heart Circ Physiol. 2009;297(3):H1109-16.

81. Tremblay JC, Stimpson TV, Pyke KE. Evidence of sex differences in the acute impact of oscillatory shear stress on endothelial function. Journal of Applied Physiology. 2018;126(2):314-21.

82. Celermajer DS, Sorensen KE, Spiegelhalter DJ, Georgakopoulos D, Robinson J, Deanfield JE. Aging is associated with endothelial dysfunction in healthy men years before the age-related decline in women. J Am Coll Cardiol. 1994;24(2):471-6.

83. Mollica MA, Smith AW, Kent EE. Caregiving tasks and unmet supportive care needs of family caregivers: A U.S. population-based study. Patient Education and Counseling. 2020;103(3):626-34.

84. Nielsen MW, Stefanick ML, Peragine D, Neilands TB, Ioannidis JPA, Pilote L, et al. Gender-related variables for health research. Biol Sex Differ. 2021;12(1):23.

85. Age, Sex at Birth and Gender Reference Guide, Census of Population. Statistics Canada. 2022.

86. Poteat TC, Divsalar S, Streed CG, Feldman JL, Bockting WO, Meyer IH. Cardiovascular Disease in a Population-Based Sample of Transgender and Cisgender Adults. American Journal of Preventive Medicine. 2021;61(6):804-11.

87. Morgan E, D'Aquila R, Carnethon MR, Mustanski B. Cardiovascular disease risk factors are elevated among a cohort of young sexual and gender minorities in Chicago. Journal of Behavioral Medicine. 2019;42(6):1073-81.

88. Mereish EH, Goldstein CM. Minority Stress and Cardiovascular Disease Risk Among Sexual Minorities: Mediating Effects of Sense of Mastery. International journal of behavioral medicine. 2020;27(6):726-36.

89. Longpré-Poirier C, Dougoud J, Jacmin-Park S, Moussaoui F, Vilme J, Desjardins G, et al. Sex and Gender and Allostatic Mechanisms of Cardiovascular Risk and Disease. Canadian Journal of Cardiology. 2022;38(12):1812-27.

90. Kramer BL, Himmelstein MS, Springer KW. Getting to the Heart of Masculinity Stressors: Masculinity Threats Induce Pronounced Vagal Withdrawal During a Speaking Task. Annals of behavioral medicine : a publication of the Society of Behavioral Medicine. 2017;51(6):846-55.

91. Burke PJ, Cast AD. Stability and Change in the Gender Identities of Newly Married Couples. Social Psychology Quarterly. 1997;60(4):277-90.

92. Mehta CM. Gender in Context: Considering Variability in Wood and Eagly's Traditions of Gender Identity. Sex Roles. 2015;73(11):490-6.

93. Fan P-L, Marini MM. Influences on Gender-Role Attitudes during the Transition to Adulthood. Social Science Research. 2000;29(2):258-83.

94. Auster CJ, Ohm SC. Masculinity and Femininity in Contemporary American Society: A Reevaluation Using the Bem Sex-Role Inventory. Sex Roles. 2000;43(7):499-528.

95. Hansson RO, Chernovetz ME, Jones WH. Maternal employment and androgyny. Psychology of Women Quarterly. 1977;2:76-8.

96. Kerr P, Barbosa Da Torre M, Giguère C, Lupien SJ, Juster RP. Occupational gender roles in relation to workplace stress, allostatic load, and mental health of psychiatric hospital workers. J Psychosom Res. 2021;142:110352.

97. Williams JS, MacDonald MJ. Influence of hormonal contraceptives on peripheral vascular function and structure in premenopausal females: a review. American Journal of Physiology-Heart and Circulatory Physiology. 2021;320(1):H77-H89.

98. Williams MR, Westerman RA, Kingwell BA, Paige J, Blombery PA, Sudhir K, et al. Variations in endothelial function and arterial compliance during the menstrual cycle. J Clin Endocrinol Metab. 2001;86(11):5389-95.

99. D'Urzo KA, King TJ, Williams JS, Silvester MD, Pyke KE. The impact of menstrual phase on brachial artery flow-mediated dilatation during handgrip exercise in healthy premenopausal women. Exp Physiol. 2018;103(2):291-302.

100. Hashimoto M, Akishita M, Eto M, Ishikawa M, Kozaki K, Toba K, et al. Modulation of endothelium-dependent flow-mediated dilatation of the brachial artery by sex and menstrual cycle. Circulation. 1995;92(12):3431-5.

101. Williams JS, Dunford EC, MacDonald MJ. Impact of the menstrual cycle on peripheral vascular function in premenopausal women: systematic review & meta-analysis. *Submitted to American Journal of Physiology - Heart and Circulatory Physiology*. 2020.

Supplementary Table 1 – Bem Sex Role Inventory Questionnaire

The following table includes the Bem Sex Role Inventory – 30 Questionnaire.

"Rate yourself on each item below, on a scale of 1 (Never or almost never true) to 7 (Almost always true)"

	1 – Never/Almost Never True	2	3	4	5	6	7 - Almost Always True
Defend my own beliefs							
Have leadership abilities							
Affectionate							
Eager to soothe hurt feelings							
Conscientious							
Secretive							
Independent							
Willing to take risks							
Sympathetic							
Warm							
Moody							
Adaptable							
Assertive							
Dominant							
Sensitive to the needs of others							

	1 – Never/Almost Never True	2	3	4	5	6	7 - Almost Always True
Tender							
Reliable							
Conceited							
Strong personality							
Willing to take a stand							
Understanding							
Loves children							
Jealous							
Tactful							
Forceful							
Aggressive							
Compassionate							
Gentle							
Truthful							
Conventional							

Supplementary Table 2 – Ethnicity & Race of Participants

Data is reported in alphabetical order:

Ethnicity of Participants	Race of Participants
African Origins (n=5, 4%)	Asian (n=48, 37%)
Asian Origins (n=54, 42%)	Black or African American (n=2, 2%)
European Origins (n=38, 29%)	Middle Eastern or North African (n=10, 8%)
North American Origins (n=3, 2%)	White or Caucasian (n=60, 46%)
Mixed Ethnicity (n=30, 23%) 　n=1 African & Asian Origins 　n=1 African & European Origins 　n=2 Asian, Caribbean, & European Origins 　n=7 Asian & European Origins 　n=1 Asian, European & Latin American Origins 　n=1 Asian, European & Other North American Origins 　n=2 Asian & North American Origins 　n=1 Canadian-Punjabi/South Asian Origins 　n=1 Caribbean & European Origins 　n=13 European & Other North American Origins	Mixed Race (n=8, 6%) 　n=6 Asian & European/Caucasian 　n=1 Aboriginal & Caucasian 　n=1 Not disclosed Prefer to Self-Disclose (n=2) 　n=1 Brown 　n=1 Brown/South Indian
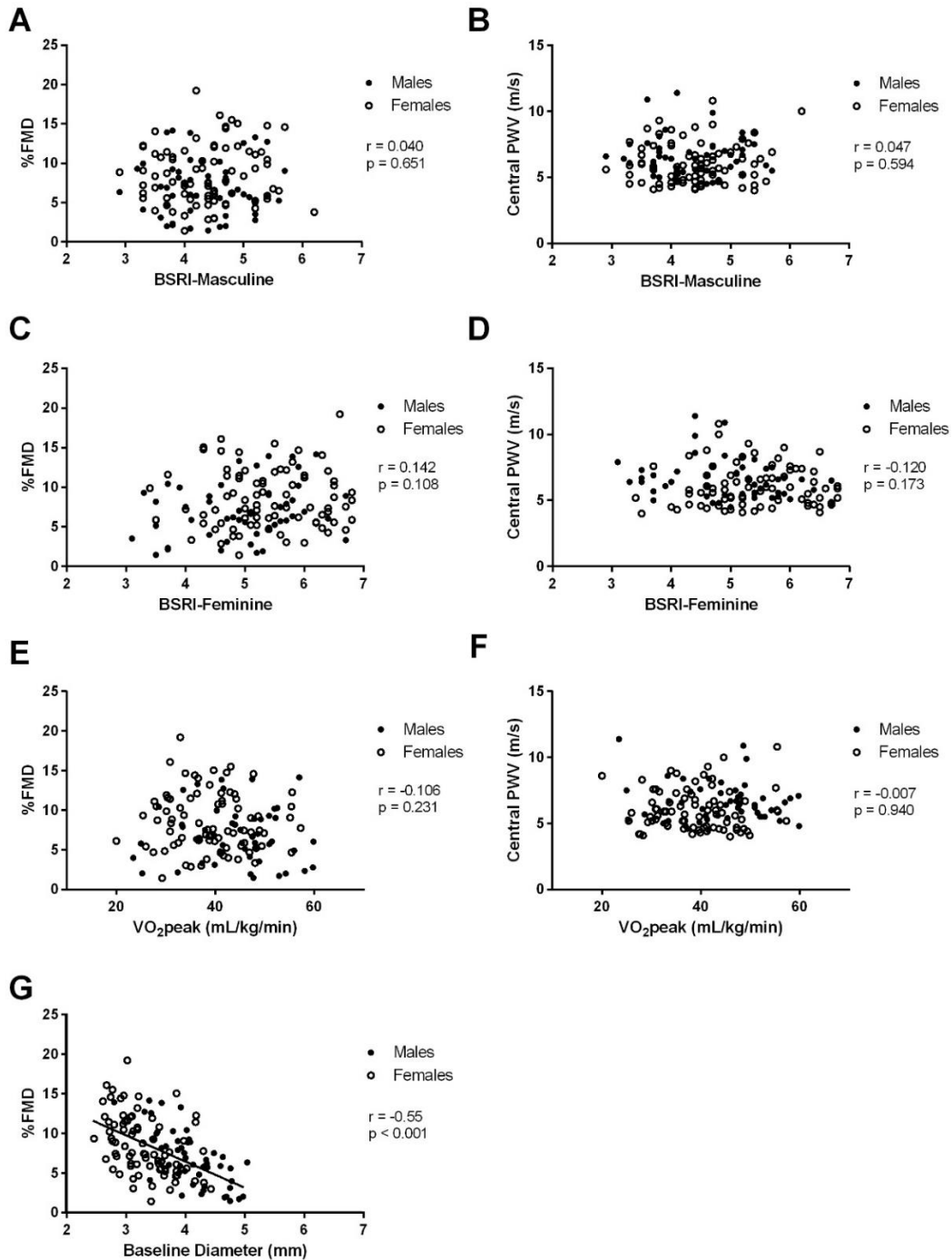
Supplementary Figure 1. Relationships between %FMD or Central PWV and BSRI-Masculine, BSRI-Feminine, and V̇O₂peak. (A) Relationship between %FMD and BSRI-Masculine; (B) Relationship between Central PWV and BSRI-Masculine; (C) Relationship between %FMD and BSRI-Feminine; (D) Relationship between Central PWV and BSRI-Feminine; (E) Relationship between %FMD and V̇O₂peak; (F) Relationship between Central PWV and V̇O₂peak; (G) Relationship between %FMD and baseline diameter.

CHAPTER 8: GENERAL DISCUSSION

The overarching purposes of this dissertation were to:

    (a) Examine the sex-specific inclusion of research participants in human vascular exercise physiology literature, including commonly cited barriers to female inclusion;

    (b) Comprehensively summarize prior literature examining menstrual cycle and hormonal contraceptive use, including cycle phase differences, on peripheral vascular function and structure;

    (c) Examine factors that may underscore differences in CVD risk in young adults, including the influence of the natural menstrual and OCP cycles, biological sex, and gender identity and expression; and

    (d) Holistically examine the influence of the natural menstrual and OCP cycles on the cardiovascular, respiratory, and skeletal muscle metabolism systems.

In this chapter, key findings from each chapter are summarized, and the overall significance of the dissertation is stated. This chapter will then provide additional context to the findings of each study, followed by an evaluation of the strengths and limitations of the studies and an overview of future areas for research. Finally, this chapter will conclude by sharing guidance for researchers in the form of the "top ten tips" to take away from this research.

1. Summary of Findings and Significance of Dissertation

Chapter 2: "Examination of sex-specific participant inclusion in vascular exercise physiology research: a systematic review." (1)

This systematic review identified 514 studies with over 25,000 participants examining human vascular endothelial function exercise physiology research questions. This study reported a male bias in the total number of participants and the number of male-only versus female-only studies. This study also identified the lower likelihood of male-only studies to report sex in the title or abstract, and to justify the exclusion on the basis of sex, compared to female-only studies. Unfortunately, rates of male bias remained relatively constant across the 20 years of publication dates examined. Finally, a qualitative analysis of the studies included in the review found four themes related to the

exclusion of participants; most strikingly was the exclusion of females based on the perceived influence of the hormonal cycle.

Chapter 3: "Impact of the menstrual cycle on peripheral vascular function in premenopausal women: systematic review & meta-analysis." (2)

This systematic review and meta-analysis identified 30 studies with over 1,300 participants and found that endothelial function was increased to a small magnitude in the late follicular, compared to early follicular, phase in the macrovasculature and not the microvasculature. In addition, endothelial function was largely unchanged from the early follicular to luteal phases (including mid-luteal phase), and unchanged across phases for vascular smooth muscle function; however, these latter findings came from very few studies (<10 studies). It was suggested that the small effect of phase on macrovascular endothelial function may be a product of differences in adherence to vascular endothelial function or menstrual cycle methodology guidelines – specifically the use of discrete versus continuous measurement of arterial diameter during an endothelial function test, or the increased inclusion of menstrual cycle methodologies (e.g., menstrual cycle tracking, ovulating testing, blood testing).

Chapter 4: "Influence of hormonal contraceptives on peripheral vascular function and structure in premenopausal females: a review." (3)

This narrative review reported that hormonal contraceptives influence macrovascular, and potentially microvascular, endothelial function depending on contraceptive generation and progestin type, the ratio of ethinyl estradiol to progestin, and the route of administration of the contraceptive (i.e., oral versus non-oral). However, this review reported that hormonal contraceptive use largely did not influence macro- and microvascular smooth muscle function, arterial stiffness, and arterial structure. This review also noted that there was a paucity of literature in this area of cardiovascular physiology, and proposed several areas of further study, with several gaps subsequently filled by research outlined in Chapter 5.

Chapter 5: "Menstrual cycle and oral contraceptive pill phase largely do not influence vascular function, arterial stiffness, and associated cellular regulation in young, healthy premenopausal females."

This observational research trial examined cardiovascular outcomes in forty-nine participants who were NAT (n=17), OCP2 (n=17) or OCP3 (n=15) across their LH and HH phases. This study found that most cardiovascular outcomes were unaltered by groups or phases, including lower limb (femoral) endothelial function, smooth muscle function in both upper and lower limbs, central and peripheral arterial stiffness, blood pressure, and cultured cell protein content of ERα and eNOS. Curiously, there was a small effect of phase in brachial artery endothelial function such that function was elevated in the HH versus LH phase. At the same time, brachial artery diameter was more constricted; however, scaling for this difference resulted in a non-significant effect of phase. There was also a small increase in HR during the HH phase compared to LH phase; together with the artery findings, this may point to an influence of the autonomic nervous system during the HH phase.

Chapter 6: "The impact of natural menstrual cycle and oral contraceptive pill phase on substrate oxidation during rest and acute submaximal aerobic exercise."

This observational research trial included the same participants from Chapter 5 (plus one extra NAT participant) to examine group and phase differences in substrate oxidation during rest and submaximal aerobic exercise. Like Chapter 5, this study reported that there were no differences across groups or phases for RER at each stage of the trial: supine rest, seated rest, or submaximal aerobic cycling exercise at 40% or 65% VO_2peak. However, while carbohydrate and lipid oxidation rates were unchanged also during stages of rest, but carbohydrate oxidation was elevated in NAT versus OCP2 during exercise at 40% and 65% VO_2peak. As in Chapter 5, HR was also consistently elevated during the HH versus LH phases with no difference across groups.

Chapter 7: "Differences in cardiovascular risk factors associated with biological sex, but not gender expression, in young, healthy adults."

This cross-sectional trial combined datasets from a variety of studies conducted in the Vascular Dynamics Lab to examine hemodynamics, arterial stiffness, and brachial artery endothelial function in 130 young, healthy adults, stratified based on biological sex, gender identity and gender expression. This study reported that, as expected, systolic blood pressure and central arterial stiffness were elevated in males and men compared to females and women. This study also found that endothelial function was elevated in males and men when differences in baseline artery diameter were accounted for using allometric scaling. Despite sex-differences (aligned with gender identity-differences), there were no differences across gender expression groups for cardiovascular outcomes. In the small proportion of gender diverse participants (n=2 non-binary), there was some evidence of cardiovascular dysfunction, warranting further research into this population in the future.

2. Influence of NAT and OCP Cycles on Cardiovascular, Respiratory, and Muscle Metabolism Outcomes

The following section will highlight contributions of this dissertation to the literature on NAT and OCP cycles and cardiovascular, respiratory, and muscle metabolism outcomes.

2.1 Vascular Outcomes

Considering first NAT cycles, Chapter 3 from this dissertation highlighted that despite early research, such as findings by Hashimoto et al (1995) (4) and Williams et al (2001) (5), observing robust alterations in %FMD across the menstrual cycle, systematic analysis supports only a small increase in %FMD from the early follicular to late follicular phase and no change in the mid-luteal phase. These findings correspond with most recent research by our lab and others who have largely observed no differences across menstrual cycle phases (6-10). This chapter also highlighted changes in the development of FMD guidelines that may be, in part, responsible for the lack of changes observed in more recent studies. Specifically, FMD guidelines since the early 2000s specified the need for continuous artery diameter assessment, rather than the use of a discrete measurement point to assess peak dilation (11-13).

Building on this research, in Chapter 5 we found no difference across groups and phases for lower limb (femoral) endothelial function but did observe a small increase in %FMD in the HH phase in upper limb (brachial) endothelial function. Allometric scaling for a more constricted vessel in the HH phase resulted in a maintained, but borderline, significant increase in %FMD. Importantly, this was a main effect of phase across all groups including OCP2, OCP3 and NAT. Forcing an interaction to examine pairwise comparisons, as is common in OCP literature (14, 15), revealed a no change from the LH to HH phase within NAT: $5.8\pm2.7\%$ to $6.1\pm2.2\%$ ($p=0.52$). Further, inclusion of this new data into Chapter 3's limited EF versus ML endothelial function assessment did not alter the results of that previously conducted meta-analysis (SMD before addition: 0.37; following addition: 0.34). The data cited in Chapter 5 is located in the middle of the spread of macrovascular studies and adjacent to previous work from our lab by Shenouda et al, finding a similar SMD of 0.13 across phases (7), and slightly above work by Rakobowchuk et al finding an SMD of -1.28 (10).

Importantly, these findings further align with more recent reports, including published conference abstracts, identifying a small or no difference in vascular outcomes, like %FMD and passive leg movement (measurement of leg microvascular endothelial function), across menstrual cycle phases (16-19). For example, a recent study by Weggen and colleagues (2023), found that there was no difference across the early to late follicular phase of the menstrual cycle for passive leg movement microvascular function of the leg and active handgrip-induced brachial artery dilation (20). Similarly, work by D'Agata and colleagues (2021) identified no difference across the early to late follicular to mid-luteal phases of the menstrual cycle for passive leg movement, however, did note that Black women had significantly lower function than White women (21). Altogether, these recent findings in both arm and leg vasculature further support our observations of lack of phase differences in NAT participants.
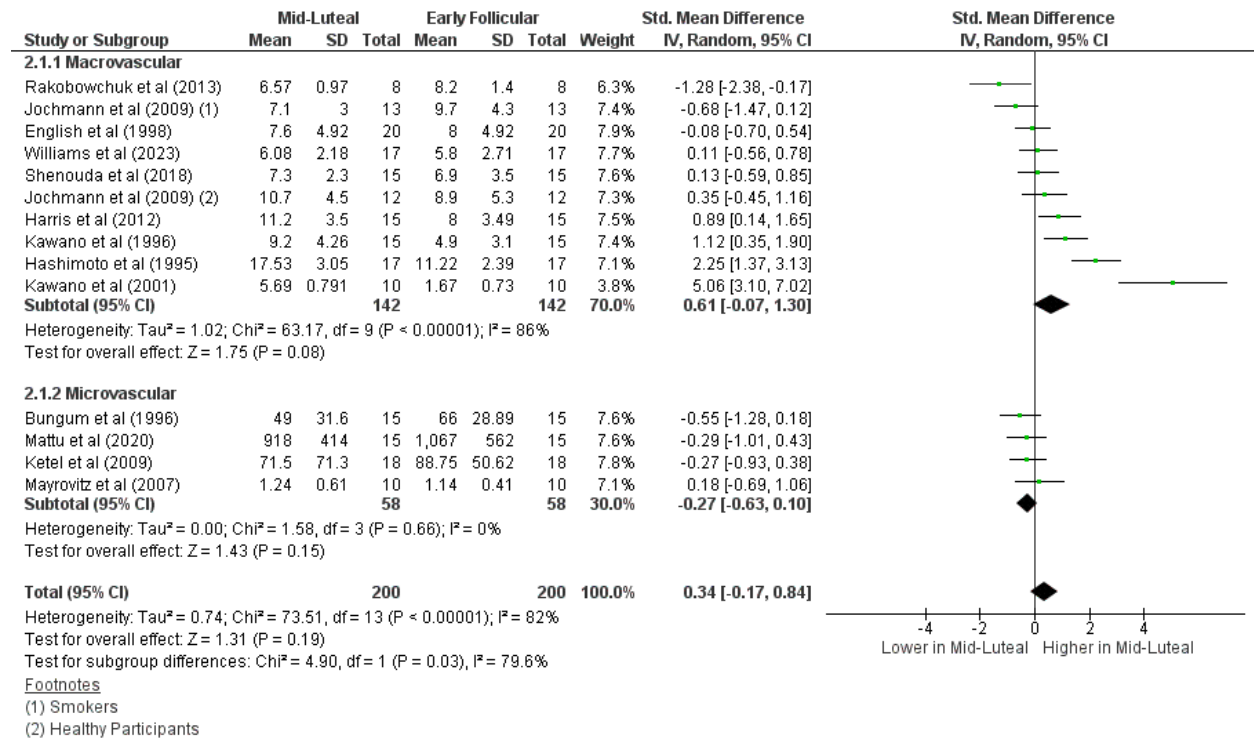
Study or Subgroup	Mid-Luteal			Early Follicular			Weight	Std. Mean Difference IV, Random, 95% CI	Std. Mean Difference IV, Random, 95% CI
	Mean	SD	Total	Mean	SD	Total			
2.1.1 Macrovascular									
Rakobowchuk et al (2013)	6.57	0.97	8	8.2	1.4	8	6.3%	-1.28 [-2.38, -0.17]	
Jochmann et al (2009) (1)	7.1	3	13	9.7	4.3	13	7.4%	-0.68 [-1.47, 0.12]	
English et al (1998)	7.6	4.92	20	8	4.92	20	7.9%	-0.08 [-0.70, 0.54]	
Williams et al (2023)	6.08	2.18	17	5.8	2.71	17	7.7%	0.11 [-0.56, 0.78]	
Shenouda et al (2018)	7.3	2.3	15	6.9	3.5	15	7.6%	0.13 [-0.59, 0.85]	
Jochmann et al (2009) (2)	10.7	4.5	12	8.9	5.3	12	7.3%	0.35 [-0.45, 1.16]	
Harris et al (2012)	11.2	3.5	15	8	3.49	15	7.5%	0.89 [0.14, 1.65]	
Kawano et al (1996)	9.2	4.26	15	4.9	3.1	15	7.4%	1.12 [0.35, 1.90]	
Hashimoto et al (1995)	17.53	3.05	17	11.22	2.39	17	7.1%	2.25 [1.37, 3.13]	
Kawano et al (2001)	5.69	0.791	10	1.67	0.73	10	3.8%	5.06 [3.10, 7.02]	
Subtotal (95% CI)			142			142	70.0%	0.61 [-0.07, 1.30]	
Heterogeneity: Tau² = 1.02; Chi² = 63.17, df = 9 (P < 0.00001); I² = 86%									
Test for overall effect: Z = 1.75 (P = 0.08)									
2.1.2 Microvascular									
Bungum et al (1996)	49	31.6	15	66	28.89	15	7.6%	-0.55 [-1.28, 0.18]	
Mattu et al (2020)	918	414	15	1,067	562	15	7.6%	-0.29 [-1.01, 0.43]	
Ketel et al (2009)	71.5	71.3	18	88.75	50.62	18	7.8%	-0.27 [-0.93, 0.38]	
Mayrovitz et al (2007)	1.24	0.61	10	1.14	0.41	10	7.1%	0.18 [-0.69, 1.06]	
Subtotal (95% CI)			58			58	30.0%	-0.27 [-0.63, 0.10]	
Heterogeneity: Tau² = 0.00; Chi² = 1.58, df = 3 (P = 0.66); I² = 0%									
Test for overall effect: Z = 1.43 (P = 0.15)									
Total (95% CI)			200			200	100.0%	0.34 [-0.17, 0.84]	
Heterogeneity: Tau² = 0.74; Chi² = 73.51, df = 13 (P < 0.00001); I² = 82%									
Test for overall effect: Z = 1.31 (P = 0.19)									
Test for subgroup differences: Chi² = 4.90, df = 1 (P = 0.03), I² = 79.6%									

Footnotes
(1) Smokers
(2) Healthy Participants

Figure 1. Re-Evaluation of Early Follicular to Mid-Luteal Endothelial Function Meta-Analysis from Chapter 3.

Given the previous lack of consensus on the topic of menstrual cycle influence on vascular function, and the considerations around research study control, a Point-Counterpoint series was developed in the *Journal of Applied Physiology* to form consensus on this topic (22, 23). Groups of researchers presented points in favour of and against "controlling" for menstrual cycle phase in vascular control studies, which were then responded to by researchers broadly (24). Our group wrote a commentary, replicated verbatim below, that illustrates the recently published Chapter 3 at that time and now aligns with the findings of Chapter 5.

> "To the editor: The debate questioning whether or not researchers should control for the menstrual cycle when studying females in vascular research (22, 23) has been controversial due in part to a key factor identified by Wenner and Stachenfeld in their Rebuttal: "…there is not yet a consensus regarding change in physiological measures such as endothelial function…across the menstrual cycle" (25). As a result, researchers often highlight studies to align with supporting (4, 5) or rejecting (6, 7) considerations for menstrual cycle phase.

Coincidentally, our group conducted a systematic review and meta-analysis (unpublished (*at the time, now published (2)*)) that examined this question. Analysis from 30 studies found a "very low" certainty of evidence (GRADE) that endothelial function increases in the LF phase versus the EF phase, with differences only observed in the macrovasculature, not the microvasculature. However, heterogeneity in macrovascular studies can be partially explained by methodological differences. Additionally, endothelial function was largely unchanged in the luteal phases versus EF phase, and smooth muscle function was unaltered across the cycle.

Given these results, researchers should consider the menstrual cycle, alongside other hormonal cycles (e.g., hormonal contraceptives) and conditions. In agreement with Wenner and Stachenfeld (22), researchers should control and/or examine the influence of fluctuations in endogenous hormones when assessing basic physiological mechanisms; however, in agreement with Stanhewicz and Wong (23), it may be less important to control for phase in clinical trials. At the very least, considering hormonal cycle reporting, alongside other participant characteristics, will improve scientific rigor and reproducibility."

The published point-counterpoint commentaries highlighted a balance between implementing reasonable participant controls – similar to other common study controls like diet, exercise, and testing environment conditions – while balancing these controls with principles of generalizability and feasibility of the study design. For example, some researchers highlighted that for acute studies or those focused on understanding a basic mechanism that may be influenced by subtle changes in sex hormones, researchers may want to control for the menstrual cycle phase. This suggestion is both feasible and allows for controls that would support the research question. Similarly, some researchers highlighted research questions or environments in which menstrual cycle controls would not be feasible (e.g., military training or high-altitude environments, repeat testing within a window that would not align with consistent menstrual cycle

phases). Some researchers also challenged the notion of testing exclusively within a specific menstrual cycle phase, if it was well known that the menstrual cycle had limited influence on the outcome of interest, as it limits the generalizability of findings to only a single phase. This debate bears striking resembles to a key theory in sex/gender-based analysis research developed in 2022 by Dr. Sarah Richardson called "sex contextualism" (26), discussed further below. This theory suggests that study design, including the consideration of sex/gender and sex/gender-related factors (e.g., sex hormones), should align with the research question; like the conclusions of the point-counterpoint debate.

Considering OCP cycles, in contrast to some findings of Chapter 4 illustrated above, Chapter 5 found no difference across OCP groups and phases in most vascular outcomes including arterial stiffness, smooth muscle function, and lower limb endothelial function. Like above, upper limb endothelial function was elevated in the HH phase compared to LH phase, but not when allometric scaling was applied. Forcing an interaction analysis, similar to previous work by Torgrimson et al (2007) (14) and Thompson et al (2011) (15) who examined paired t-test comparisons within each group, revealed a small increase in %FMD in OCP2 but no change in OCP3 [OCP2: 8.1±3.6% to 9.0±3.9% (p=0.04) versus OCP3: 7.3±3.5% to 7.9±3.7% (p=0.15)]. The use of a two-way ANOVA in Chapter 5 and 6 was selected for its ability to compare both across groups and within phases, unique to the research questions posed. Despite the small change in OCP2 observed with forcing pairwise comparisons, the clinical relevance of an acute 0.9% increase in %FMD across phases is unknown, especially alongside the lack of impact of long term OCP2 use on %FMD, reported in Chapter 5. In addition, this small %FMD increase observed in the OCP2 group runs counter to previous literature identified in Chapter 4, with studies identifying either no effect or a negative effect of acute and long-term OCP2 use on %FMD (3). Previous work by our group identified no difference across OCP2 phases for %FMD (7) or arterial stiffness (27), but a negative association between the duration of use of OCP2 on %FMD. It is unclear why this the current study did not corroborate previous acute and long-term findings of OCP2 use. Similarly, it is also unclear why this study did not observe changes in OCP3 across

phases, unlike other studies demonstrating small improvements in %FMD (15, 28). One speculation is that similar to NAT cycle phase differences across studies, individual variability in sex hormone fluctuations may be in part responsible for the small differences across phases.

Variability in both menstrual cycle characteristics (e.g., lengths of cycles, lengths of periods and phases) along with fluctuations in endogenous hormones provide complexity to, and may confound, small differences in phases masked by this inter- and intra-variability. For example, recent work by Soumpasis et al (2020) used data from ovulation testing from over 30,000 women in the US and UK and found that only ~12% of users had a standard 28-day cycle; most instead were deemed eumenorrheic with a cycle of 23-35 days (29). In addition, considering cycle-to-cycle variability, this study reported that 52% of users' cycles varied more than 5 days (29). Connecting to vascular assessments, findings by Liu and colleagues (2021) investigated the changes in 17β-estradiol and associated %FMD from the early follicular to late follicular phases across two consecutive menstrual cycles (9). This study reported that changes across phases in one cycle did not predict changes in the subsequent cycle; likewise, there was no relationship between the change in 17β-estradiol across phases in the first cycle to the second cycle. Considering the variability observed in the Chapter 5 study, with 17β-estradiol levels ranged from 102 to 257 pmol/L in the early follicular phase to 223 to 936 pmol/L in the mid-luteal phase. Similarly, progesterone levels, which rise in the mid-luteal phase, ranged from 0.3 to 8.5 nmol/L in the early follicular phase to 3.3 to 63.6 nmol/L in the mid-luteal phase. While this study followed hormonal cycling guidelines in attempting to tightly control for sex hormone fluctuations (30, 31), there is well known inter- and intra-cycle variability in hormone levels. In addition to menstrual cycle fluctuations, there are also challenges raised in objectively assessing exogenous sex hormones, like ethinyl estradiol, levonorgestrel, desogestrel, and norgestimate. Technical limitations in the availability of assessment methods for exogenous hormone greatly limits the capacity to confirm phases. Given the lack of commercially available assays, researchers typically interpret the suppression of endogenous hormones in OCP users during the active phase (with similar 17β-estradiol and progesterone levels

during the placebo phase) as confirmation of cycle phase. As seen in Chapter 5, there was a small, but evident, suppression of 17β-estradiol during the active phase in both OCP2 and OCP3 groups. Uniquely, for the first time in OCP vascular research, an assessment of levonorgestrel was attempted using a newly developed commercial assay by Thermo Fisher Scientific. Using this novel assay, we were able to document a rise in levonorgestrel levels from the placebo to active pill phase in OCP2, again with variability in measures: 16.9 to 230.4 pg/mL in the placebo phase to 13.6 to 1920.8 pg/mL in the active phase. Altogether, these hormonal concentration fluctuations may be responsible for the small differences between studies noted above.

In addition to the general lack of significant findings in vascular outcomes across NAT and OCP cycles, we found a similar lack of differences in eNOS and ERα protein content in our companion serum cell exposure studies. This finding runs counter to previous work by Gavin and colleagues (2009) who reported that ERα was elevated in the late follicular compared to early follicular phase by 30%, and that ERα was positively associated both with %FMD, eNOS, and phosphorylated eNOS in the larger dataset which included postmenopausal women (32). These discrepant findings may be the product of differences in methodology, with our study using serum-exposed female HUVECs and Western blotting for protein content, while the latter study used a J-scrape of venous endothelial cells from participants and immunofluorescence of single cells. It is also possible that relationships between eNOS, ERα, and %FMD exist, but only in circumstances where there is a wider distribution of outcomes, such as in data sets including both pre- and post-menopausal participants. Indeed, examining the ERα and eNOS protein content data from the study in Chapter 5, there is minimal variability within a phase or between phases to probe for associations between measures. Finally, the study by Gavin and colleagues (2009) examined premenopausal females in the early and late follicular phases (only an increase in 17β-estradiol) (32), while the present study in Chapter 5 examined the mid-luteal phase with an increase in both 17β-estradiol and progesterone. For example, there is some evidence that progesterone antagonizes estradiol (33-35), which may reduce the estradiol-induced elevation in ERα and

subsequently result in eNOS maintaining its early follicular phase levels (34), as seen in the present study.

Altogether, these findings from Chapters 3-5 report that NAT and OCP cycles have little-to-no effect on the majority of vascular outcomes. These findings suggest that physiological examinations across the NAT and OCP cycle may not require as stringent controls, and that any hormonal cycle controls should be balanced alongside other aspects of the research study design, in agreement with the theory of sex contextualism. However, there are a few areas in which there may be evidence of hormonal cycle influences on vascular outcomes that are relatively understudied. For example, there is evidence of NAT cycle fluctuations during periods of dynamic stressors. Work by Luca and colleagues (2016) identified an impairment in FMD associated with an ischemia reperfusion stressor during the early follicular phase, but a preservation during the late follicular phase when 17β-estradiol levels were elevated (36). Similarly, Restaino and colleagues (2022) noted a lower forearm vascular conductance response to handgrip exercise in obese, compared to normal weight, women during the early follicular phase; a difference that was lessened in the late follicular phase (37). However, this observation is not true for all forms of stressors, particularly not for metabolic stressors. For example, work by Harris and colleagues (2012) identified that premenopausal females were protected against the deleterious effects of a high-fat meal on FMD compared to males, and this observation was consistent across the early follicular, late follicular, and mid-luteal phases of their menstrual cycle (38). Previous research I conducted as part of my Master's degree found no difference in acute hyperglycemia-induced impairments in FMD across the early and late follicular phases of the menstrual cycle (8). In summary, there may be some physiological stressors for which small fluctuations in vascular function associated with NAT or OCP cycles are observed; however, further research is needed to fully explore this hypothesis.

2.2 Respiratory & Skeletal Muscle Metabolism Outcomes

The cardiovascular system has reciprocal relationships with other organ systems, including the respiratory and skeletal muscle organs. To extend the observations of Chapters 3-5, Chapter 6 explored the impact of NAT and OCP cycles on respiratory and skeletal muscle metabolism outcomes during rest (supine and seated) and different intensity stages of submaximal aerobic cycling exercise in the same population as Chapter 5. This study reported that there were no differences in RER across groups or phases in any stage of rest or exercise, although there was slightly elevated carbohydrate oxidation during exercise in NAT versus OCP2.

In previous research there is some evidence that NAT and OCP cycles influence RER and substrate oxidation. For example, recent work by Mattu and colleagues (2020) found that RER was elevated in the mid-follicular and inactive pill state of OCP2 and OCP3 compared to the mid-luteal and active pill state during a 30min maximal lactate steady-state test (39). Similarly, early work by Hackney and colleagues (1994) observed higher RER in the mid-follicular versus mid-luteal phases at lower (35% and 60% VO_2max) but not at higher (75% VO_2max) exercise intensities (40). However, there are many studies that have found no phase or group effects of NAT or OCP cycles on RER or substrate oxidation (41-45). Like the discrepancies in the vascular literature, these conflicting results may point to differences in research methodology, exercise intensity examined, or hormonal phase (and variability) considered.

Given these mixed findings, we pursued a systematic review and meta-analysis to collate all data examining substrate oxidation and NAT or OCP cycles (PROSPERO registry: https://www.crd.york.ac.uk/prospero/display_record.php?RecordID=237018). A total of 55 studies with 928 participants were included in the review, with 25 studies examining RER at rest in NAT (n=487 participants) and 25 studies examining RER during exercise in NAT (n=483). The meta-analysis reported that there was a very low certainty of evidence that RER was not different across NAT phases during rest (SMD: 0.05, 95% CI: -0.15, 0.25, p=0.63), or during exercise (SMD: -005, 95%CI: -0.13, 0.23, p=0.57). We also found a total of 7 studies examining RER in OCP cycles during

exercise (n=51 participants), with too few studies examining RER at rest to make meta-analytical conclusions. Similar to NAT cycles, there was a low certainty of evidence that RER was not different across placebo and active OCP phases (SMD: -0.36, 95% CI: -0.76, 0.03, p = 0.07), although more research is needed. These findings were recently submitted for publication in an international collaborative review entitled: "The menstrual cycle and oral contraceptive use in exercise physiology: big deal, minor inconvenience, or nothing to worry about?", which further argued that the impact of NAT and OCP cycles on vascular, exercise performance, skeletal muscle hypertrophy, and metabolism is "small or non-existent".

Another curious finding in Chapter 6 was the elevated HR in the HH versus LH phase, with no difference across groups. This finding was also consistent with findings in Chapter 5. Interestingly, while these measures involved the same set of participants, they were taken on different days, and with different HR monitors – Chapter 5 used a single-lead ECG while Chapter 6 used a Polar HR monitor. As a result, the speculation that this finding is due to measurement error or day-to-day variability is limited. Instead, there is support that there is altered autonomic nervous system activity during the HH phase when progesterone is elevated, compared to LH phase. For example, examining data from large datasets (e.g., subscribers to health applications), there is some evidence that resting heart rate increases from LH to HH phases in NAT and OCP (46). Similarly, a recent meta-analysis examining cardiac vagal activity as a combination of parasympathetic measurements, observed a decrease in vagal activity from the follicular to luteal phase of the NAT cycle with a medium effect size (47). Similarly, a recent review by Stadler and colleagues (2019) also identified a "sympathetic predominance", or more parasympathetic vagal withdrawal to the heart, during the luteal phase compared to follicular phase (48). To further add to these reports, research by Carter and Lawrence (2007) found similar resting and mental-stress induced hemodynamic changes in HR, MAP and MSNA across NAT phases, but a prolonged MSNA activation during the recovery phase post-mental stress in the mid-luteal phase (49). We speculate that the balance of parasympathetic and sympathetic outflow on the heart may be altered towards the latter during the HH phase when progesterone is

elevated. The impact of a small (~2bpm), but consistent, elevation in HR during this phase is unknown but may be related to increased demands on the heart related to preparing the uterus for possible implantation, post-ovulation.

3. Importance of Sex-Inclusion in Exercise Physiology Research

Considerations to sex-inclusion in exercise physiology have gained prominence in the last decade with researchers, advocacy bodies, government organizations, and the public questioning why women are understudied in basic and clinical research. Chapter 2 reported on a systematic review in which over 500 studies with over 25,000 participants in exercise physiology trials involving vascular endothelial function measures was conducted to examine the number of male, compared to, female participants, and the number of male-only, female-only and mixed-sex trials conducted, along with other sex/gender questions. This review identified a male bias in vascular exercise physiology trials, with 2/3 of participants as males and a higher prevalence of male-only studies compared to female-only studies conducted; unfortunately, these findings have remained consistent over the last ~20 years of research, despite policy and advocacy efforts.

While the findings presented in Chapter 2 are novel to the field of vascular physiology, they are not new in exercise science research, or in other fields like animal endocrinology (50), thermoregulation (51), nutrition (52), blood flow restriction (53), and cardiac and nephrology research (54). For example, early work by Costello and colleagues (2014) identified an under-representation of women in sports and exercise medicine journals of similar levels to those reported in Chapter 2 (~39%) (55). Similarly, more recent work by Cowley and colleagues (2021) examined studies from 2014-2020; these authors found a continuation of male-bias with only 34% of participants female and only 6% of studies conducted as female-only (compared to 31% as male-only cohorts) (56). Similar work in the cardiovascular field by Patel and colleagues (2021) also found in studies using high-dose exercise with cardiovascular outcomes, 50% excluded females and only 3% included a female-only cohort. Furthermore the authors reported a representation of only 18% females in these trials (57).

Despite the burgeoning literature that has reported sex-bias towards male inclusion in physiology trials, there are some researchers who continue to question the existence of and mechanisms underlying this bias. For example, an opinion piece by Nuzzo (2021) suggested that volunteer bias, over investigator bias, is the root cause of the lack of female inclusion in exercise and sports science research (58). This researcher argued that sex-differences in disease prevalence, physical activity engagement, and personality traits may be more so responsible for the lack of female inclusion in research. While this opinion piece included several claims that offered minimal objective research justification, one claim made by Nuzzo regarding prior sex-inclusion reviews was the attribution of the intentional exclusion of females due to menstrual cycle influences on outcomes, without providing evidence of this observation. The novelty of Chapter 2 is that it asked, for the first time, "why"? By investigating the qualitative rationales that researchers disclosed in their manuscripts, we were able to capture four core reasons for the lack of inclusion of females in this area of research. Aligned with commentary by Nuzzo (58), there were challenges associated with recruitment of females for studies in which there are known sex-differences in prevalence or challenges with seeking female engagement in exercise trials (e.g., cardiac rehabilitation trials). However, most strikingly were the perceptions that the NAT/OCP cycle would influence the outcomes of interest, as detailed in the discussion above, and the perception that researchers must maintain the male norm to facilitate comparison to prior literature. These qualitative findings challenge, in part, the opinions of Nuzzo by pointing to investigator bias, over volunteer bias, as a source of female exclusion from trials.

Similarly, another claim offered by Nuzzo in this commentary (58), and later supported by a recent objective assessment by Nuzzo and Deaner (2023), was sex-differences in the interests and willingness to engage with exercise science research (59). This latter study found that women were more likely to engage with exercise trials that involved stretching and group-based aerobics (e.g., yoga, dancing, walking, stretching), and online exercise interventions compared to men (59). This study also found that women

were more likely to engage with studies involving women's health questions, and likewise men were more likely to engage with men's health studies, and research in the areas of sports medicine, and muscle health (59). The latter finding that men were more likely to engage with muscle health trials aligns in part with the observation that the male-bias in participant inclusion in Chapter 2 was more prominent in exercise trials involving resistance training (often with muscle health outcomes) than with aerobic training (1). Importantly, this study found that while there were sex-differences in some exercise procedures, such as men being more likely to engage in an acute high intensity exercise test, nerve stimulation of muscles, strength training, sleep deprivation for 48h, and taking muscle supplements, there were also several procedures in which women were more likely to engage (e.g., group aerobics exercise, home-based exercise programming, survey methods) (59). There were research procedures in which there was no sex-dominance, such as those involving blood draws, biopsies, bone x-ray, immobilization, and saliva sampling among others (59). Most importantly, and in contrast to the commentary proposed, there was no procedure in which women were not willing to participate. We would assert that the presence of a sex-difference in participation rates and indicators of willingness and interest does not mean that *all* women are not willing or interested. We suggest that volunteer bias (or challenges with recruiting females) alongside investigator bias in the intentional exclusion of females within research studies contribute jointly to the rates of male bias in sport and exercise science research.

Building on the question of "why", the question of "who" is also relevant. There has been recent discourse around who is conducting female-inclusive research. Recent research by Laxdal (2023) identified that out of ~2000 authors of female-only studies in the same dataset as the Cowley paper detailed above (56), found that 42% were female, with an "overrepresentation" of females in first and last authorship roles (60). The author concluded that females were more likely to pursue research on females compared to males and argued that this sex gap "should not fall solely on the shoulders of females" and that all researchers pursuing studies that consider sex/gender inclusion. However, there were several methodological concerns in this paper that are illustrated further in

our letter to the editor (61), including the conflation of sex and gender terminology and lack of comparison to demographic shifts in female pursuit of graduate-level study, along with an alternative hypothesis presented. Building on this work, there has been a ~0.5% increase in the proportion of female first authors over the last 20 years, which holds promise for further inclusion of females in trials (62). Beyond the consideration to "who" pursues female-only research, the question of "who" is in positions of leadership must be considered. While unsurprising, there is an underrepresentation of women in positions of journal leadership on editorial boards within exercise and sport medicine research (62). Focusing on improving female-inclusion in sports and exercise science research is a responsibility of all researchers; however, there are clear indicators that the inclusion of more females in positions of research leadership (either as authors or editors) may help advance this goal. The hope is to conduct the study presented in Chapter 2 again in 20 years and observe shifts in participant inclusion towards greater sex parity.

4. Importance (and Challenges) of Sex/Gender Inclusion in Cardiovascular Research

As discussed in Chapter 7, sex/gender and their related factors are integral to further understanding both basic science mechanisms and health outcomes in humans. One interesting finding from Chapter 2 was the lack of previous studies examining gender (<1%) in cardiovascular exercise physiology research, despite ongoing calls for action by researchers, policymakers, and journals (63-67). One mechanism of sex/gender inclusion that researchers are eagerly awaiting results from is the decision of journals like the American Journal of Physiology – Heart and Circulatory Physiology to require inclusion of both males and females in research trials and discussions around gender where applicable (68, 69). As noted in Chapter 7, consideration to both sex and gender allows for a more robust analysis of factors driving physiological differences across groups; in Chapter 7, we found that there were differences in cardiovascular outcomes between biological sexes (aligned with gender identity in 99% of participants) but no influence of gender expression in our cohort of young, healthy adults. As the first study

224

to investigate sex and gender in early risk factors for cardiovascular disease, this study will provide some foundational thinking for consideration in future trials.

Despite these calls for inclusion of sex/gender and related factors in physiology research, there are two challenges that limit the feasibility of these calls that must be addressed. The first challenge, raised in Chapter 7, is the limitation of the current tools available for gender assessment. The primary tool recommended by the Institute of Gender and Health within the Canadian Institutes of Health Research is the Genesis Praxy questionnaire (70), which includes several gender-based questions including the Bem Sex Role Inventory (BSRI) (71). However, many of the questions included in the Praxy questionnaire may not be appropriate for all young adults (e.g., primary earner status, income range, children and caregiving responsibilities). In addition, there is no published methodology for how to analyze the Praxy questionnaire, posing challenges for its utility. The study in Chapter 7 used the Bem Sex Role Inventory to examine feminine and masculine gender expression, which has been previously validated in a young, adult (university-aged) population (71). However, a major concern of the BSRI is that it relies on the gendered stereotypes of traits identified and validated in the 1970s, despite shits in gender roles within society such as greater engagement of women in the workforce, education, politics and public spaces, and shifts away from sole responsibility of childcare and household tasks (72-74). Notably a meta-analysis by Donnelly and Twenge (2017) identified that scores of masculinity in the BSRI in women have increased over time since the 1870s, while femininity in women and both masculinity and femininity in men have remained unchanged (75).

Building on this work, a component to the Chapter 7 study (not presented in this dissertation) was to examine the perception of the BSRI-30 traits in a young, healthy population and examine whether there are gender-related differences in trait perception. This study asked participants to classify the BSRI-30 traits on a scale of 1 "Always or almost always a masculine trait" to 7 "Always or almost always a feminine trait". We found that BSRI-feminine scores were higher in women compared to men, however, BSRI-masculine scores were not different between men and women. In addition, there

were several traits in which the perception from the participants shifted away from the BSRI categorization, including five out of ten BSRI-masculine traits being perceived as neutral (defend my own beliefs, have leadership abilities, independent, strong personality, willing to take a stand) and two BSRI-feminine traits being perceived as neutral (understanding, compassionate). Given the results of this study, there are concerns about the utility of the BSRI in evaluating gender expression in future. However, the newly developed Nielsen questionnaire, which was developed based on a review of gender methods to capture seven gender-related variables and has now been validated in a young adult cohort (76). This new questionnaire removes the gendered nature of feminine and masculine traits and instead groups traits together by common gendered areas like "caregiver strain", "work strain", "risk-taking", "independence", "emotional intelligence", "social support", and "discrimination" (76). The authors, in their discussion, specifically reference that it may be that caregiver strain, rather than "femininity" that is related to acute coronary syndrome recurrence, connected to previous work by Pelletier and colleagues (2016) who first used femininity and masculinity scoring in CVD research (77). Finally, the future usability of this new questionnaire is strong, given the detailed methodology and analysis provided in the study manuscript (76).

Another challenge in the field of sex/gender research is the oversimplification of sex/gender, such as approaching sex and gender from a binary perspective (male versus female, man versus woman) or not pursuing questions that explore sex/gender-related factors underlying sex/gender-differences. Connecting back to sex contextualism (26), which suggests that research questions and their associated methodologies should deeply explore sex/gender and consider the mechanisms that underlie sex/gender-differences rather than group-level differences solely. Often sex/gender categories can be representative of deeper mechanistic questions that are represented within these categories . For example, recent work by Junker and colleagues (2022) looking at sex/gender in mitochondrial research, considers the overlap in biological sex hormone characterizations and considers moving away from binary sex models to variations of sex characteristics using genetics and sex hormones

(78). Similarly, this paper challenges the notion of using gender categories to explore gender-related exposures, and instead encourages the use of the exposures themselves to look at mitochondrial health – like diet, physical activity, social capital, role strain, healthcare utilization…etc. (78). Finally, this paper illustrates the primary approaches to exploring sex/gender by examining categories or spectrums of gender, or by using specific mechanistic variables to consider sex/gender (78). Another example of breaking down sex/gender binaries is in the article by Richardson on sex contextualism (26), where they argue that there may be four "sexes" that consider 17β-estradiol levels, and biological sex defined by sex chromosomes. The specificity of considering sex at birth and sex hormones as distinct categories is also represented in recent work by Lalande and colleagues (2021) who examined differences in FMD in response to ischemia-reperfusion injury between males and females who had similar 17β-estradiol levels (79). Although the comparison of males and females during a period where sex hormones are relatively equal is commonplace, the focus in the language used in this text is an example of how specificity of sex and sex-related variables can add nuance to sex/gender considerations in physiology trials.

This dissertation is not without critique: the methodologies employed throughout did primarily use categorical variables of sex/gender rather than mechanistic variables to examine physiological responses, apart from an exploration of hormonal variations in females. However, with the development of new sex/gender methods including contextual approaches like the Nielsen questionnaire (76) and the factor analysis outlined by Junker and colleagues (78), more sophisticated analyses of sex/gender in cardiovascular research is anticipated.

5. Strengths and Limitations of Dissertation

There are several strengths and limitations of this dissertation worth highlighting. One major strength of this dissertation research is the initial synthesis of vascular function and arterial stiffness research (Chapters 3 & 4) that provide context for the follow-up observational research by our lab. Considering the observational study on NAT and OCP cycles and cardiovascular outcomes (Chapter 5), this study had a relatively large

sample size with appropriately disaggregated OCP generations. As a result, we were able to draw OCP generation-specific conclusions. In addition, this dissertation aimed to provide a comprehensive evaluation of the impacts of NAT and OCP cycles on three organ systems: cardiovascular, respiratory, and skeletal muscle metabolism (Chapters 5 & 6). Examining multiple organ systems recognizes that the cardiovascular system does not operate in a vacuum and has influence within and from other systems of interest. Another key strength of Chapter 5 was the inclusion of *in vivo* cellular research models to provide a truly integrative physiology approach to addressing the research questions. The ability to connect macro-level vascular function and arterial stiffness measures within each participant to the cellular protein responses of ERα and eNOS through HUVEC serum exposure further reaffirms the observed lack of NAT and OCP influences on the cardiovascular system. A final strength from this dissertation was the exploration of gender identity and gender expression on cardiovascular outcomes, as the first of its kind of study in this area. This study will provide foundations for future research aimed at examining both sex and gender in early indicators of cardiovascular disease, an area study that is increasing in focus.

In addition to strengths, there are several limitations and challenges faced with this dissertation. First, while the initial chapters of this dissertation included OCP and non-oral contraceptive options, the latter observational trial only included two generations of OCPs. This was in part due to changing prescription patterns in the available participant pool along with shifts in insurance company coverage policies away from non-oral contraceptives (i.e., rings, patches) to the use of intrauterine devices (IUDs). With growing popularity of IUDs, our lab will be focusing on these contraceptive options in the coming years. In addition, while the initial review chapters discussed microvascular function measures, the observational trial was limited to understanding upper and lower limb macrovascular endothelial and smooth muscle function. The rationale for the exclusion of microvascular function was simply limitations in timing and concerns around participant burden. Testing for the observational trial, including both the vascular testing days and substrate oxidation exercise testing days, resulted in nearly 8 hours of participant testing. Adding in microvascular outcomes would have extended testing days

by ~30min-1 hour per vascular session. In addition, reviews from Chapters 3 and 4 highlighted that the microvasculature has not been influenced largely by NAT and OCP cycles; therefore, it is unlikely the addition of microvascular outcomes would have added considerably to the vascular trial's conclusions. Another limitation outlined in Chapter 7 (sex/gender and cardiovascular outcomes) was the lack of participants who came from gender-diverse groups. While the population was generally representative of the number of non-binary participants we would have expected (~<1%), there was an absence of transgender participants. Further research is necessary in these groups, who may be more prone to early vascular dysfunction.

As with any graduate student pursuing research in 2020 (and beyond), the COVID-19 pandemic had a major impact on the research presented in this dissertation. Our lab was shutdown for 16 months, in the middle of early testing for the observational trial presented in Chapters 5 & 6. While this shutdown delayed progress in initial research plans, these restrictions provided the motivation for major "pivots" to continue to pursue curious research questions. During this time, we pursued cell-based research, which was permitted at our university when human participant testing was still restricted, to understand the connections between macro-level vascular outcomes and micro-level cellular pathways. In addition, this period provided the time for additional considerations of sex and gender as factors in research, which ultimately drove the development of Chapter 7. While this period could be perceived as a major challenge for the dissertation, it is instead a strength that supported the comprehensive building of the research.

6. Areas for Future Research

Based on the findings from this dissertation, there are several areas that warrant future research. First, Chapter 5 focused on cardiovascular outcomes in two generations of OCPs. Research is needed to explore cardiovascular outcomes in other forms of hormonal contraceptives, specifically non-oral routes of administration such as through IUDs. In addition, participants in our studies had been using OCPs on average for 2-3 years; as a result, further research is necessary to examine longer term effects of OCP

use on cardiovascular outcomes specific to each generation of OCPs. Finally, and most curiously, a recent case study reported in a female using a 4th generation OCP for ~11-12 years, a substantial improvement in microvascular endothelial function following cessation of OCP use (80). As a result, studies examining short- and long-term cessation of OCP use are necessary to fully understand the physiological responses to OCP use and cessation of use.

Second, Chapter 6 focused on respiratory outcomes at rest and during submaximal exercise in NAT and OCP users. While this study provided a picture of alterations in substrate oxidation from supine rest to seated rest to two stages of submaximal exercise, many studies exploring substrate oxidation in recreationally active to elite athletes have used longer-duration acute exercise interventions (e.g., 65% VO_2peak for 90 minutes) (81-84). It is plausible that NAT or OCP influence may only become apparent in longer-duration exercise interventions where small differences in substrate oxidation become measurable, as seen in work by Devries and colleagues (81). Further, it is also possible that whole-body outcomes (e.g., RER measured from breath at the mouth) does not provide sufficient sensitivity to detect alterations occurring at the skeletal muscle-level. As a result, more invasive, fuel-specific methods, such as metabolic tracers or arterio-venous blood sampling in the exercising legs, may be necessary to observe group- or phase-level differences.

Third, Chapter 7 focused on sex and gender associations with cardiovascular outcomes, reporting differences in blood pressure, arterial stiffness and endothelial function associated with biological sex (and gender identity matched in 99% of participants), but not in gender expression. A major area of future research is in exploring further gender and gender-related variables across the aging spectrum, where gender expression and gender roles may shift through life events. Similarly, a limitation of the assessment of gender has been in limitations to the methodological tools available, such as limitations to the BSRI as detailed above. New methodologies have been proposed, with many researchers developing tools to further help quantify gender constructs. Exploration of cardiovascular outcomes in gender minority populations, such

as non-binary and transgender populations, is needed alongside investigations into mechanisms underlying gender-specific cardiovascular dysfunction like gender minority stress and allostatic load.

7. Guidance for Researchers

To concluding this dissertation, and based on the findings of and experiences associated with this dissertation, the following section outlines the "top ten tips" for researchers considering NAT/OCP cycles and sex/gender considerations in human physiology research, along with lessons learned from this dissertation:

(1) *Include female participants in research studies. Period.*

Our sex-inclusion research identified a male bias in cardiovascular exercise physiology trials (1), similar to sex-inclusion findings in other fields. Increasing inclusion of females in studies will have resounding impacts on our understanding of basic female physiology, sex-differences, and clinical outcomes. Further, it is now a requirement of most funding agencies to consider both male and female participants in basic and clinical studies (85, 86).

(2) *Do I need to control for the menstrual and/or oral contraceptive pill cycle? It depends.*

Our findings from the cardiovascular, respiratory, and skeletal muscle metabolism systems showed that the NAT and OCP cycles have minimal impact on these outcomes. As a result, study design controls that select a phase of the NAT or OCP cycle to test within may not be necessary. However, given the paucity of research on females, there may be some physiological systems that are impacted or conditions in which small effects become more prominent – such as during metabolic or psychological stress. The need for controls for NAT and OCP cycle ultimately depends on the research question and outcomes, balanced with the challenges with coordinating cycle phase-specific research.

(3) *Record female-specific reproductive health data.*

Even if researchers are not "controlling" for NAT/OCP cycle phase, they should include details about reproductive health data as it is important for providing context to the population studied. In the same way that a researcher may report on the number of hours slept, eaten, or exercised before testing, reporting menstrual cycle length, the phase tested in (even if not controlled for), and standardized definitions of menstrual cycle regularity are important. More details on reporting on this data can be found in a recent guidance document by Elliott-Sale *et al* (31).

(4) *Be specific in recruitment of contraceptive users (or at least report).*

One of the major flaws of contraceptive research is the conflation of all contraceptives as similar, and thus having similar effects on physiological systems. In recruiting for human research trials, be specific in the types of contraceptive options recruited, and attempt to stratify based on contraceptive type (e.g., OCP generations, IUDs) to avoid missing out on contraceptive-specific findings. Where not possible to stratify based on contraceptive type, such as in studies with small sample sizes, examining females or sex-differences, reporting on contraceptive use and phase-specific details will help provide context of findings to the reader.

(5) *What about sex hormone measurements in females?*

Along with other guidance above, sex hormone measurements in females (and males) provides additional context to a research project and can allow for further disaggregation of "sex-differences" into hormone-level specific differences (see below on sex/gender factors). However, hormone assessments must be balanced with the time, participant burden (for blood draws), and financial cost associated with measurement. In addition, while there are clinical assays that can measure 17β-estradiol, progesterone, and testosterone, availability of reliable commercial assays for endogenous hormones is limited. Similarly, there is an absence of exogenous hormone assessment methods (e.g., ethinyl

estradiol, levonorgestrel, desogestrel); though this opens a major opportunity for research and development.

(6) *Measure and report sex and gender in human trials.*

Aligned with calls to action by research bodies, journals, researchers, and advocacy groups, measuring and reporting sex and gender in human trials is necessary. While measurement of sex is well defined in basic and clinical research, assessments of gender are still being developed; a recent breakthrough in gender methodology may be the recently published gender questionnaire by Nielsen and colleagues (2021) (76).

(7) *Explore beyond sex and gender outcomes to sex and gender related factors.*

A growing area of research is building from generally binary and dichotomous approaches to sex/gender research to thinking about factors underlying sex/gender differences, such as hormone levels, anatomical differences, psychosocial factors, gendered traits and roles, and gender equity variables. For example, instead of considering differences across biological sex, some researchers are instead stratifying based on the mechanism underlying a biological sex difference – such as enzyme levels, hormone levels, or stature – to explain the differences observed. Building from dichotomous variables (e.g., male versus female) into continuous variables (e.g., enzyme levels) contributes to a richness of data and may lead to subsequent discoveries.

(8) *Consider comprehensive assessments rather than singular measurements.*

One major strength of this dissertation was the comprehensive nature of the assessments. Considering multiple cardiovascular system specific assessments (e.g., heart rate, blood pressure, arterial stiffness, hemodynamics, endothelial function, smooth muscle function), and across two vascular beds (upper and lower limbs) allowed for a richer evaluation of the cardiovascular system responses.

(9) *Collaborate to build additional comprehensive evaluations of physiology.*
Along with #8, building in collaborations to add additional organ systems or outcomes contributes to comprehensively assessing physiology. For example, in this dissertation, we explored the respiratory and skeletal muscle metabolism systems, along with drawing connections between *in vivo* and *in vitro* cardiovascular measures using cell culture research.

(10) *No effect does not necessarily mean there is no effect in <u>all circumstances</u>.*
While this dissertation largely found little to no effect of NAT/OCP cycles on cardiovascular, respiratory, and muscle metabolism outcomes, and no effect of gender expression on cardiovascular risk factors, that does not mean there is an absence of effect in all circumstances. It is possible that under different situations, like periods of metabolic or psychological stress, or in older or clinical populations, there may be an effect. Further studies building on this work should continue to challenge these findings in other populations and under stressors.

8. Significance of the Dissertation

The findings from this dissertation add to the burgeoning literature on female vascular, respiratory, and muscle physiology, by considering the influence of NAT and OCP cycles on prevalent outcomes. Specifically, this dissertation challenges the notions provided in Chapter 2 that the NAT or OCP cycles have a strong influence on vascular (and other body system) outcomes. In contrast, research from this dissertation (Chapters 3-6) along with several collaborative additional projects identified in this discussion but not included in the thesis have identified that the NAT and OCP cycles have minimal or absent influence on the majority of outcomes examined. In addition, these findings will aid in the advancement of females in vascular physiology trials to combat the sex-exclusion identified in Chapter 2. In addition, Chapter 7 highlighted the importance of considering sex, gender identity, and gender expression into basic and clinical research, recognizing current limitations of gender methodologies available for researchers to use. Altogether, this dissertation will advance the understanding of female physiology considering NAT/OCP cycles and inclusion of sex/gender variables in cardiovascular physiology research.

References

1. Lew LA, Williams JS, Stone JC, Au AKW, Pyke KE, MacDonald MJ. Examination of Sex-Specific Participant Inclusion in Exercise Physiology Endothelial Function Research: A Systematic Review. Frontiers in sports and active living. 2022;4:860356.

2. Williams JS, Dunford EC, MacDonald MJ. Impact of the menstrual cycle on peripheral vascular function in premenopausal women: systematic review & meta-analysis. *Submitted to American Journal of Physiology - Heart and Circulatory Physiology.* 2020.

3. Williams JS, MacDonald MJ. Influence of hormonal contraceptives on peripheral vascular function and structure in premenopausal females: a review. American Journal of Physiology-Heart and Circulatory Physiology. 2021;320(1):H77-H89.

4. Hashimoto M, Akishita M, Eto M, Ishikawa M, Kozaki K, Toba K, et al. Modulation of endothelium-dependent flow-mediated dilatation of the brachial artery by sex and menstrual cycle. Circulation. 1995;92(12):3431-5.

5. Williams MR, Westerman RA, Kingwell BA, Paige J, Blombery PA, Sudhir K, et al. Variations in endothelial function and arterial compliance during the menstrual cycle. J Clin Endocrinol Metab. 2001;86(11):5389-95.

6. D'Urzo KA, King TJ, Williams JS, Silvester MD, Pyke KE. The impact of menstrual phase on brachial artery flow-mediated dilatation during handgrip exercise in healthy premenopausal women. Exp Physiol. 2018;103(2):291-302.

7. Shenouda N, Priest SE, Rizzuto VI, MacDonald MJ. Brachial artery endothelial function is stable across a menstrual and oral contraceptive pill cycle but lower in premenopausal women than in age-matched men. Am J Physiol Heart Circ Physiol. 2018;315(2):H366-H74.

8. Williams JS, Stimpson TV, Tremblay JC, Fenuta AM, Pyke KE. The influence of acute hyperglycemia on brachial artery flow-mediated dilatation in the early and late follicular phases of the menstrual cycle. Exp Physiol. 2019.

9. Liu KR, Lew LA, McGarity-Shipley EC, Byrne AC, Islam H, Fenuta AM, et al. Individual variation of follicular phase changes in endothelial function across two menstrual cycles. Exp Physiol. 2021;106(6):1389-400.

10. Rakobowchuk M, Parsloe ER, Gibbins SE, Harris E, Birch KM. Prolonged low flow reduces reactive hyperemia and augments low flow mediated constriction in the brachial artery independent of the menstrual cycle. PLoS One. 2013;8(2):e55385.

11. Thijssen DH, Black MA, Pyke KE, Padilla J, Atkinson G, Harris RA, et al. Assessment of flow-mediated dilation in humans: a methodological and physiological guideline. Am J Physiol Heart Circ Physiol. 2011;300(1):H2-12.

12. Thijssen DHJ, Bruno RM, van Mil A, Holder SM, Faita F, Greyling A, et al. Expert consensus and evidence-based recommendations for the assessment of flow-mediated dilation in humans. Eur Heart J. 2019;40(30):2534-47.

13. Harris RA, Nishiyama SK, Wray DW, Richardson RS. Ultrasound assessment of flow-mediated dilation. Hypertension. 2010;55(5):1075-85.

14. Torgrimson BN, Meendering JR, Kaplan PF, Minson CT. Endothelial function across an oral contraceptive cycle in women using levonorgestrel and ethinyl estradiol. American Journal of Physiology - Heart and Circulatory Physiology. 2007;292(6):H2874-H80.

15. Thompson AK, Przemska A, Vasilopoulou D, Newens KJ, Williams CM. Combined oral contraceptive pills containing desogestrel or drospirenone enhance large vessel and microvasculature vasodilation in healthy premenopausal women. Microcirculation. 2011;18(5):339-46.

16. Weggen JB, Hogwood AC, Decker KP, Darling AM, Chiu A, Richardson J, et al. Examining Vascular Responses to Passive Movement in Premenopausal Females: Comparisons Across Sex and Menstrual Cycle Phase. The FASEB Journal. 2022;36(S1).

17. Derella CC, Bieck MR, Blackburn M, Harris RA. Diabetes Attenuates the Increase in Estrogen-Mediated Endothelial Function. The FASEB Journal. 2020;34(S1):1-.

18. Lauren E. Eagan, Catalina A. Chesney, Sara E. Mascone, Ninette Shenouda, Ranadive. SM. Natural fluctuations in progesterone do not impact enodthelial function in healthy premenopausal women. . International Journal of Exercise SCience. 2019;9(8).

19. Khaksar A, Adler TE, Coovadia Y, Usselman CW. Effect of the Menstrual Cycle on Vasodilatory Responses to Standard and Enhanced Flow- Mediated Dilation Stimuli. The FASEB Journal. 2019;33(S1):541.10-.10.

20. Weggen JB, Hogwood AC, Decker KP, Darling AM, Chiu A, Richardson J, et al. Vascular Responses to Passive and Active Movement in Premenopausal Females: Comparisons across Sex and Menstrual Cycle Phase. Med Sci Sports Exerc. 2023;55(5):900-10.

21. D'Agata MN, Hoopes EK, Berube FR, Hirt AE, Kuczmarski AV, Ranadive SM, et al. Evidence of reduced peripheral microvascular function in young Black women across the menstrual cycle. Journal of Applied Physiology. 2021;131(6):1783-91.

22. Wenner MM, Stachenfeld NS. Point: Investigators should control for menstrual cycle phase when performing studies of vascular control that include women. Journal of Applied Physiology. 2020;129(5):1114-6.

23. Stanhewicz AE, Wong BJ. Counterpoint: Investigators should not control for menstrual cycle phase when performing studies of vascular control that include women. Journal of Applied Physiology.0(0):null.

24. Giersch GEW, Charkoudian N, Pereira T, Edgell H, Freeberg KA, Craighead DH, et al. Commentaries on Point:Counterpoint: Investigators should/should not control for menstrual cycle phase when performing studies of vascular control. J Appl Physiol (1985). 2020;129(5):1122-35.

25. Wenner MM, Stachenfeld NS. Rebuttal from Wenner. Journal of Applied Physiology.0(0):null.

26. Richardson S. Sex Contextualism. Philosophy, Theory, and Practice in Biology. 2022;14(2).

27. Priest SE, Shenouda N, MacDonald MJ. Effect of sex, menstrual cycle phase, and monophasic oral contraceptive pill use on local and central arterial stiffness in young adults. Am J Physiol Heart Circ Physiol. 2018;315(2):H357-h65.

28. Meendering JR, Torgrimson BN, Miller NP, Kaplan PF, Minson CT. Ethinyl estradiol-to-desogestrel ratio impacts endothelial function in young women. Contraception. 2009;79(1):41-9.

29. Soumpasis I, Grace B, Johnson S. Real-life insights on menstrual cycles and ovulation using big data. Human Reproduction Open. 2020;2020(2).

30. Sims ST, Heather AK. Myths and Methodologies: Reducing scientific design ambiguity in studies comparing sexes and/or menstrual cycle phases. Exp Physiol. 2018;103(10):1309-17.

31. Elliott-Sale KJ, Minahan CL, de Jonge X, Ackerman KE, Sipilä S, Constantini NW, et al. Methodological Considerations for Studies in Sport and Exercise Science with Women as Participants: A Working Guide for Standards of Practice for Research on Women. Sports Med. 2021;51(5):843-61.

32. Gavin KM, Seals DR, Silver AE, Moreau KL. Vascular endothelial estrogen receptor alpha is modulated by estrogen status and related to endothelial function and endothelial nitric oxide synthase in healthy women. J Clin Endocrinol Metab. 2009;94(9):3513-20.

33. Jayaraman A, Pike CJ. Progesterone attenuates oestrogen neuroprotection via downregulation of oestrogen receptor expression in cultured neurones. J Neuroendocrinol. 2009;21(1):77-81.

34. Cox MW, Fu W, Chai H, Paladugu R, Lin PH, Lumsden AB, et al. Effects of progesterone and estrogen on endothelial dysfunction in porcine coronary arteries. The Journal of surgical research. 2005;124(1):104-11.

35. Wassmann K, Wassmann S, Nickenig G. Progesterone Antagonizes the Vasoprotective Effect of Estrogen on Antioxidant Enzyme Expression and Function. Circulation Research. 2005;97(10):1046-54.

36. Luca MC, Liuni A, Harvey P, Mak S, Parker JD. Effects of estradiol on measurements of conduit artery endothelial function after ischemia and reperfusion in premenopausal women. Can J Physiol Pharmacol. 2016;94(12):1304-8.

37. Restaino RM, Cradock K, Barlow MA. Effects of the Follicular Menstrual Phase on Forearm Vascular Conductance in Abdominal Obese Premenopausal Women During Graded Handgrip Exercise. Artery Research. 2022;28(2):79-88.

38. Harris RA, Tedjasaputra V, Zhao J, Richardson RS. Premenopausal women exhibit an inherent protection of endothelial function following a high-fat meal. Reprod Sci. 2012;19(2):221-8.

39. Mattu AT, Iannetta D, MacInnis MJ, Doyle-Baker PK, Murias JM. Menstrual and oral contraceptive cycle phases do not affect submaximal and maximal exercise responses. Scandinavian Journal of Medicine & Science in Sports. 2020;30(3):472-84.

40. Hackney AC, McCracken-Compton MA, Ainsworth B. Substrate Responses to Submaximal Exercise in the Midfollicular and Midluteal Phases of the Menstrual Cycle. International Journal of Sport Nutrition. 1994;4(3):299-308.

41. Casazza GA, Jacobs KA, Suh SH, Miller BF, Horning MA, Brooks GA. Menstrual cycle phase and oral contraceptive effects on triglyceride mobilization during exercise. J Appl Physiol (1985). 2004;97(1):302-9.

42. Horton TJ, Grunwald GK, Lavely J, Donahoo WT. Glucose kinetics differ between women and men, during and after exercise. Journal of Applied Physiology. 2006;100(6):1883-94.

43. Redman LM, Scroop GC, Westlander G, Norman RJ. Effect of a synthetic progestin on the exercise status of sedentary young women. J Clin Endocrinol Metab. 2005;90(7):3830-7.

44. Isacco L, Duche P, Boisseau N. Influence of Hormonal Status on Substrate Utilization at Rest and during Exercise in the Female Population. Sports Medicine. 2012;42(4):327-42.

45. Horton TJ, Miller EK, Glueck D, Tench K. No effect of menstrual cycle phase on glucose kinetics and fuel oxidation during moderate-intensity exercise. American journal of physiology Endocrinology and metabolism. 2002;282(4):E752-E62.

46. Stacy TS, Laura W, Emily RC. Patterns of endogenous and exogenous ovarian hormone modulation on recovery metrics across the menstrual cycle. BMJ Open Sport &amp; Exercise Medicine. 2021;7(3):e001047.

47. Schmalenberger KM, Eisenlohr-Moul TA, Würth L, Schneider E, Thayer JF, Ditzen B, et al. A Systematic Review and Meta-Analysis of Within-Person Changes in Cardiac Vagal Activity across the Menstrual Cycle: Implications for Female Health and Future Studies. Journal of Clinical Medicine. 2019;8(11):1946.

48. Stadler A, Weidlinger S, Stute P. Impact of endogenous and exogenous progesterone exposure on stress biomarkers: a systematic review. Climacteric. 2019;22(5):435-41.

49. Carter JR, Lawrence JE. Effects of the menstrual cycle on sympathetic neural responses to mental stress in humans. J Physiol. 2007;585(Pt 2):635-41.

50. Smiley KO, Lipshutz SE, Kimmitt AA, DeVries MS, Cain KE, George EM, et al. Beyond a biased binary: A perspective on the misconceptions, challenges, and implications of studying females in avian behavioral endocrinology. Frontiers in Physiology. 2022;13.

51. Hutchins KP, Borg DN, Bach AJE, Bon JJ, Minett GM, Stewart IB. Female (Under) Representation in Exercise Thermoregulation Research. Sports Medicine - Open. 2021;7(1):43.

52. Devries MC, Jakobi JM. Importance of considering sex and gender in exercise and nutrition research. Applied Physiology, Nutrition, and Metabolism. 2021;46(6):iii-vii.

53. Counts BR, Rossow LM, Mattocks KT, Mouser JG, Jessee MB, Buckner SL, et al. Let's talk about sex: where are the young females in blood flow restriction research? Clinical Physiology and Functional Imaging. 2018;38(1):1-3.

54. Lea Merone KT, Darren Russell, and Cate Nagle. Mind the Gap: Reporting and Analysis of Sex and Gender in Health Research in Australia, a Cross-Sectional Study. Women's Health Reports. 2022;3(1):759-67.

55. Costello JT, Bieuzen F, Bleakley CM. Where are all the female participants in Sports and Exercise Medicine research? European Journal of Sport Science. 2014;14(8):847-51.

56. Cowley ES, Olenick AA, McNulty KL, Ross EZ. "Invisible Sportswomen": The Sex Data Gap in Sport and Exercise Science Research. Women in Sport and Physical Activity Journal. 2021;29(2):146-51.

57. Patel R, Kemp CL, Hafejee M, Peckham N, Jain V, McCann GP, et al. The Underrepresentation of Females in Studies Assessing the Impact of High-Dose Exercise on Cardiovascular Outcomes: a Scoping Review. Sports Medicine - Open. 2021;7(1):30.

58. Nuzzo J. Volunteer Bias and Female Participation in Exercise and Sports Science Research. Quest. 2021;73(1):82-101.

59. Nuzzo JL, Deaner RO. Men and women differ in their interest and willingness to participate in exercise and sports science research. Scandinavian Journal of Medicine & Science in Sports. 2023;n/a(n/a).

60. Laxdal A. The sex gap in sports and exercise medicine research: who does research on females? Scientometrics. 2023.

61. Williams JS, Stone JC, Ritz SA, MacDonald MJ. Letter to the editor: Laxdal (2023) "The sex gap in sports and exercise medicine research: who does research on females?". Scientometrics. 2023;128(7):4155-60.

62. Martínez-Rosales E, Hernández-Martínez A, Sola-Rodríguez S, Esteban-Cornejo I, Soriano-Maldonado A. Representation of women in sport sciences research, publications, and editorial leadership positions: are we moving forward? Journal of science and medicine in sport. 2021;24(11):1093-7.

63. Clayton JA, Tannenbaum C. Reporting Sex, Gender, or Both in Clinical Research? Jama. 2016;316(18):1863-4.

64. Pacheco C, Mullen K-A, Coutinho T, Jaffer S, Parry M, Van Spall HGC, et al. The Canadian Women's Heart Health Alliance Atlas on the Epidemiology, Diagnosis, and Management of Cardiovascular Disease in Women; Chapter 5: Sex- and Gender-Unique Manifestations of Cardiovascular Disease. CJC Open. 2022;4(3):243-62.

65. Bhargava A, Arnold AP, Bangasser DA, Denton KM, Gupta A, Hilliard Krause LM, et al. Considering Sex as a Biological Variable in Basic and Clinical Studies: An Endocrine Society Scientific Statement. Endocrine Reviews. 2021;42(3):219-58.

66. Greaves L, Ritz SA. Sex, Gender and Health: Mapping the Landscape of Research and Policy. Int J Environ Res Public Health. 2022;19(5).

67. Norris CM, Yip CYY, Nerenberg KA, Clavel MA, Pacheco C, Foulds HJA, et al. State of the Science in Women's Cardiovascular Disease: A Canadian Perspective on the Influence of Sex and Gender. Journal of the American Heart Association. 2020;9(4):e015634.

68. Lindsey ML, Carter JR, Ripplinger CM, Kassiri Z, Hansell Keehan K, Brunt KR, et al. Sex still matters in cardiovascular research. American Journal of Physiology-Heart and Circulatory Physiology. 2022;324(1):H79-H81.

69. Lindsey ML, LeBlanc AJ, Ripplinger CM, Carter JR, Kirk JA, Hansell Keehan K, et al. Reinforcing rigor and reproducibility expectations for use of sex and gender in cardiovascular research. American Journal of Physiology-Heart and Circulatory Physiology. 2021;321(5):H819-H24.

70. Pelletier R, Ditto B, Pilote L. A composite measure of gender and its association with risk factors in patients with premature acute coronary syndrome. Psychosom Med. 2015;77(5):517-26.

71. Bem SL. Bem sex role inventory. Journal of personality and social psychology. 1981.

72. Auster CJ, Ohm SC. Masculinity and Femininity in Contemporary American Society: A Reevaluation Using the Bem Sex-Role Inventory. Sex Roles. 2000;43(7):499-528.

73. Hansson RO, Chernovetz ME, Jones WH. Maternal employment and androgyny. Psychology of Women Quarterly. 1977;2:76-8.

74. Kerr P, Barbosa Da Torre M, Giguère C, Lupien SJ, Juster RP. Occupational gender roles in relation to workplace stress, allostatic load, and mental health of psychiatric hospital workers. J Psychosom Res. 2021;142:110352.

75. Donnelly K, Twenge JM. Masculine and Feminine Traits on the Bem Sex-Role Inventory, 1993–2012: a Cross-Temporal Meta-Analysis. Sex Roles. 2017;76(9):556-65.

76. Nielsen MW, Stefanick ML, Peragine D, Neilands TB, Ioannidis JPA, Pilote L, et al. Gender-related variables for health research. Biol Sex Differ. 2021;12(1):23.

77. Pelletier R, Khan NA, Cox J, Daskalopoulou SS, Eisenberg MJ, Bacon SL, et al. Sex Versus Gender-Related Characteristics: Which Predicts Outcome After Acute Coronary Syndrome in the Young? Journal of the American College of Cardiology. 2016;67(2):127-35.

78. Junker A, Juster R-P, Picard M. Integrating sex and gender in mitochondrial science. Current Opinion in Physiology. 2022;26:100536.

79. Lalande S, Hemingway HW, Jarrard CP, Moore AM, Olivencia-Yurvati AH, Richey RE, et al. Influence of ischemia-reperfusion injury on endothelial function in men and women with similar serum estradiol concentrations. American Journal of Physiology-Regulatory, Integrative and Comparative Physiology. 2021;321(2):R273-R8.

80. Turner CG, Stanhewicz AE, Nielsen KE, Wong BJ. Microvascular endothelial function following cessation of long-term oral contraceptive pill use: A case report. Exp Physiol. 2023;108(1):5-11.

81. Devries MC, Hamadeh MJ, Phillips SM, Tarnopolsky MA. Menstrual cycle phase and sex influence muscle glycogen utilization and glucose turnover during moderate-intensity endurance exercise. Am J Physiol Regul Integr Comp Physiol. 2006;291(4):R1120-8.

82. Tarnopolsky LJ, MacDougall JD, Atkinson SA, Tarnopolsky MA, Sutton JR. Gender differences in substrate for endurance exercise. J Appl Physiol (1985). 1990;68(1):302-8.

83. Phillips SM, Atkinson SA, Tarnopolsky MA, Macdougall JD. Gender differences in leucine kinetics and nitrogen-balance in endurance athletes. Journal of Applied Physiology. 1993;75(5):2134-41.

84. Carter SL, Rennie C, Tarnopolsky MA. Substrate utilization during endurance exercise in men and women after endurance training. American journal of physiology Endocrinology and metabolism. 2001;280(6):E898-907.

85. Haverfield J, Tannenbaum C. A 10-year longitudinal evaluation of science policy interventions to promote sex and gender in health research. Health Research Policy and Systems. 2021;19(1):94.

86. Stranges TN, Namchuk AB, Splinter TFL, Moore KN, Galea LAM. Are we moving the dial? Canadian health research funding trends for women's health, 2S/LGBTQ + health, sex, or gender considerations. Biology of Sex Differences. 2023;14(1):40.