Curtin School of Allied Health

Examining Female Representation in Vascular Physiology: Exercise and hormone effects on arterial morphology and function in females

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This thesis is presented for the Degree of

Master of Research (Biomedical Science)

of

Curtin University

October 2023

Author Declaration

To the best of my knowledge and belief this thesis contains no material previously published by any other person except where due acknowledgement has been made.

This thesis contains no material which has been accepted for the award of another degree or diploma at any university.

The research presented and reported in this thesis was conducted in accordance with the National Health and Medical Research Council National Statement on Ethical Conduct in Human Research (2007) - updated 2018. The proposed research study received human research ethics approval from the Curtin University Human Research Ethics Committee (ECOO262).

Approval Number HREC2021-0245.

Signed by the candidate: Sarah Thompson

Date: 04/10/2023

Acknowledgement of Country

I acknowledge that Curtin University works across hundreds of traditional lands and custodial groups in Australia, and with First Nations people around the globe. I wish to pay my deepest respects to their ancestors and members of their communities, past, present, and to their emerging leaders. My passion and commitment to work with all Australians and peoples from across the world, including our First Nations peoples are at the core of the work I do, reflective of my institutions' values and commitment to the role as leaders in the Reconciliation space in Australia.

Acknowledgements

Thank you to my amazing supervisors. I would not have been able to complete this thesis without your help and support both academically and personally. I am forever grateful for everything you have done for me over the past few years. Thank you to my parents, friends, and partner for being my emotional support outside of university. I would not have been able to get to this point without your constant love and support.

Statement of Contribution

This thesis contains works in preparation for publication, all of which have been co-authored. Using the CRediT (Contributor Roles Taxonomy) author statement, the candidate's individual contribution is recognised and described for each of the following chapters:

Chapter 2: Review of the Literature

Term	**Candidate Individual Contribution**
Conceptualisation	Formulation of systematic review research question, aims and hypotheses
Methodology	Development of systematic review methodology including search strategy; pre-registration with Open Science Framework
Formal analysis	Synthesis and analysis of study data
Investigation	Database searching; data screening; data extraction
Resources	PRISMA-P development; data extraction template creation
Writing - Original Draft	Preparation, creation and presentation of the work, specifically writing the initial draft.
Writing - Review & Editing	Preparation, creation and presentation of the work, specifically critical review, commentary or revision
Visualisation	Preparation, creation and presentation of the work, specifically visualisation/ data presentation
Project administration	Management and coordination responsibility for the research activity planning and execution

Chapter 3: Experimental Study

Term	**Candidate Individual Contribution**
Conceptualisation	Formulation of aims and hypothesis
Methodology	Development of experimental study methodology including creation of participant information sheets and online consent forms
Formal analysis	Data processing of arterial scans obtained from data collection
Investigation	Data collection across two testing sessions
Resources	Recruitment of participants and use of data collection ultrasound technology
Writing - Original Draft	Preparation, creation and presentation of the work, specifically writing the initial draft.
Writing - Review & Editing	Preparation, creation and/or presentation of the work, specifically critical review, commentary or revision
Visualisation	Preparation, creation and presentation of the work, specifically visualisation/data presentation
Project administration	Management and coordination responsibility for planning and execution of the experimental study including scheduling of data collection sessions

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Thesis Abstract

Several areas of exercise physiology have primarily studied males, with female populations largely excluded based on the potential influence of ovarian hormones on physiological outcomes. In vascular physiology, circulating oestrogen in premenopausal females exerts cardioprotective effects. Exercise is known to independently affect conduit artery diameter and function and is often influenced by exercise modality. Specifically, chronic endurance-based exercise results in larger artery diameter when compared to resistance-based exercise, with these findings often localised to the exercising limbs. Paradoxically, artery function measured using the flow-mediated dilation (FMD) technique is not improved with chronic exercise, a consequence of the associated structural enlargement. However, most studies have been conducted in male athletic participants, hence the synergistic effects of ovarian hormones and exercise on arterial size and function has yet to be fully appreciated in the female athlete.

This thesis presents findings from two discrete studies: a systematic review and experimental study. The systematic review included studies assessing exercise and arterial function using FMD in healthy (non-clinical) adults. An audit framework of best-practice methodologies for studying female populations was then applied to quantify female representation in the literature and assess methodological quality. Of the 212 studies included for review, 53% included at least one female participant, however only 38% of all participants studied were female. Less than 5% of studies implemented best-practice methodologies assessing menstrual status which was limited to menopausal and pregnant female cohorts. These findings highlight the need for not only greater quantities of vascular studies in female participants, but also improved methodological quality to appreciate the potential impacts of ovarian hormones on key outcome measures. The experimental study included three distinct female athlete groups (powerlifters, cyclists, and runners) and measured brachial and femoral artery diameter and FMD at two different hormone phases, specifically low and high hormone. Cyclists and

runners had the highest $\dot{V}O_2$max while powerlifters had the largest upper body lean mass. Brachial and femoral artery function did not differ between the groups or phases. Femoral artery size was greatest in the endurance-based groups, regardless of hormone phase, suggesting a localised effect of exercise on artery adaptation. Brachial artery diameter in powerlifting athletes was larger in the high hormone phase, however only during assessment of vasodilatory capacity. This suggests vascular reactivity may be enhanced when ovarian hormone concentration is high, however further evaluation is required to confirm these findings. The study objectively measured ovarian hormones, which differed between measurement timepoints, along with a sound methodological approach in females. Findings from this experimental study suggest arterial parameters of female athletes may be more responsive to chronic training rather than hormone effects per se, however this warrants further exploration. Overall, this thesis has identified that high-quality data pertaining to vascular responses to exercise in female-specific cohorts, which adequately account for menstrual status, are lacking. Arterial function is consistent between different female athlete groups, while lower-limb endurance exercise appears to be a potent stimulus for morphological adaptation.

List of Abbreviations

A/G – android-gynoid percent fat ratio

BM – body mass

BMD – bone mineral density

BMI – body mass index

BSA – body surface area

DBP – diastolic blood pressure

DBS – dried blood spot

eNOS – endothelial nitric oxide synthase

E2 - oestradiol

FAindex – free androgen index

FMD% – flow mediated dilation

HC – hormonal contraception

HighH – high hormone phase

HRT – hormone replacement therapy

LARC – long-acting reversible contraception

LH – luteinising hormone

LowH – low hormone phase

MAP – mean arterial pressure

MC – menstrual cycle

mOCP – monophasic oral contraception

MVC – maximal voluntary contraction

NO – nitric oxide

OCP – oral contraception

PRISMA P - Preferred Reporting Items for Systematic Reviews and Meta-Analyses

PWR – power-to-weight ratio

P4 - progesterone

RHR – resting heart rate

SBP – systolic blood pressure

SHBG – sex hormone binding globulin

T - testosterone

VC% - vasodilatory capacity

$\dot{V}O_2max$ – maximal oxygen consumption

$\dot{V}O_2peak$ – peak oxygen consumption

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Review of the Literature

Experimental Study

Chapter 1: General Introduction

Most of what we understand in exercise and sports physiology has been derived primarily from research conducted on the 'typical' male participant described as Caucasian, 70 kg, otherwise-healthy and university-aged (~18 - 22 years) (Huxley et al., 2007). Despite progress towards achieving gender-parity to address this underrepresentation, comparatively less is known about females, particularly regarding the potential influence of ovarian hormones on human body systems, and the interaction with exercise-induced adaptation in premenopausal individuals (Costello et al., 2014; Bruinvels et al., 2017; Cowley et al., 2021). Understanding females and the potential impact hormones may have on cardiovascular health is vitally important with cardiovascular disease being the leading cause of death in female populations, with 29% of Australian females dying from cardiovascular disease in 2016 (Australian Institute of Health and Welfare, 2019; Vogel et al., 2021). In the field of vascular physiology, the available literature on exercise or physical activity and artery function favours male participants, a notable sex-bias that has remained unchanged over the past two decades (Lew et al., 2022).

Due to an abundance of oestrogen receptors found on endothelial tissue, circulating oestrogen increases endothelium-mediated vasodilation through regulation of the nitric oxide (NO) pathway, thereby assisting with the mitigation of vascular injury and atherosclerotic plaque development (Chambliss & Shaul, 2002; Gavin et al., 2009; Shenouda et al., 2018; Novella et al., 2019). There are suggestions that oestrogen prevents decline in vascular function, which is evident in postmenopausal females with an absence of oestrogen (Chambliss & Shaul, 2002). The risk of cardiovascular disease vastly increases in females aged > 55 years as this age is commonly associated with menopause and an absence of exogenous hormones (Prabakaran et al., 2021). Although mixed results have been produced regarding the magnitude of this effect on naturally menstruating reproductive-aged females (Adkisson et al., 2010; Shenouda et al., 2018). There is also a common observation that sex hormone binding globulin (SHBG) concentrations will be higher in oral contraceptive users (OCP-users) compared to naturally menstruating females (Glintborg et al., 2014). This

hormone assists in the control of sex hormone concentrations in the body (Glintborg et al. 2014). As OCP-use indicates a suppression of endogenous hormones, it is not uncommon for OCP-users to have higher levels of SHBG (Glintborg et al. 2014).

Regular exercise exerts independent effects on the endothelium, resulting in increased NO production and bioavailability that improves endothelial function (Green et al., 2017) with the mode, type and duration of exercise having an impact on both arterial structure and function (Churchill et al., 2020). While animal studies report enhanced cardiovascular function in exercising females, the purported synergistic effects of exercise and oestrogen on the endothelium in female humans remain unexplored (Konhilas et al., 2004; Rogers & Sherriff, 2014). It is crucial to understand the impact of ovarian hormones and exercise on the health and athletic performance in athletic and non-athletic female populations. This is due to the chronic underrepresentation of females in vascular literature (Lew et al., 2022). In humans, the available evidence supporting endocrine hormone (oestrogen and progesterone) impacts on artery function is conflicting (Adkisson et al., 2010; D'Urzo et al., 2018; Shenouda et al., 2018). A recent systematic review and meta-analysis in premenopausal, naturally menstruating females explored the impact of menstrual cycle phase on endothelial vascular function in 29 eligible studies (Williams et al., 2020). Authors reported a moderate effect size and "very low" certainty of evidence to support improved macrovascular function during the late follicular phase, consistent with the pre-ovulatory rise in endogenous oestrogen (oestradiol) (Williams et al., 2020). However, the review acknowledged that substantial heterogeneity observed between studies could be explained, at least partially, by inconsistency in endothelial measurement methods, menstrual cycle assessment methods or both. As such, studies employing best-practice methodological approaches for assessing endothelial function when researching females should be prioritised. It should be recognised that female athletes are a specific population with their own physiology affected by various factors (Elliot-Sale et al. 2021). In particular, ovarian hormones have different impacts on their physiology which has

implications for training and performance not just their vasculature (Elliott-Sale et al. 2021). For example, athletes may be impacted by low energy availability which may lead to REDs as well as other menstrual cycle irregularities such as anovulation or luteal phase dysfunction which may impact vascular structure and function (Elliott-Sale et al. 2021).

Flow-mediated dilation (FMD) is a non-invasive imaging technique which uses high-resolution ultrasonography to quantify endothelial function in humans (Harris et al., 2010; Thijssen et al., 2019). The technique measures the dilatory response of a conduit artery, usually the brachial artery, to a reactive hyperaemic stimulus following a period of occlusion using a pneumatic cuff. Flow-mediated dilation represents an endothelial-dependent, NO-mediated response to increase shear stress and is typically expressed as a percentage change from baseline, or pre-occlusion artery diameter (FMD%) (Thijssen et al., 2019). First introduced in the early 1990's, technological advancements in imaging and computing have contributed to the popularity of FMD as a research tool to assess the impacts of physiological interventions with independent prognostic value for predicting cardiovascular disease events (Ras et al., 2013; Holder et al., 2021). Considerable variability exists between laboratory measurement (e.g., timing, cuff placement and occlusion pressure) and analysis procedures (e.g., automated software with continuous edge-detection versus manual calliper measurement) which can limit the reproducibility of results and complicate the interpretations of findings (Thijssen et al., 2019; Williams et al., 2020). International expert-consensus evidence-based guidelines were developed by Thijssen et al. (2019) to address these methodological limitations. In addition to technical guidelines, the authors recognise the importance of practical considerations pertaining to participant preparation for valid and reproducible assessment. Recommendations for assessing premenopausal, naturally menstruating females include assessment during the early follicular phase of the menstrual cycle, when ovarian hormone concentrations are typically at their lowest (Thijssen et al., 2019). While this approach allows for standardisation, it negates the ability to discern whether, and to what extent, endothelial function

is impacted by the fluctuation in hormones over a menstrual cycle, the merits of which have been the subject of much debate (Stanhewicz & Wong, 2020; Wenner & Stachenfeld, 2020). Excluding females from studies assessing endothelial function purely based on the potential influence of sex hormones (Naylor et al., 2021) not only perpetuates the 'male norm' (Hay et al., 2019; Boidin et al., 2021) but is also in breach of national ethics statements and research policies (NHMRC, 2020; Lew et al., 2022). Better representation of females in research could profoundly impact the vascular health of this population, to address sex inequalities and enable optimal prevention, management, and treatment of cardiovascular disease.

From previous vascular studies conducted in athletic male populations, it was determined that sporting modality has an influence on the structure and function of conduit arteries (Churchill, 2020). Different types of exercise are suggested to induce changes in the vasculature and can be classified as static or dynamic according to the sports classification continuum created by Mitchell and colleagues (1994). This continuum classifies sports according to their static and dynamic exercise components. Static exercise is determined by the percentage of maximal voluntary contraction (MVC). Sports which require a large percentage of MVC (> 50%) are classified as high static, including weightlifting and powerlifting (Mitchell et al. 1994). Dynamic exercise can be described in terms of the percentage of maximal oxygen uptake ($\dot{V}O_2max$), with sports producing a higher $\dot{V}O_2max$ described as high dynamic including long-distance running (Mitchell et al., 2005). Previous studies in male cohorts discovered that resistance-based (static) exercise produces little to no change in arterial diameter or function but an increase in artery wall thickness (Green et al., 2017). The mechanism which stimulates this adaptation is an increased afterload (the intra-arterial resistance when blood is ejected from the heart in each beat) (Spence et al., 2013; Green et al., 2017). An increase in afterload increases blood pressure at the onset and throughout exercise causing differences in cell growth rate of the endothelium thus impacting arterial wall thickness by causing it to increase (Spence et al., 2013; Green et al., 2017).. In male athletes, high dynamic exercise has resulted in increases in arterial

size but no change in arterial function suggesting a paradox (the athlete's artery) in which function increases prior to structural changes (Tinken et al., 2008; Churchill, 2020). The mechanism responsible for an increase in arterial diameter is repeated shear stress which at the onset of exercise causes the increased release of NO to stimulate acute vasodilation thus promoting an increase in arterial diameter (Green et al., 2017). Exercise which produces high amounts of force over a long period of time are described as high static high dynamic and include sports such as rowing, triathlon and cycling (Mitchell et al., 2005).

There is a suggestion that in female athletes, arterial structure and function increases in a similar fashion to male athletes in magnitude and direction (Moe et al., 2005). There is limited literature available relating to female athletes and the effects of exercise mode on arterial structure and function. The available literature concludes there is no difference in brachial artery endothelial function in both endurance-based athletes and sedentary controls (Moe et al., 2005). Yoshida and colleagues (2006) assessed arterial structure and function in female volleyball players in comparison to sedentary controls and found athletes to have had a lower endothelial function compared to controls. However, in this study athletes were amenorrhoeic which impacts the transference of these findings to non-amenorrhoeic athletes (Yoshida et al., 2006). Previous literature related to vascular structure and function in athletes is largely conducted in an ageing cohort, typically postmenopausal populations (DeVan & Seals, 2012). This review highlighted the limited available information in females compared to males, however noted that brachial artery endothelial function (measured by FMD) is enhanced in some but not all female endurance master's athletes compared to age-matched controls and acknowledges the response may be activity-dependent (DeVan & Seals, 2012). There is a tendency for studies in female athletes, and the wider female population, to be conducted in postmenopausal females to mitigate the potential effects on outcome variables of the cyclical changes in ovarian hormone availability across the menstrual cycle (Costello et al., 2014; Lew et al., 2022). As such, studies in premenopausal females should also be included

to appreciate the potential impacts of menstrual status, chronic exercise, and vascular responses. Throughout this thesis gendered terms such as ‘men’ and ‘women’ have not been used as I understand that not all people who identify as women were assigned female at birth (Lew et al. 2022). The biological definition of female for this thesis has been used with the assumption that these participants are born with two X chromosomes (XX) and have reproductive and endocrine characteristics associated with the female sex.

Thesis Aims and Hypothesis

Vascular studies in athletic populations have predominantly been conducted in male cohorts, with available female athlete data being limited (Churchill, 2020). It is necessary to include females, particularly athletes, and properly account for the potential effects reproductive hormones may have on the vasculature whilst also considering the impact of exercise on arterial structure and function (Turner et al., 2020). This is due to the ever-increasing prevalence of public health campaigns focussed on women and growing female participation in sport. Female athlete participation in the Olympics will reach 50% in the 2024 Summer Olympic Games compared to 35% at the 2000 games (Olympics, 2022; Cowley et al., 2023). There is a need for understanding the gaps in the current literature to assist in directing research and resources into priority areas and to appreciate the bidirectional effects of athletic performance on arterial structure, function, and overall female health. The aim of this thesis is to determine the representation of females in vascular exercise studies and audit the methodological quality of studies in females as per the framework of Smith and colleagues (2022). The secondary aim is to characterise vascular phenotype in female athletes participating in different categories of sports and to determine the effect of menstrual cycle phase on measures of endothelial function (using FMD). We hypothesise there to be underrepresentation of females compared to males in vascular studies and the quality of the methodologies used to be poor. We also hypothesise there to be a difference in endothelial function between different sporting groups and across the menstrual cycle. In the experimental study we hypothesised there would be a divergent arterial phenotype dependent on the primary exercise

mode of the athletes and that hormone phase would influence the magnitude of arterial diameter and functional adaptations particularly in the high hormone phase.

Chapter 2: Review of the Literature

This chapter is based on a systematic review prepared for submission as:

Thompson SL, Brade CJ, Naylor LH and Spence AL. (2023). Vascular adaptation to exercise: A systematic review and audit of female representation. *Manuscript in preparation.*

ABSTRACT

Representation of sex is a salient factor to appreciate vascular physiological responses to exercise. While a male bias is apparent in the literature, methodological quality of available studies in females is not yet known. This systematic review primarily aimed to describe studies that assess the impacts of exercise interventions and chronic training on endothelial function, measured using flow-mediated dilation, in otherwise healthy individuals and athletes. A standardised audit quantified the representation of female participants, with a tiered grading system applied to studies that meet best-practice recommendations for conducting physiological research in females. A total of 210 eligible studies in 5,992 participants were identified, with athletes comprising 18% of the sample. The primary exercise intervention mode and type were aerobic (49%) and acute (61%) respectively, with the brachial artery being assessed in 82% of studies. While 53% of studies (n = 112) included at least one female, female participants accounted for only 38% of the total study population but 49% of the athlete population. Majority (49%) of studies in females were conducted in premenopausal cohorts (n = 55). A third of studies (34%) did not report any menstrual status of female participants. No studies in naturally menstruating, hormonal contraceptive users or in participants experiencing menstrual irregularities met all best-practice recommendations. Very few studies (~ 5%) achieved best-practice methodological guidelines for studying females and those that did were limited to menopause and pregnant cohorts. These findings suggest that in addition to the limited quantity of female participants in studies assessing arterial responses to exercise, there remains limited high-quality evidence with acceptable methodological control of ovarian hormones. Researchers should provide sufficient evidence of menstrual status when studying females in vascular and exercise contexts, to improve the overall methodological quality of evidence and fully appreciate potential endocrine effects on vascular responses to exercise.

INTRODUCTION

While sex-specific data continues to emerge in the exercise science literature (Cowley et al., 2021), reproductive aged-females are often excluded due to the cyclical nature of ovarian hormones (Lew et al., 2022). Owing to the complexity of female physiological systems, research in this population can be logistically challenging and resource-intensive which is compounded by sex-specific barriers to study participation, leading to difficulty in recruiting female participants (Matthews et al., 2023; Nuzzo & Deaner, 2023), which often results in female exclusion altogether (Lew et al., 2022). Similarly, a potential age-bias may also be apparent when studying vascular outcomes in females, with a preference for postmenopausal cohorts due to the decline in reproductive hormones reducing endocrine variability which mitigates the effect of ovarian hormones on endothelial function (Shenouda et al., 2018; Holder et al., 2019). While the relative lower incidence of cardiovascular disease observed in premenopausal females may warrant less clinical emphasis (Holder et al., 2019).

A common method used to measure arterial function is the non-invasive FMD technique (Thijssen et al., 2019). This approach uses high-resolution ultrasonography to examine endothelial-dependent, shear-mediated change in arterial diameter following ischaemia. Current consensus guidelines indicate that assessing FMD in premenopausal females should be standardised to a phase of the menstrual cycle as ovarian hormones may influence endothelial function, especially when repeated FMD measures are obtained (Thijssen et al., 2019). Studies assessing the effectiveness of exercise interventions often employ FMD as a prognostic indicator of cardiovascular risk reduction (Green et al., 2011) however the logistical and methodological considerations of assessing reproductive-aged females during standardised menstrual cycle phase may preclude female participation in exercise-based studies.

Pathways by which oestrogen potentially affects endothelial function are complex and not yet fully understood (Novella et al., 2019). It is likely that

oestrogen impacts vasodilation via oestrogen receptors located on the endothelium that affect the NO pathway, which in turn increases vasoreactivity (Gavin et al., 2009). In terms of FMD, this mechanism would theorise that in premenopausal, otherwise healthy, young females, FMD will be enhanced when compared to males and postmenopausal populations, an observation that has been previously reported and attributed to the effects of oestrogen (Holder et al., 2019). However, appreciating the available female-specific evidence in exercise contexts is needed to enable practitioners to optimise interventions for both health and performance benefits. Previous literature in non-athletic female participants suggests a potential increase in arterial function during the late follicular phase, corresponding with the rise in oestrogen prior to ovulation (Williams et al., 2001; Adkisson et al., 2010), although meta-analytic evidence indicates a 'very low' certainty of evidence to support this (Williams et al., 2020). Few studies detail the effects of exercise on endothelial function in athletic female populations, with some studies suggesting exercise-associated amenorrhea negatively influences endothelial function (Rickenlund et al., 2005; Yoshida et al., 2005). While controlling for ovarian hormone concentration remains a topic of debate in vascular physiology, including additional methodological considerations when conducting physiological research in females is necessary (Smith et al., 2022).

With public health campaigns focussed on female participation in sport continuing to increase, understanding the current gaps in the literature can assist in directing resources and research priority areas. The aim of this review was therefore to assess studies that have evaluated the effects of exercise on endothelial function (assessed using FMD) in otherwise healthy (non-clinical) populations and athletic individuals. The available literature was then audited with an existing methodological framework (Smith et al., 2022) to determine the representation of female participants and evaluate the methodological quality of studies using a systematic approach.

METHODS

The Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA-P) guidelines were used to guide this systematic review which was pre-registered on October 27 2022 via the Open Science Framework (https://osf.io/nau27) (Page et al., 2021).

Search Strategy

The protocol followed for study selection was i) identification of potential studies, ii) duplicates eliminated, iii) titles and abstracts screened, iv) relevance to the review objective was analysed and, v) studies were explored further in full-text form. Systematic electronic searches of four key databases were undertaken from inception to November 22, 2022: Medline and Scopus resulted in 1,429 and 737 articles respectively, with Web of Science and CINHAL PLUS resulting in 3,835 and 165 articles respectively. The following search strategy was used for all databases searching title, abstract and key words for a combination of exercise OR training OR intervention OR athlete* OR sport AND endothelial AND function OR flow-mediated dilation OR vascular AND function OR FMD. Studies were limited to the English language, full-text articles and those conducted in humans. After search completion, the studies were imported to Research Screener, a web-based machine-learning program, to identify articles based on title and abstract which met the inclusion criteria for extraction (Chai et al., 2021). Two authors (ST and AS) reviewed the studies independently for eligibility with discrepancies between authors resolved using the third author (CB) resulting in consensus. We also completed backward and forward searches on September 21, 2023, via reference lists and citations respectively, of articles identified as eligible from the primary database searches. Ethical approval was not required due to the nature of the study type (i.e., systematic review) (Page et al., 2021).

Study Eligibility Criteria

Studies were included if they met the following criteria, developed using a PICO framework approach (Richardson et al., 1995). The population were otherwise healthy (non-clinical) adults (aged 18 years and older), defined as having no diagnosed clinical condition(s). Also, athletic individuals, defined using the approach from Araujo and Scharhag (2016), were included either as comparators or control participants. Studies with participants undergoing an exercise or physical activity intervention were included. Studies must assess, and report conduit artery endothelial function measured using FMD methods consistent with established guidelines (Corretti et al., 2002; Harris et al., 2010; Thijssen et al., 2011; Thijssen et al., 2019) in either the brachial, femoral and/or popliteal arteries.

Data Extraction, Quality Assessment and Audit

Data was extracted by one reviewer (ST) into a pre-prepared data extraction sheet template, modified from that proposed by Smith and colleagues (2022). Outcomes of interest included data related to a) general publication information (i.e., title, authors and year of publication) b) research theme (adaptation, ageing, haemodynamics and mechanistic considerations, nutrition intervention, sedentary behaviours, thermoregulation, vascular health), c) study design (i.e., case-control, controlled intervention, cross-sectional study, observational cohort study, pre-post intervention with no control), d) study population metrics and sample size (i.e., male only, female only, mixed cohort, male vs female design, male vs female subanalysis), e) The Participant Classification Framework (McKay et al., 2022), exercise/training intervention duration (i.e. acute, chronic, both or other) and mode (i.e., aerobic, resistance, both aerobic and resistance, other sport, or physical activity). Data was extracted relating to FMD including the protocol followed (protocols included in methods) and the conduit artery assessed (brachial, femoral, and/or popliteal artery). A subset of extracted studies was checked by a second reviewer (AS). To assess the quality of the studies and risk of bias, the NIH Study Quality Assessment Tool relevant for each study

design was used with studies rated as ‘good’, ‘fair’ or ‘poor’ quality (National Heart, Lung and Blood Institute, 2021).

The most crucial quantitative aspect of studies in females for audit methodology included the characteristics of menstrual status, specifically as menstrual cycle consideration, hormonal contraception and menstrual irregularities. If studies had insufficient information to enable robust menstrual status categorisation, these studies were labelled as unclassified. Studies with a categorisation were then graded as gold, silver, bronze or ungraded depending on the methodological quality presented in the research study (Smith et al., 2022). This audit methodology was used specifically to assess key features of the study design and standardises the quantification of athletic female participation in sport science research. In the present review, we included studies in non-athletic individuals, assessing FMD in female participants across the reproductive lifespan (reproductive-age, pregnancy, and menopause) hence the development of additional audit categories for pregnancy and menopause-related studies. Key criteria for these categories were derived from the methodological considerations for conducting exercise science research in females as proposed by Elliott-Sale and colleagues (2021). A gold grade for studies including premenopausal females was awarded if participants were eumenorrheic; had a known MC length between 21 and 35 days; evidence of an LH surge; used blood serum hormone analysis for a correct hormonal profile; no HC use for 3 months prior to recruitment; tracking of MC characteristics has occurred for at least 2 months prior to testing and outcome measures are repeated in a second cycle (Smith et al., 2022). For studies in the menopause category, gold grade could be achieved if they defined participant menopausal status and length of status e.g., postmenopausal defined as greater than 1 year of amenorrhea; status was supported by hormonal measures; and detailed information on any hormone replacement therapy (HRT) was provided. In studies categorised as pregnancy-related, studies were eligible for gold grade if detailed information regarding the stage of pregnancy (weeks and trimester), parity, gravidity and whether singleton or multiple pregnancy was provided.

Deviations from Pre-Registered Protocol

We deviated from our pre-registered PRISMA-P as follows: we did not include ProQuest Dissertations and Thesis Global database as preliminary searches found the database contained dissertations or articles in pre-print not readily available peer-reviewed journals. Further, we included studies which assessed FMD using all available methodological guidelines such as those reported by Thijssen and colleagues (2011; 2019), the Brachial Artery Reactivity Task Force (Corretti et al., 2002), Harris and colleagues (2010) and originally proposed FMD techniques by Celemajer and colleagues (1992). The rationale behind including these protocols was agreement with the most recent consensus guidelines (Thijssen et al., 2019) regarding the need to be cognisant of the menstrual cycle phase female participants are assessed, with the early follicular phase highlighted as a proposed standard phase in premenopausal female participants when FMD should be assessed to mitigate any potential influence of ovarian hormones on endothelial function.

Data Analysis

Qualitative data were extracted into the data extraction spreadsheet to capture relevant information from the selected studies (as mentioned above). Frequency based metrics for key outcomes are represented as a proportion of total studies, a proportion of a sub-set of studies with a particular outcome of interest (i.e., proportion of the total female population included) and the number of studies including various exercise intervention types, study designs and other participant features (training status, The Participant Classification Framework, menstrual status and artery bed assessed) were examined and reported. Studies that include mixed menstrual status cohorts (e.g., naturally menstruating and postmenopausal) is represented as 0.5 as there are two different menstrual statuses included in one study. All data were analysed using open-source statistical software package jamovi (The jamovi project (2002). Jamovi, Version 2.3. Retrieved from https://www.jamovi.org) with GraphPad Prism (Version 10.0.2 for Windows, GraphPad Software, Boston,

Massachusetts USA, www.graphpad.com) used for generating frequency-based figures.

RESULTS

Following the removal of duplicates, a total of 6,035 articles from four databases underwent title and abstract screening. Title and abstract screening resulted in 5,792 articles being excluded primarily due to inclusion of clinical populations and FMD not used to assess vascular function. Full-text review and backwards and forwards searching revealed a total of 210 articles to be included in the systematic review. A flowchart outlining the screening process and exclusions are presented in **Figure 1**, courtesy of a Shiny app for generating PRISMA 2020-compliant diagrams (Haddaway et al., 2022).

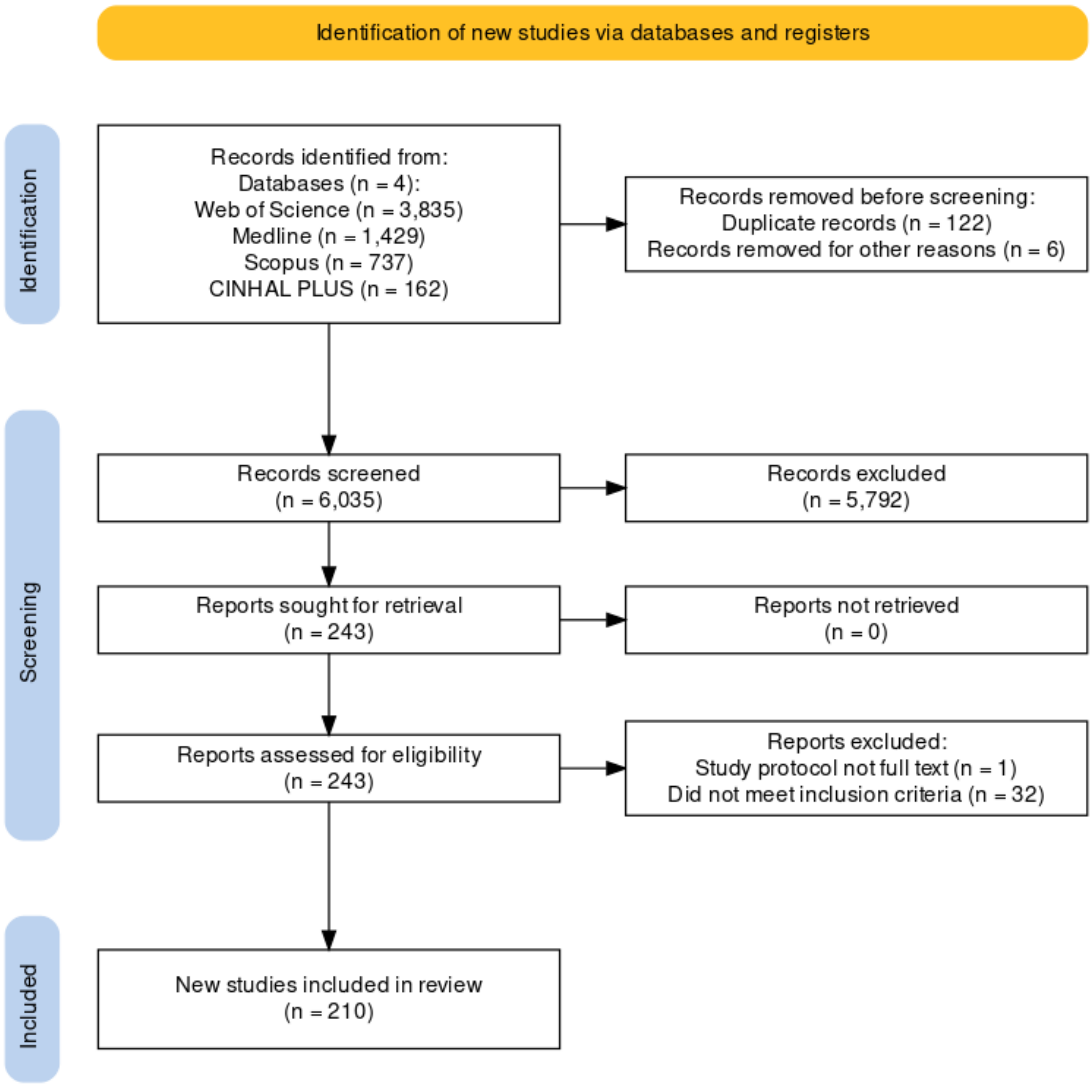

Figure 1. Systematic Review PRISMA Flow Diagram (Haddaway et al., 2022)

Study Characteristics

The total number of healthy (non-clinical) participants included in this review was 5,992 with the division between males and females in the included studies being 59% and 38% respectively. There were two studies which did not report participant sex. There were 112 studies (53%) which included at least one female participant. Studies included in this review were published between 1999 and 2023, with 42% (n = 89) of studies published in the last 5-year period. In 2003, the first study of female participants was published, with 2022 having the most studies including female participants (either as female-only, male vs female design or mixed-cohort studies, n = 11, 5%). Male vs female design is defined as studies which have been designed to investigate response to an intervention between sexes, including a statement in the aims (Smith et al.,

2022). Male vs female sub-analysis is defined as studies which include a sex-based comparison within statistical procedures (Smith et al. 2022). Detailed information relating to year of publication and sex-representation is provided in **Figure 2A**. Studies were categorised according to a research theme relevant to each study with most studies (n = 74, 35%) relating to adaptation in response to exercise. Other identified research themes included haemodynamic (n = 40, 19%) and mechanistic changes (n = 33, 15%), effects of ageing (n = 18, 8%), nutrition interventions (n = 18, 8%), sedentary behaviours (n = 11, 5%), vascular health (n = 9, 4%) and thermoregulation (n = 7, 3%).

Study Type and Quality (Risk of Bias)

Of the 210 studies, there was a single case-control study, 23% (n = 49) were controlled intervention studies, 4% (n = 8) were cross-sectional studies and 9% (n = 19) were observational studies. Majority (63%, n = 133) of studies were a pre-post intervention with no control group. In terms of quality assessment, most studies (55%) were classified as ‘fair’, with 33% classified as ‘good’ quality and the remining 12% being classified as ‘poor’ quality. Quality of each study design is represented in **Figure 2B**.

The Participant Classification Framework

Of these 5,992 participants, most participants (n = 3,830, 64%) were studied in the context of an exercise intervention with 1,297 participants acting as controls. Both athletes and non-athletes were classified according to training and athletic status using The Participant Classification Framework (McKay et al., 2022). There was a total of 4,913 non-athletic participants sampled in 139 (66%) studies. These participants were classified as either sedentary (Tier 0; n = 87, 41%) or recreationally active (Tier 1; n = 52, 24%). Of the 210 included studies, 48 included athletes with 1,079 (18%) athletic participants included. These athletes were classified as trained/developmental (Tier 2; n = 36, 17%), highly trained/national level (Tier 3; n = 9, 4%) or elite/international level (Tier 4; n = 3, 1%). There were no world class (Tier 5) athletes identified in any

studies. In 53 studies (25%), participant training status was not reported with sufficient detail and therefore remained unclassified. Athletes were involved in 23% of studies and were included as either the intervention group undergoing an intervention (n = 15, 31%) or the control group (n = 1, 2%). Of the total number of athletes, most (75%) were a comparator in relation to sedentary controls. Information related to the breakdown between male and female participants classified according to training status has been represented in **Figure 3A**.

Exercise Intervention Mode, Type and Duration

Most studies (83%, n = 176) utilised an exercise or physical activity intervention. There were 36 studies which did not include an exercise intervention, 88% of these studies included a comparison between an athletic or exercising group and sedentary or non-exercising controls. Three studies did not specify an exercise intervention per se, however included a non-exercise intervention (i.e., nutrition) in an athlete comparator and control group. Of the 176 studies which included an exercise intervention 60% (n = 105) were aerobic, 31% (n = 55) were resistance, 6% (n = 10) were a combination of resistance and aerobic while 3% (n = 6) were related to a physical activity intervention (e.g., sitting or standing). The type of exercise intervention was defined as either acute (one session with pre-post assessment of FMD) or chronic (more than one session). Majority (62%, n = 109) of studies involved an acute intervention, with 37% (n = 65) of studies including a chronic intervention with varying durations. There were two studies which included more than one study type (both acute and chronic). Of these 65 chronic studies, the duration of interventions most assessed were 12 weeks (n = 18, 28%) and 8 weeks (n = 16, 25%) spanning 5 sessions and 52 weeks. Exercise intervention mode and type is represented in **Figure 3B** with exercise intervention type and the population studied provided in **Figure 3C**.

Flow-Mediated Dilation Outcomes

The artery beds assessed included the brachial artery (n = 173, 82%), popliteal artery (n = 6, 3%) and femoral artery (n = 11, 5%). There were a smaller number of studies (n = 20) which assessed multiple artery beds in the same participant group. This included brachial and femoral artery (n = 12, 6%), brachial and popliteal artery (n = 7, 3%) and the brachial and radial artery (n = 1, > 1%). Details regarding the measurement of artery beds according to participant sex is represented in **Figure 3D**.

Menstrual Status of Participants

The 210 studies were characterised by participant groupings as female-only (n = 37, 17%), male-only (n = 97, 46%), male vs. female design features (n = 5, 2%), male vs female sub-analysis (n = 2, 1%) and mixed-cohort (n = 68, 32%). There were two studies which did not specify the sex of their participants. A total of 2,263 (38% of the total population sampled) healthy (non-clinical) female participants were sampled in this review from the 112 studies including female populations. Female participants were categorised according to their reported menstrual status. Of the 112 studies in female participants, 34% (n = 38) did not provide sufficient information to classify the menstrual status of participants. In the articles which provided sufficient information to classify menstrual status, female participants were described as either naturally menstruating (menstrual cycle), hormonal contraceptive (HC) users, experiencing menstrual irregularities, relating to menopause or pregnancy. A quarter (25%) of female studies exclusively included naturally menstruating participants with only one study conducted exclusively in HC-users.

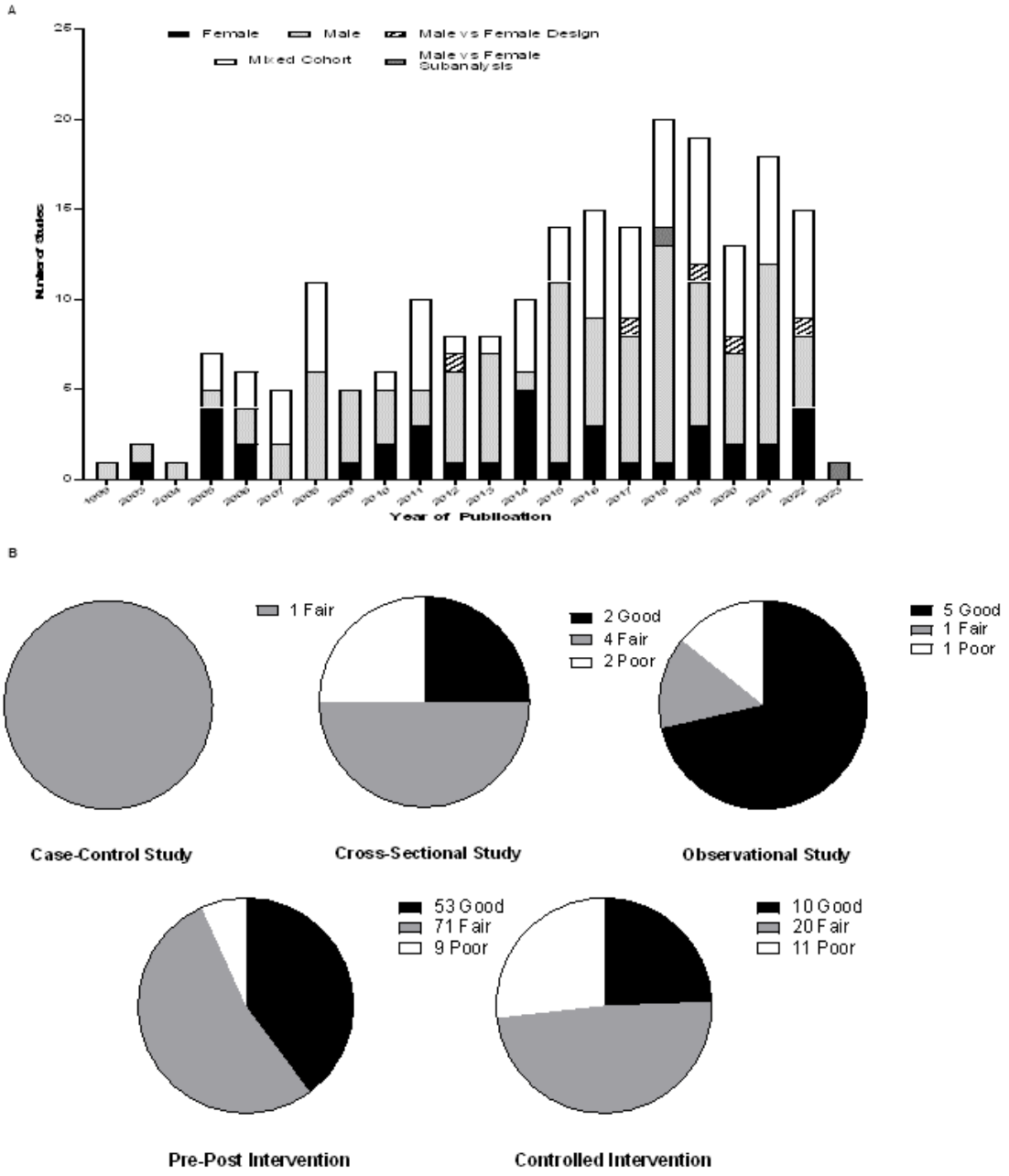

Figure 2 Study Characteristics A. Year of publication and population included in published studies. Male vs female design is defined as studies which have been designed to investigate response to an intervention between sexes, including a statement in the aims. Male vs female sub-analysis is defined as studies which include a sex-based comparison within statistical procedures (Smith et al., 2022). B. Quality of study design (risk of bias) using the relevant NIH Study Assessment Tool for each study design.

There were 14 (13%) studies assessing menopausal females and two studies conducted in pregnant cohorts. There were 27 (24%) studies which included mixed menstrual status, which was described as more than one group of female participants with different menstrual status. As each of these mixed studies included two groups of female participants with different menstrual status, each study is represented by 0.5 grading. The studies within this review with mixed menstrual status included naturally menstruating and HC-users (n = 5.5, 10%), naturally menstruating and menstrual irregularities (n = 2.5, 4%), naturally menstruating and menopausal females (n = 5, 9%) and naturally menstruating and pregnant cohorts (n = 0.5, 1%). All mixed menstrual status studies included a naturally menstruating group meaning 49% (n = 41.5) of all included female studies assessed naturally menstruating females.

Audit of Best-Practice Methodologies for Female Participants

Each of the 112 studies conducted in female participants were audited using the tiered grading system as proposed by Smith and colleagues (2021), dependent on specific criteria defining menstrual status and the best-practice recommendations used to account for menstrual status. Studies were awarded a grade of gold, silver, bronze or ungraded depending on the detail of methodological information provided (**Figure 4**). Of the total studies (n = 55, 49%) assessing naturally menstruating females (including mixed studies), no studies were graded as gold, 1.5 studied achieved silver grading, 9 studies were graded bronze and the remaining 31 studies were ungraded. In HC-users there were no gold or silver graded papers, 0.5 bronze and 6 ungraded studies. In the 5 studies which assessed female participants with menstrual cycle irregularities (amenorrhea or oligomenorrhea), there were no gold graded papers, however 1.5 papers achieved silver grade with 1 graded bronze. There were 24 studies which assessed FMD in menopause category with 3.5 studies providing sufficient detailed information to be awarded a gold grade. The remaining menopausal studies were classified as 4.5 silver, 8 bronze and 3 ungraded. There were 3 studies which assessed pregnant females with 66% (n = 2) of these studies awarded gold. The remaining study in a pregnant population was awarded bronze (n = 0.5). Regarding the methodologies,

majority (74%) of the studies in naturally menstruating participants were assessed during the early follicular phase (typically between days 1 - 8) of the menstrual cycle while the HC-users were commonly assessed when taking the inactive/placebo pill of the OCP pseudo-cycle. In the naturally menstruating participants, only 9 studies (8%) collected blood serum samples to confirm correct hormonal profile. There were 6 studies which explicitly outlined that the study did not control for the menstrual cycle, although they all mentioned menstrual status of their female participants.

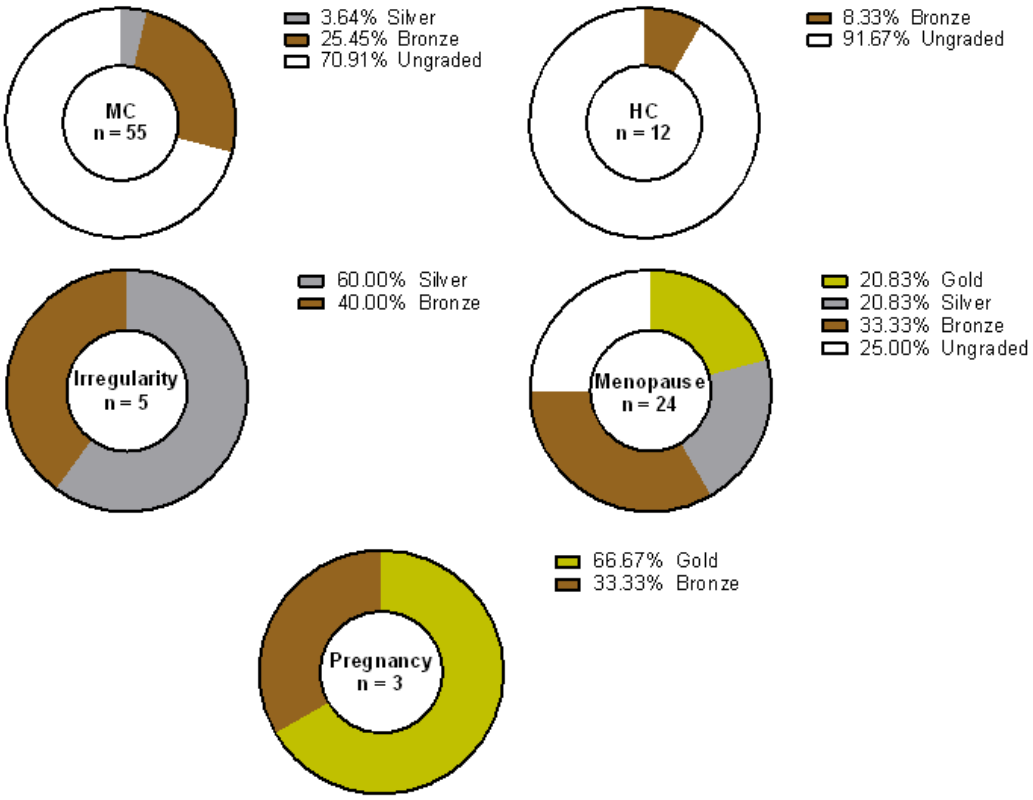

Figure 4 Grading of studies with female participants of differing menstrual status. Abbreviations: MC, menstrual cycle; HC, hormonal contraception.

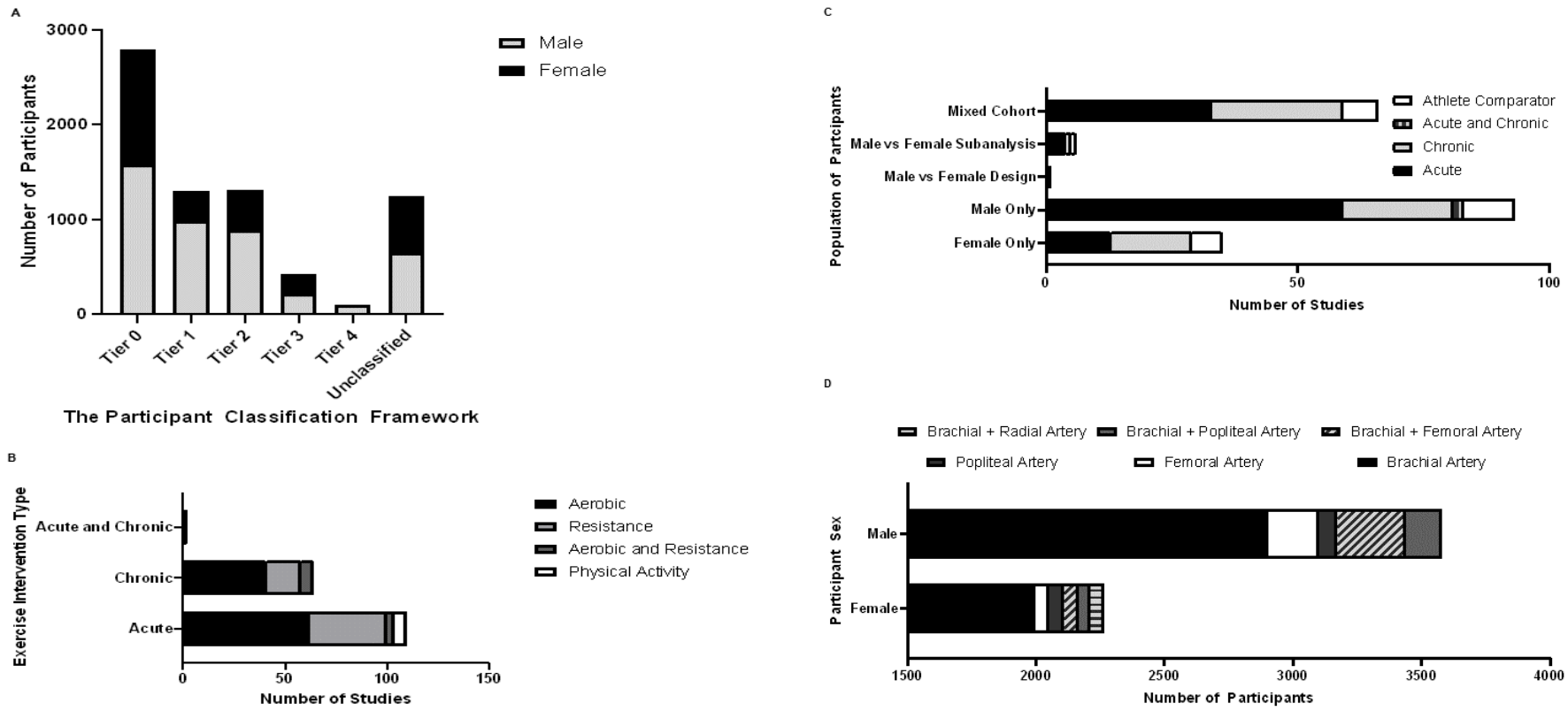

Figure 3 Studies evaluating flow-mediated dilation in response to exercise interventions by sex and participant cohort A. The Participant Classification Framework (McKay et al., 2022) according to sex: Tier 0 (sedentary), Tier 1 (recreationally active), Tier 2 (training/developmental), Tier 3 (highly trained/national), Tier 4 (elite/international), unclassified (did not provide information regarding participant training status). B. Mode and type of exercise interventions included in the review. C. Population group by exercise intervention type. D. Artery beds assessed via flow-mediated dilation (FMD) in males and females.

DISCUSSION

Main Findings

The primary purpose of this review was to systematically evaluate studies that report effects of exercise on endothelial function assessed using FMD in otherwise healthy (non-clinical) athletic and non-athletic individuals. These studies underwent an audit and systematic evaluation of the representation of female participants and the methodological quality which were graded using an available audit framework (Smith et al., 2022). Studies were published between 1999 and 2023 with six different study designs included. Majority of these study designs were deemed to be of ‘fair’ or ‘good’ quality. We found most studies assessed FMD following an acute exercise intervention in both male and female cohorts, particularly in sedentary populations. There were 48 studies which assessed athletic populations who were either involved in an intervention, as a control or a comparator to sedentary controls. The brachial artery was the primary artery for assessing FMD compared to both femoral and popliteal artery beds. Of the 112 studies which included female participants, 72 mentioned the menstrual status of their participants and specifically in which phase of the menstrual cycle FMD was assessed. No studies conducted in naturally menstruating females were classified as gold, meaning they did not meet the recommended best-practice guidelines for assessing females. The only gold gradings were awarded to studies conducted in menopause and pregnancy cohorts.

Study Characteristics

The current review differs from previous reviews in the following ways. First, in this review, the effect of exercise on endothelial function in otherwise healthy males and females, over the entirety of their reproductive lifespan (reproductive-aged, pregnancy, menopause) were represented. In comparison, previous systematic reviews have primarily focussed on endothelial function and exercise in a clinical group (e.g., Type 2 Diabetes, hypertension or heart failure) at a certain point in the lifespan (e.g., older adults) (Pearson et al., 2017; Lee et al., 2018). This resulted in more studies

and a larger participant cohort included in the current systematic review in comparison to the clinical systematic reviews which had few studies assessed (k = 16, N = 529 and k = 8, N = 306, respectively). Also, there was no mention of menstrual status of female participants included in these clinical cohort reviews, nor in literature combined with healthy (non-clinical) cohorts (Ashor et al., 2015; da Silva et al., 2022; Tao et al., 2023).

There were 210 studies included in this review with 38% of the participants sampled being female, despite the number of studies including female participants exceeding 50% of eligible studies. As such, these findings suggest that while researchers are cognisant of including females, often in response to several grant-body and journal requirements (Williams et al., 2020), there remains an underrepresentation of females in absolute participant numbers. This is in comparison to previous systematic reviews determining the representation of female participants in exercise physiology studies, as well as vascular research specifically (Costello et al., 2014; Cowley et al., 2021; Lew et al., 2022) which reported 39%, 34% and 36% respectively. Only 24% of the total female population included in this review were defined as athletes with none of these athletes being at the elite/international level (Tier 4) or world class (Tier 5; McKay et al., 2022), however, female athletes achieved near-parity (49%) with their male athletic counterparts. Together, this highlights two key points; first, there is a need for further research to include elite female athletes classified as Tier 4 and above to understand how chronic exercise at the elite level affects FMD, including during and following their athletic careers. Secondly, researchers may have prioritised assessment of vascular function in female athletes susceptible to the deleterious effects of relative energy deficiency in sport (Mountjoy et al 2023) limiting further application to otherwise healthy female athletes.

Publication range for studies within this review were from 1999 to 2023 with the inclusion of females in studies increasing from 2003 onwards. This likely represents the advancement of in-vivo imaging technology and

implementation of FMD as an outcome measure. However there remains a maximum of 6 vascular studies with female-only participants published in a single year, in comparison to an average of 10 male-only studies published each year. These results suggest a male-bias and preference for studying males in exercise vascular research, which agrees with previous literature (Lew et al., 2022). It should also be noted that the resource-intensive nature of studying reproductive-aged females is perhaps another contributing factor to the observed sex-disparity.

While the collective study quality (risk of bias) for studies including female participants were predominantly 'fair' (n = 54) and 'good' (n = 42), this was not necessarily reflected when assessing the quality of female-specific methodologies used to account for ovarian hormones. There were only 5.5 studies which received a gold grading, and these were limited to the menopause and pregnancy cohorts. Our findings contradict the NIH study quality tool used in the present review as it would be expected that 'good' quality study designs would implement equally high-quality methodologies to account for the ovarian hormones in females, however this was not the case. Future studies in females should aim to follow best-practice guidelines (Elliott-Sale et al., 2021) to ensure that the study outcomes accurately reflect the impact exercise has on female participants (Cowley et al., 2023).

Exercise Intervention Mode, Type and Duration

The primary exercise intervention type and mode utilised in 29% of studies within this review were acute aerobic interventions. The most common duration of chronic interventions was 8 weeks and 12 weeks, but this ranged from as few as 5 sessions to as long as 52 weeks. Chronic exercise, especially continuous aerobic exercise of relatively longer duration (> 12 weeks) has been shown to improve endothelial function (Tao et al., 2023). A recent meta-analysis determined that aerobic, resistance or a combination of both are also deemed to be effective strategies to improve endothelial function in adults (Shivgulam et al., 2023). Although we did not meta-analyse the results of these

studies and the effect different exercise interventions had on FMD there is suggestion that the type, duration, and intensity of exercise produces different FMD responses (Dawson et al., 2013). Acute exercise may produce an initial decrease immediately post exercise bout before returning to normal levels in healthy participants, termed the 'exercise paradox' (Dawson et al., 2013). However, in clinical cohorts (e.g., type 2 diabetes, hypertension) there is suggestion FMD is increased following exercise, thus exercise has a positive effect on endothelial dysfunction (Pearson et al., 2017; Lee et al., 2018). In terms of athletic participants there were a substantial number of studies (n = 36) which assessed athletes as a comparator to sedentary or non-exercising controls. These studies did not include an exercise intervention as the athletes were currently training. In some physiological studies comparing athletic populations to sedentary controls or other athletes is taking a within-species "comparative physiology" approach to better appreciate adaptation to exercise (Joyner et al., 2022).

Flow-Mediated Dilation methodologies

Studies included in this review assessed endothelial function using a FMD protocol consistent with previously published measurement recommendations (Celemajer et al., 1992, Corretti et al., 2002, Harris et al., 2010, Thijssen et al., 2019). Each of these protocols propose minor variations in cuff occlusion pressure, duration of imaging post cuff occlusion and position of cuff relative to conduit artery (i.e., proximal vs distal). These guidelines also mention that conducting FMD in premenopausal females should be limited to a standardised phase of the menstrual cycle to control for potential effects of ovarian hormones. For example, Harris and colleagues (2010) proposes that premenopausal cohorts are assessed during the early follicular phase (between days 1-7) to mitigate the potential influence of ovarian hormones on endothelial function. This phase is associated with the onset of menses typically making it the most easily identified menstrual cycle phase. However, a menstrual bleed is not necessarily reflective of a typical ovulatory menstrual cycle, as anovulatory cycles can produce withdrawal bleeding that is indistinguishable from menses (Habiba & Benagiano, 2023). As such, the

resulting outcome measure may not be reflective of a typical, ovulatory menstrual cycle necessitating that additional objective measures of cycle phase determination are required (i.e., cycle day tracking, serum hormone analysis to quantify hormone profile, luteinising hormone (LH) measurement for ovulation confirmation) (Schaumberg et al., 2017). Other guidelines provide minimal (Corretti et al., 2002) or no reference (Celemajer et al., 1992) to a particular menstrual phase merely recommending that researchers be aware of the potential implications.

In terms of which menstrual phase female participants' FMD was assessed, early follicular phase was most common, which aligns with recommendations for assessing FMD in premenopausal females (Thijssen et al. 2019). The four studies which assessed female participants during the luteal phase provided no clear reasoning for this decision, although it was kept consistent between participants (Weissberger et al., 2010; Pyke & Jazuli, 2011; Seager et al., 2015; Park et al., 2022). One study assessed female participants in different phases of the menstrual cycle, with a proportion of participants assessed during the follicular phase, some during menses and the remaining participants assessed during the luteal phase (King et al., 2015). However, these phases were controlled for with respect to the within-participant study design for repeated measures (King et al., 2015). Consistent reporting of menstrual phase terminology between studies should be prioritised to avoid the same menstrual cycle phase being referred to with different terms i.e., menses and early follicular phase (Elliott-Sale et al., 2021).

In the current review, the brachial artery was the primary conduit artery bed assessed in majority of the included studies, which is reflective of consensus guidelines and associated ease of assessing the upper limb. There were only a small number of studies which assessed multiple artery beds, indicating a missed opportunity to ascertain potential localised arterial adaptations to exercise depending on the mode of intervention and exercising muscle group(s) involved (Rowley et al., 2011). Several groups have provided meta-

analytic evidence suggesting exercise results in systemic adaptations in FMD in older and clinical populations (Campbell et al., 2019; Fuertes-Kenneally et al., 2023). While we did not meta-analyse the results of exercise and FMD in our review and are so are unable to comment on localised or systemic effects in the corresponding artery beds, future studies should investigate this, particularly in female-focused participant groups.

Menstrual Status and Audit of Methodologies

There were no studies graded gold in naturally menstruating or HC-using females or cohorts with menstrual irregularities. There were a small number of studies in postmenopausal and pregnant populations, despite half of all eligible studies in females assessing naturally menstruating participants. Our results echo those previously conducted in other disciplines which have applied this audit framework to grade methodological quality in female participants. Kuikman and colleagues (2023) audited studies relating to carbohydrate fuelling strategies used during exercise and determined there were no studies graded as gold with very few (9%) graded silver. Further, Smith and colleagues (2022b) audited performance supplementation in female athletes and determined that 3 of 614 studies achieved a gold grade. While we are unable to directly compare our findings for the menopause or pregnancy classifications included in the present review, we propose inclusion of these categories to reflect female participants across the lifespan, inclusive of pregnancy. Taken together, it is clear that there is need for more studies in female participants, both athletic and non-athletic, that employ high quality methodologies to account for ovarian hormone effects and cover the spectrum of exercise-induced vascular responses.

There were six articles in the current review which explicitly stated that menstrual cycle effects were intentionally not controlled. Previous reviews have suggested there to be minimal control for the menstrual cycle in female participants which resulted in females being excluded from physiological studies (Costello et al., 2014; Wilson et al., 2020; Cowley et al., 2021; Lew et

al., 2022). However, the results of the current review disagree with this sentiment, as only a small proportion of studies including female populations did not control for the menstrual cycle (8%). This raises the previous debate as to whether there is a need to control for the menstrual cycle in exercise physiology studies at all. Wenner and Stachenfeld (2020) argued for the need to control for menstrual cycle phase as there is potential to miss the direct or indirect influences of the cycling ovarian hormones on endothelial function. Against controlling for the menstrual cycle, Stanhewicz and Wong (2020) reasoned there is no need to control for menstrual phase unless the research question specifically highlights the need to address ovarian hormones. In this case, controlling for ovarian hormones will increase the external validity of the study and to gain the true potential differences in female sex-related data (Stanhewicz & Wong, 2020). A previous systematic review and meta-analysis detailed that the effects of ovarian hormones on FMD may potentially be impacted by discrepancies in FMD protocols used or methodologies used to account for the menstrual cycle (Williams et al., 2020). If studies in female participants fail to employ a highest possible quality methodology to account for ovarian hormones, within the available resourcing constraints, results are unable to be applicable to the wider female population (Smith et al., 2022).

Study Limitations and Future Research Directions

This systematic review applied and expanded an existing audit framework that assesses both menstrual status and best-practice methodologies used to assess the impact of ovarian hormones on endothelial function in female participants undergoing exercise interventions. The audit tool used was developed from specific guidelines related to research studies in females (Elliott-Sale et al., 2021; Smith et al., 2022). We expanded the audit framework to include menopause and pregnancy, developed from the same methodological quality guidelines (Elliott-Sale et al., 2021) which enabled a lifespan approach to understanding FMD responses to exercise in females which has not been studied previously.

While this review is limited to studies which assessed endothelial function using best-practice FMD guidelines (Corretti et al., 2002, Harris et al., 2010, Thijssen et al., 2011, Thijssen et al., 2019), future research needs to further interrogate protocols used to measure endothelial function via the use of FMD, particularly as FMD methodological differences may be more discriminatory than those relating to menstrual cycle phase (Wilson et al., 2015). Also, we did not include clinical cohorts, which may identify additional gaps in the data pertaining to methodological quality in female individuals. A further limitation to this review is the exclusion of studies assessing hormonal contraception methods other than the oral contraceptive pill (OCP). In terms of contraception, this review only reports results on studies which included the use of OCP, of which only 12 were eligible. Further investigations in female populations should include a variety of contraception methods (such as long-acting reversible contraception; LARC) and the potential effects this may have on endothelial function. No definitive conclusions can be drawn relating to how exercise influences FMD however, several other groups (Campbell et al., 2019; Tao et al., 2023) have provided insight into these findings, albeit not specific to females.

Best-practice approaches in females to account for ovarian hormones can be time consuming, have a significant cost and provide a substantial burden on participants to provide the requisite information regarding hormonal profile [i.e., luteinising hormone (LH) surge and length of menstrual cycle] (Elliott-Sale et al., 2021; Smith et al., 2022). Developing lower-cost and less invasive approaches to blood sampling that use available technology could be explored further, for example dried blood spot (DBS) sampling used for anti-doping applications. The DBS process involve capillary sampling of the finger or heel, meaning minimal training, transferring a small blood spot sample onto sampling paper which is considerably less blood than from a venous blood sample, requires fewer storage requirements, and maintains stability in ambient temperatures (Trifonova et al., 2019). This technology allows smaller blood samples to be collected and storage of samples is easier. Applying this

process to hormonal profiling could considerably reduce the cost and burden placed on participants when accounting for the ovarian hormones.

CONCLUSION

In conclusion, current literature highlights a lack of female representation in vascular exercise studies with only 38% of the total population being female, despite more than half of eligible studies including at least one female participant. We found no studies in premenopausal cohorts that achieved all best-practice recommendations to account for ovarian hormones. These findings are perhaps reflective of the resource-intensive nature of conducting high-quality research in female populations, as well as sex-specific barriers to research participation. Future research should focus on more affordable and less burdensome methodologies and address the potential volunteer-bias in vascular exercise research. Achieving parity of female participants in exercise physiology, in particular vascular studies, will enhance the collective understanding of exercise effects on vascular function which has potential impacts for the health and performance of female athletic and non-athletic populations.

SUPPLEMENTARY MATERIALS

Table 1 Data Extraction – Title and Author

Table 2 Data Extraction – Audit Framework

Chapter 3: Experimental Study

This chapter is based on an experimental study prepared for submission as: Thompson SL, Brade CJ, Naylor LH and Spence AL. (2023). The female athlete's artery: Characterising arterial phenotype across distinct exercise modalities. *Manuscript in preparation.*

ABSTRACT

Athletes participating in distinct modalities of exercise hypothetically present with specific arterial morphological and functional phenotype. However, this has yet to be explored in a female athlete population, while accounting for fluctuating ovarian hormones. Using a repeated measure, cross-sectional study design, 21 athletes from distinct sporting modalities (powerlifters, triathletes/cyclists/rowers, long-distance runners) (age = 27.1 ± 4.8 y, height = 166.9 ± 6.1 cm, body-mass = 65.2 ± 8.1 kg) attended two testing sessions determined by hormone concentration [low (LowH) or high (HighH) hormone]. Cycle tracking, serum hormone analysis and urinary ovulation testing were used for phase determination in naturally menstruating participants, while pill type (active or placebo) determined phases in oral contraceptive users. Brachial and femoral artery diameter, flow-mediated dilation (FMD%) and ischaemic hand-gripping exercise representing vasodilatory capacity (VC%) were assessed. Femoral artery baseline diameter was larger in runners (+ 14.6%, p = 0.019) and cyclists (+ 15.6%, p = 0.014) in comparison to powerlifters. There were no differences between athlete group in brachial artery outcome measures. Interestingly, resting brachial diameter during VC% assessment was higher in the HigH phase compared to the LowH phase in powerlifters (+ 4.2% increase from LowH p = 0.004). There were no interaction differences between group and hormone phase on all vascular outcome measures. In conclusion, brachial and femoral artery function remains unchanged between LowH and HighH phases with no apparent differences between distinct female athlete groups. Considering the methodological quality of vascular studies in female athletes will enable findings to be better applied to the broader female athletic population.

INTRODUCTION

Female involvement in sport and the professionalisation of females in sport is ever increasing with female participation at the 2020 Summer Olympic Games reaching 49% compared to 35% at the 2000 Olympics (Olympics, 2022). Yet, exercise physiological studies have historically excluded female athletes, on the basis that the cyclical nature of ovarian hormones and the potential impacts on physiological systems remaining unknown (Costello et al., 2014; Turner et al., 2020). In vascular physiology, oestrogen exerts both genomic and non-genomic effects to modulate endothelial function due to oestrogen receptors (ERα) located in endothelial and smooth muscle tissues that impact the NO pathway (Simoncini et al., 2004; Turner et al., 2020). As such, oestrogen has positive, antiatherogenic effects on the vasculature and promotes vasodilation via direct activation of endothelial nitric oxide synthase (eNOS) among other mechanisms (Mendelsohn, 2002). However, few studies have directly assessed the impact of oestrogen availability on the vasculature, specific to female athletes.

Like the hypothesised ‘athlete’s heart’, the athlete’s artery is a colloquialism used to describe arterial structural and functional adaptations due to chronic exercise exposure (Green et al., 2012). Evidence supporting the notion of the athlete’s artery has been limited to male athletic populations participating in high-dynamic sports (producing a $\dot{V}O_2$max greater than 70%), with studies indicating an increased artery lumen diameter in comparison to sedentary controls (Mitchell et al., 2005; Green et al., 2012; Hellsten & Nyberg, 2015; Wilson et al., 2015). This increase in arterial diameter has been attributed to repeated shear stress stimulus on the arterial walls during dynamic exercise (Tinken et al., 2010; Black et al., 2016). Contrary to this, studies conducted in athletes participating in high-static sports (producing > 50% maximal voluntary contraction) are limited with conflicting findings that suggest increased arterial diameter compared with non-athletic controls may be localised to exercising limbs (Spence et al., 2013; Black et al., 2016; Naylor et al., 2021).

Not surprisingly, there is little evidence to describe the impact of exercise in otherwise healthy reproductive-aged female athletic cohorts. Yoshida et al. (2005) studied arterial function (represented by flow-mediated dilation or percentage change in diameter from baseline in response to an ischaemic stimulus [FMD%]) in female athletes and non-athlete controls and reported impaired FMD% in athletes. However, female athletic participants had exercise-induced amenorrhea which may have altered oestrogen availability and resulting vascular function. Others have reported similar findings in professional ballet dancers with low energy availability (Hoch et al., 2011) and amenorrhoeic athletes compared to eumenorrheic athlete controls (Hoch et al., 2010; Augustine et al., 2016). Taken together these studies suggest that menstrual irregularities may impact FMD%, independent of chronic exercise effects, due to reduced endogenous oestrogen availability in amenorrhoeic participants. Interestingly, in endurance-trained eumenorrheic females compared to sedentary controls, brachial FMD% was not different (Moe et al., 2005; O'Donnell et al., 2014; Kyte et al., 2022) although participants in these studies were measured during one phase of the menstrual cycle (early follicular). In terms of artery size, regularly menstruating athletic females have reportedly larger brachial arteries than controls (Moe et al., 2005; Rickenlund et al., 2005) but this finding is inconsistent (O'Donnell et al., 2014; Kyte et al., 2022). Current knowledge pertaining to vascular structure and function in female athletes is limited to endurance athletes measured during a single phase of the menstrual cycle.

Studies in non-athletes have assessed whether FMD% changes across the menstrual cycle, with conflicting results. Shenouda et al. (2018) and D'Urzo et al. (2017) found no change in FMD% across a natural menstrual cycle, which contrasts findings from Adkisson et al. (2010) who found an increased FMD% in late follicular phase which corresponded with peak measurement of oestradiol. Although these studies measured and reported serum oestrogen, inconsistencies in cycle phase length between studies as well as confirming that measurement cycles were ovulatory may have confounded results. Results from previous literature regarding the influence of ovarian hormones

and menstrual cycle phase on arterial morphology appears to be more consistent such that artery diameter remains unchanged across phases of the menstrual cycle (Williams et al., 2001; Adkisson et al., 2010; D'Urzo et al., 2018, Shenouda et al., 2018). Few studies have investigated the impact of monophasic oral contraceptive (mOCP)-use on arterial structure and function, with no consensus reached for FMD%. In particular. Heidarzadech et al. (2014) found that FMD% was lower in mOCP-users compared to naturally menstruating controls whereas Shenouda et al. (2018) found no difference between users and non-users nor any difference in FMD% across the active and placebo/inactive pill phases. However, artery diameter did not differ between users and non-users (Heidarzadech et al., 2014; Shenouda et al., 2018) nor were there any changes in artery size across active and inactive pills (Heidarzadeh et al., 2014; Shenouda et al., 2018). Importantly, no studies have evaluated FMD% responses in highly trained athletic participants using mOCP. Considering the popularity of hormonal contraceptives in athletic cohorts (Larsen et al., 2020) appreciating the potential impacts of exogenous synthetic oestrogen on vascular outcomes is necessary.

Therefore, the primary aim of this study was to determine whether chronic exercise of differing modalities results in a distinct structural and functional vascular phenotype, specifically in a population of female athletes. The secondary aim was to determine if arterial morphology and functional measures differ depending on ovarian hormone availability in Phase 1 of the menstrual cycle or when using an inactive/placebo mOCP pill [low hormone (LowH)] versus Phase 4 of the menstrual cycle or when using active mOCP pill [high hormone (HighH)]. We hypothesised there would be a divergent arterial phenotype dependent on the primary exercise mode of the athletes and that hormone phase would influence the magnitude of arterial diameter and functional adaptations.

METHODS

Participants

We recruited 21 highly trained female athletes from different sporting modalities. These participants were aged between 18 to 35 and premenopausal. A mean sample size of n = 10 was needed per group to achieve sufficient power. This was calculated *a priori* assuming a small=moderate mean effect size of 0.4, assuming the difference between the groups was significant ($p \leq 0.05$). Athletes were categorised as Tier 2, Tier 3 and Tier 4 according to The Participant Classification Framework (McKay et al., 2022). Using the sport categorisation matrix by Mitchell et al. (2005) we applied the following classification of sports: high-static, low-dynamic: powerlifting (POW; n = 8), high-static, high-dynamic: triathlon/rowing/cycling (CYC: n = 9) and low-static, high-dynamic: long-distance running (RUN; n = 4). Participants were either naturally menstruating (n = 18) or using mOCP (n = 3) for more than 3 months with the following brand names and formulations: *Zoely*, nomegestral acetate 2.5 mg/estradiol 1.5 mg (n = 1); *Valette*, ethinylestradiol 30 µg/dienogest 2.0 mg (n = 1); *Microgynon*, ethinylestradiol 30 µg/levonorgestrel 150 µg (n=1). All mOCP-users took the inactive pill phases during the study to enable measurement during a LowH phase. Participants were excluded from the study if they were using other forms of contraception (e.g., intrauterine device, implant), were planning to conceive, were pregnant in the previous six months or pregnant during this study, had a diagnosed menstrual cycle disturbance (e.g., polycystic ovarian syndrome or endometriosis), had a training age < 2 years, had a musculoskeletal injury which prevented them from exercising, used any medications or smoked. Prior to the commencement of testing, informed consent was obtained from all participants. The study received institutional ethics approval from Curtin University Human Research Ethics Committee (HRE2021-0245).

Study Design and Overview

In this repeated measures, cross-sectional study, participants attended two separate testing sessions according to menstrual phase and mOCP pill type

(active and placebo/inactive). Using consensus nomenclature for describing menstrual cycle phase (Elliott-Sale et al., 2021) participants were studied in the following phases: LowH visit: Phase 1 (early follicular, during menses) and HighH visit: Phase 4 (mid-luteal) as outlined in **Figure 1**. Phases were confirmed using a three-method validation process (Schaumberg et al., 2017) outlined below. Participants using mOCP were tested when taking the inactive/placebo pill (LowH) and between the active pills 7 - 14 (HighH). Vascular measures of morphology and function were obtained during both hormone phases using high-resolution ultrasound as described (see Vascular Measures). During LowH, participants completed a once-off graded exercise test to determine peak oxygen consumption ($\dot{V}O_2peak$) and whole-body dual energy x-ray absorptiometry (DXA) scan to assess body composition. These measures were taken to describe participants' characteristics.

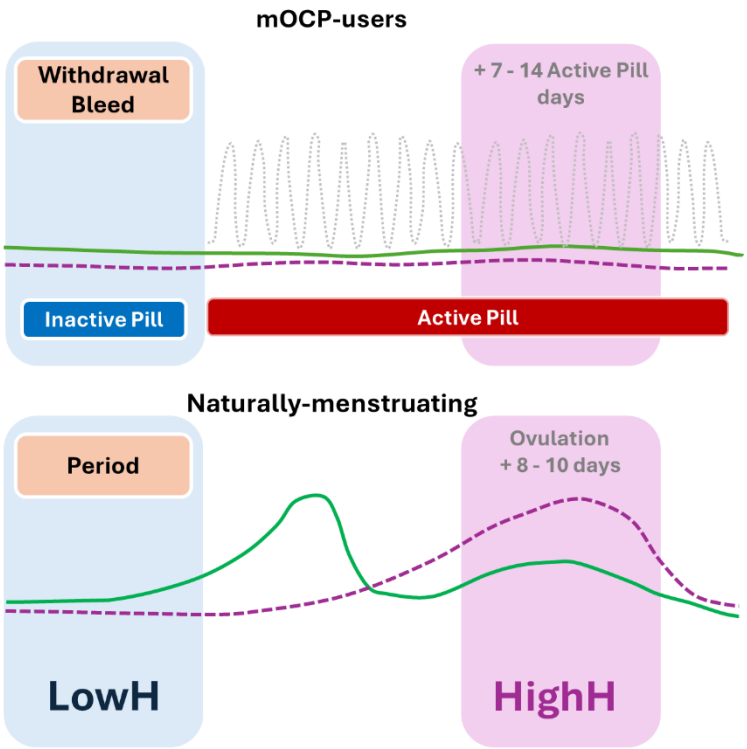

Figure 1 Study design: Repeated measures were obtained at low hormone phase (LowH) and high hormone phase (HighH) in monophasic oral contraceptive pill (mOCP)-users (n = 3), during the inactive pill and between active pills 7-14, respectively. In naturally menstruating (n = 18) participants,

during Phase 1 (early follicular, during menses) and Phase 4 (mid-luteal), respectively.

Menstrual Cycle Phase Determination

Naturally menstruating participants had a cycle length between 21 - 35 days, having nine or more periods a year when completing the pre-screening questionnaire (self-reported). These participants had not used any form of contraception for 3 months prior to being involved in the study. For the entirety of a testing cycle (approximately 4 to 8 weeks), participants tracked their cycle (calendar date, cycle day, and any associated menstrual cycle symptoms) using an online diary adapted from a menstrual cycle verification study (Prior et al., 1987). Five participants were tested across the duration of two cycles due to logistics and equipment availability. Tracking determined cycle length and established the timing of testing visits with the LowH testing visit scheduled between days 1 - 5 (menstruation) for naturally menstruating participants.

At each testing visit, a phlebotomist (ST) collected a venous blood sample of approximately 5 mL drawn from the antecubital vein using standard phlebotomy procedures. This sample was immediately transported to the state pathology laboratory to be commercially analysed to measure the levels of oestradiol (E2, pmol/L) and progesterone (P4, nmol/L) to confirm hormone phase. Concentrations of total testosterone (T, nmol/L), sex hormone binding globulin (SHBG, nmol/L) and free androgen index (FAindex, nmol/L) were also measured. Analyses were completed using liquid chromatography-tandem mass spectrometry.

Urinary hormone ovulation testing kits (Pregnancy Planning Kit, OvuPlan, Key Pharmaceuticals, Australia) were provided to naturally menstruating participants with clear instructions for at home-use during the testing cycle to measure for a surge in LH to confirm ovulation. Participants collected daily urine samples, with the start day dependent on individual cycle length (e.g., day 10 for a 27-day cycle length), with photos of the results uploaded to the daily online cycle diary which were verified by the researchers, until a positive

result was recorded. This positive result indicated the rise in LH which occurs 24 - 36 hours prior to ovulation (Schaumberg et al., 2017). The HighH testing visit was scheduled for 8 - 10 days post-positive LH test result, to coincide with Phase 4 of the menstrual cycle (Schaumberg et al., 2017). In a subset of participants (n = 5), where no positive LH test result was obtained, the HighH testing visit was estimated for 8-10 days after the cycle midpoint (predicted ovulation) of their typical cycle length (e.g., day 15 of a 30-day cycle).

Vascular Measures

The protocols used for assessing arterial FMD% followed current expert consensus guidelines as outlined by Thijssen and colleagues (2019). Participants were required to abstain from food or drink besides water (overnight fast, or for at least 6 hours), exercise, caffeine, alcohol, or vitamin C for 10 hours prior to their testing visit. These protocols differ slightly from the Thijssen et al. (2019) consensus guidelines but have been adapted from the earlier Corretti et al. (2002) guidelines as the recruited athletes were unable to abstain from training for more than 24 hours.

Brachial artery diameter was assessed following supine rest (~20 minutes), using a 10-MHz frequency linear array probe and high-resolution duplex ultrasound (Terason t3200, Burlington, Massachusetts, USA). The B-mode arterial images were optimised for all vascular scans with simultaneous recording of continuous Doppler velocity with the insonation angle ≤ 60°. Baseline brachial arterial measurement was recorded for 1-minute prior to the inflation of an occlusion cuff placed approximately 2 cm below the antecubital fossa. Using a rapid cuff inflator (Hokinson, Bellevue, Washington, USA), the cuff was inflated to 220 mmHg for 5-minutes, recording resumed 30 seconds prior to cuff deflation and continued for 3-minutes post-deflation. The post-deflation recordings were used to measure FMD%, the change from baseline to peak arterial diameter, which represents endothelial function and peak arterial diameter. Following this assessment, the same protocol was repeated

to assess the superficial femoral artery with the occlusion cuff placed approximately 5 cm above the knee (Thijssen et al., 2019).

Brachial artery vasodilatory capacity (VC%), a surrogate measure of arterial structural remodelling (Naylor et al., 2021), was assessed following the femoral artery assessment. A washout period of at 20-minutes occurred to ensure brachial VC% was not influenced by preceding brachial FMD. Like the FMD% procedure, baseline (pre-occlusion) measurements were recorded for 1-minute prior to rapid inflation of the occlusion cuff (placed in the same position as the brachial artery FMD assessment). The occlusion cuff was inflated to 220 mmHg for 5-minutes, with participants required to squeeze a hand grip device in time with a metronome set at 50 bpm (approximately 25 contractions per minute) during the middle 3-minutes of cuff inflation. Recording of brachial artery diameter and blood velocity began 30 seconds prior to cuff deflation and continued for 4-minutes post-deflation (Naylor et al., 2021).

Resting blood pressure and heart rate were monitored throughout the arterial assessment with discrete measures taken at 2-minutes pre-brachial artery FMD scan, 1-minute post-brachial artery FMD scan, 2-minutes pre-vasodilatory capacity scan, and 1-minute post-vasodilatory capacity scan. These values were averaged to provide a single session measure for resting systolic (SBP), diastolic blood pressure (DBP), mean arterial pressure (MAP) and resting heart rate (RHR).

Vascular Processing

Validated investigator-independent, custom designed edge-detection and wall tracking software (FMD/Blood Flow 6.0, LabView 17.0f2, National Instruments, Austin, Texas, USA) was used to analyse arterial outcome measurements. Simultaneous regions of interest were identified on both the B-mode and Doppler images to analyse arterial diameter and blood velocity profile, respectively. Blood flow [the product of cross-sectional area and Doppler velocity determined with the equation: blood flow = ((mean blood velocity) x (π

× (vessel radius^2)) x 60)] and shear rate [((4 × velocity)/diameter), s^{-1}] were calculated at 30 Hz. An automated algorithm determined peak diameter following cuff deflation, with the percentage change in artery diameter from baseline to peak calculated as FMD% (for brachial and femoral artery) and VC% (brachial artery only). The time to peak dilation (seconds) was calculated as the duration from cuff deflation to post-deflation peak diameter for each artery bed and assessment. Processing of the arterial scans was conducted by a blinded analyser and results from this analysis were used in the statistical analysis. To reduce the variability of measures, the same sonographer collected all scans analysed within the experimental study.

Body Size and Composition

Body composition was analysed via whole body DXA scan (Lunar Prodigy, GE Medical Systems, WI, USA) to determine lean body mass (BM; kg), fat mass (kg), total body fat percentage and the regional breakdown of leg and arm total and lean mass (kg). Measures of body fat distribution represented as android (%) and gynoid (%) and bone mineral density (BMD, $g.cm^{-2}$) were also obtained. Total body mass (Seca 360 Wireless, Seca, Birmingham, United Kingdom) and height (Harpenden stadiometer, Holtain Limited, Crymych, United Kingdom) were collected to calculate body mass index (BMI, $kg.m^{-2}$) as well as body surface area (BSA, m^2) which was calculated using the Du Bois formula (Du Bois & Du Bois, 1916). These measurements were taken to account for differences in body size between participants in reference to arterial diameter.

Peak Oxygen Consumption

A graded exercise test using an electromagnetically braked stationary ergometer (Lode Corvial, Groningen, Netherlands) was used to assess peak oxygen consumption ($\dot{V}O_2$peak). A ramp protocol was followed beginning at a workload of 25 W, which increased each minute by 25 W. The test was terminated if participants were unable to maintain the required intensity or reached volitional exhaustion. Heart rate and participant subjective Rating of

Perceived Exertion (RPE; Borg 6-20) (Borg & Dahlstrom, 1962) measurements were taken each minute. To determine $\dot{V}O_2$peak, expired air was analysed at 30-second epochs for oxygen and carbon dioxide concentrations by a metabolic gas analysis system (TrueOne 2400, Parvo Medics, Salt Lake City, Utah, USA) with the highest value in the final workload reported.

Statistical Analysis

All data were analysed using open-source statistical software package jamovi (The jamovi project (2002). Jamovi, Version 2.3. Retrieved from www.jamovi.org) with GraphPad Prism (Version 10.0.2 for Windows, GraphPad Software, Boston, Massachusetts USA, www.graphpad.com) used for generating figures. Statistical significance was set at $p \leq 0.05$. Demographic differences between the sport groups were assessed using a one-way ANOVA, with Tukey's and Games-Howell post-hoc assessment to identify pairwise comparisons. To determine the effect of sport group (POW, CYC and RUN), visit (HighH and LowH) and the interaction of group*visit on menstrual status and outcome variables, a linear mixed-effects model was used. Group and visit were fixed effects, participants were random effects and mOCP-use was included as a covariate in the model, owing to the low sample of mOCP-users in the present study. Post-hoc assessments of between- and within-group difference between visits were based on the estimated marginal means, presented with 95% confidence intervals (95%CI). To confirm whether individuals had an ovulatory cycle (rise in P4 at HighH), a second linear mixed-effects model was used with positive LH test results and hormone phase as fixed factors. Due to assay detection limits for serum hormone E2 (< 40 pmol/L), P4 (< 1 nmol/L) and FAindex (< 1.0), variables below the detection limit were substituted with a numeric value and analysed as described. To account for missingness in the data, we used predictive mean matching with multiple data imputation. We assigned the mean of the imputed values to missing values to avoid exclusion of participants with missing data (see Results: Missing data). All other data are expressed as mean ± SD, unless indicated.

RESULTS

Missing Data

A total of 21 participants were recruited for the study. One naturally menstruating participant in the CYC group did not complete the study following the LowH visit due to unexpected exogenous hormone use. Due to poor image quality, femoral images were not obtained in 2 participants at either visit and 2 participants in the HighH visit only. Due to technical difficulties, femoral images from 1 participant in the HighH visit, 3 participants in the LowH visit and brachial images for 2 participants in the HighH visit were also not obtained. Also, blood was not collected in 3 participants from both the LowH and HighH and 1 participant from the HighH visit due to difficulties in obtaining an adequate blood sample.

Participant Characteristics

Demographic characteristics for each group are presented in **Table 1**. There were no differences in age, training age, height or BM between the groups (ANOVA $p > 0.05$). Body mass index was lowest in the CYC group, which was statistically different only from the POW ($p < 0.05$). Lean mass of the arms was greatest in POW and this was significantly different only from the RUN group ($p < 0.05$). Endurance athletes (CYC and RUN) had significantly higher absolute and relative aerobic fitness ($\dot{V}O_2peak$) compared to the POW group ($p < 0.001$ and $p < 0.05$, respectively). Following post-hoc testing, peak power output (absolute) and peak power-to-weight ratio achieved during fitness testing was greatest in CYC, which was statistically different only to the POW ($p < 0.001$).

Menstrual Status, Hormone Phase Confirmation and Serum Hormone Analysis

Pooled and group-based menstrual status and cycle characteristics for all naturally menstruating participants (n = 18) are presented in **Table 2.** There

were no significant differences in average self-reported cycle length or period length between the groups (ANOVA p = 0.868 and p = 0.195, respectively). Positive LH tests were obtained in approximately 70% of all athletes. Of those that achieved a positive LH test result, the average day of the cycle (pooled data: 15 ± 2 days) was not statistically different between the groups (ANOVA p = 0.135). A significant main effect for hormone phase was observed for both serum E2 and P4 (p = 0.002 and p = 0.003, respectively). Both E2 and P4 were significantly greater in HighH compared to LowH (pooled mean difference ± standard error E2: 182 ± 51.1 pmol/L, p = 0.002; P4: 11.8 ± 4.38 nnmol/L, p = 0.0015, **Figure 2**). Neither E2 nor P4 were significantly different between athlete groups (main effect for group p = 0.660 and p = 0.859, respectively). There were significant main effects for LH test result (p = 0.027) and hormone phase (p = 0.002) as well as a significant interaction effect (p = 0.011). Those athletes that achieved a positive LH test (n = 12) had significantly higher P4 in the HighH phase compared to those that did not achieve a positive test result (n = 5; estimated marginal mean ± 95%CI positive vs estimated marginal mean ± 95%CI no positive LH: LowH 0.99 ± 8.12 vs. 3.24 ± 12.12 nnmo/L; HighH: 36.17 ± 9.94 vs 8.00 ± 14.06 nnmo/L, p < .001).

A significant main effect for group was observed for SHBG (p = 0.047) with post-hoc analysis indicating POW were significantly greater than CYC (pooled estimated marginal means ± 95%CI: 126.7 ± 30.3 vs 78.8 ± 29.2 nmol/L). Also, a significant main effect was observed for mOCP-use such that users (n = 3) had significantly higher SHBG levels compared to naturally menstruating participants irrespective of hormone phase (mean ± SD LowH vs HighH: mOCP: 125.0 ± 56.0 vs. 158.7 ± 175.8; naturally menstruating: 65.6 ±29.3 vs. 59.6 +27.4 nmol/L, p = 0.003). However, further inspection by athlete group showed that pooled SHBG in the mOCP-user (n = 1) in CYC (70.5 nmol/L) was lower than both mOCP-users in POW (179.5 nmol/L) and RUN (175.5 nmol/L), respectively. No main effects for either athlete group or hormone phase were observed for T (p = 0.241 and p = 0.173) or FAindex (baseline brachial artery diameter during assessment of VC% was significantly larger during the HighH

compared to LowH phase (p = 0.003), specifically in the POW athletes (+ 4p = 0.093 and p = 0.071, respectively).

Vascular Measures

All vascular measures are presented in **Table 3**. There were no significant differences between mOCP-users and naturally menstruating participants for any vascular outcome measures. In the brachial artery, no significant group or hormone phase differences were found for any measures, specifically baseline diameter, peak diameter, FMD% (raw and shear-normalised) and time to peak diameter. However,.2% increased from LowH p = 0.004). Peak diameter during VC, VC% (absolute and shear-normalised) and time to peak diameter did not differ statistically between athlete group or hormone phase.

In the femoral artery, diameter at baseline was significantly greater in both CYC and RUN compared to POW (+ 14.6%, p = 0.019, + 15.6%, p = 0.014, respectively). No significant differences were observed between hormone phase for femoral artery baseline diameter. Peak diameter of the femoral artery, FMD% and normalized FMD did not differ significantly between athlete group or hormone phase. Time to peak diameter in the femoral was significantly different between hormone phases (p = 0.49) with post-hoc analysis indicating time to peak was longer in the HighH compared to LowH phase (p = 0.03), particularly in the RUN group (p = 0.023).

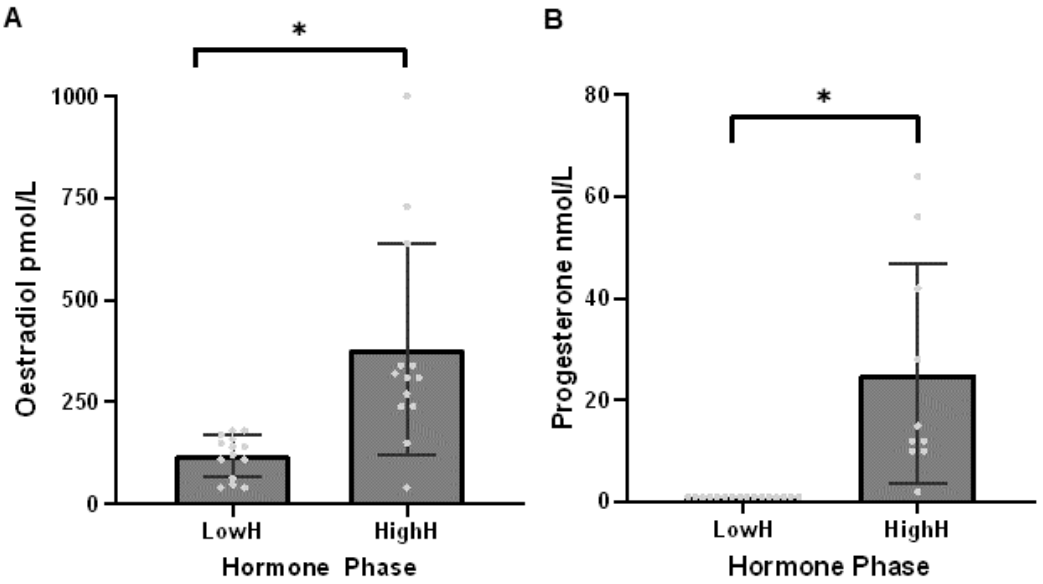

Figure 2. A. Pooled data for serum concentrations of oestradiol (E2, pmol/L) in low hormone (LowH) and high hormone (HighH) phases. B. Pooled data for serum concentration of progesterone (P4, nmol/L) in low hormone (LowH) and high hormone (HighH) phases. Data presented as mean ± standard deviation. Assay detection limits for E2 and P4 are < 40 pmol/L and < 1 nmol/L, respectively. * $p < .05$ significant to low hormone (LowH) phase

Table 1. Participant characteristics for each athlete group, including anthropometry, dual-energy x-ray absorptiometry (DXA)-derived body composition and fitness outcomes.

Characteristic	Powerlifters n = 8	Cyclists n = 9	Runners n = 4
Age, years	29 ± 6	26 ± 5	28 ± 4
Training age, years	3.2 ± 1.1	3.9 ± 2.8	5.8 ± 3.0
Anthropometry			
Height, cm	164.0 ± 7.4	169.0 ± 5.7	169.0 ± 4.4
Body mass, kg	70.3 ± 6.8	64.8 ± 9.7	66.7 ± 7.4
BMI, kg.m^{-2}	26.1 ± 2.4	22.5 ± 2.4*	23.4 ± 2.7
BSA, m^2	1.79 ± 0.11	1.74 ± 0.16	1.77 ± 0.10
Body composition			
Lean mass, kg	47.1 ± 4.9	44.5 ± 5.8	45.1 ± 4.4
Fat mass, kg	20.6 ± 4.5	16.1 ± 4.8	18.2 ± 4.2
Body fat, %	30.3 ± 5.2	27.5 ± 4.3	28.5 ± 3.6
Arm lean mass, kg	5.4 ± 0.7	4.8 ± 1.2	4.6 ± 0.1 *
Total arm mass, kg	7.8 ± 0.9	6.9 ± 1.8	6.8 ± 0.5
Leg lean mass, kg	16.9 ± 2.2	15.7 ± 2.1	15.5 ± 1.5
Total leg mass, kg	27.9 ± 2.9	24.3 ± 4.0	24.1 ± 3.2
Android fat, %	25.6 ± 9.9	21.1 ± 8.0	23.3 ± 5.4
Gynoid fat, %	36.4 ± 4.6	32.8 ± 4.8	32.9 ± 4.1
A/G ratio	0.69 ±0.20	0.63 ± 0.18	0.71 ± 0.13
BMD, g.cm^{-2}	1.26 ± 0.12	1.20 ± 0.06	1.18 ± 0.09
Aerobic Fitness			
VO_2 peak, L.min^{-1}	1.95 ± 0.35	3.25 ± 0.66 **	2.97 ± 0.30*
VO_2peak, mL.kg.min^{-1}	27.8 ± 4.5	50.8 ± 11.4 **	45.0 ± 6.8*
Peak Power Output, W	188.0 ± 23.1	256.0 ± 36.1 **	225.0 ± 20.4
Peak PWR, W.kg^{-1}	2.67 ± 0.29	4.01 ± 0.78 **	3.40 ± 0.40

Data presented as mean ± standard deviation. Bold font: ANOVA <0.05. Abbreviations: BMI, body mass index; BSA, body surface area; A/G Ratio, android-gynoid percent fat ratio; BMD, bone mineral density; VO_2 peak, peak oxygen consumption; Peak PWR, peak power-to-weight ratio. * significant compared to powerlifters ($P < 0.05$); ** significant compared to powerlifters ($P < 0.001$).

Table 2. Group and pooled menstrual status for all naturally menstruating participants. Data is presented as mean ± standard deviation.

Characteristic	Powerlifters n = 7	Cyclists n = 8	Runners n = 3	Pooled n = 18
Cycle length, days	29 ± 3	29 ± 5	30 ± 2	29 ± 3
Period length, days	4 ± 1	5 ±1	5 ± 1	5 ± 4
Positive LH test, %				
Yes	71	62.5	67	66.6
No	29	25	33	27.4
Missing^	0	12.5	0	6
Positive LH test, cycle day	15 ± 2	16 ± 2	13 ± 3	15 ± 2

^ n = 1 participant did not complete LH testing. Abbreviations: LH, [urinary] luteinizing hormone. No significant differences between athlete groups.

Blood Pressure and Heart Rate at Rest

No significant differences in resting SBP, DBP, MAP or RHR were observed across the two hormone phases or between different athlete groups (**Figure 3**).

Table 3. Upper and lower limb vascular outcome measures, represented by athlete group and hormone phase (visit).

Variable	Powerlifters n=8		Cyclists n=9		Runners n=4		Group *p*	Visit *p*	Interaction *p*
	Low	High	Low	High	Low	High			
Brachial artery									
Baseline diameter, mm	3.3 (2.9, 3.8)	3.3 (2.9, 3.7)	3.4 (3.0, 3.8)	3.3 (2.9, 3.7)	3.6 (3.1, 4.2)	3.6 (3.1, 4.1)	0.535	0.397	0.921
Peak diameter, mm	3.6 (3.2, 4.1)	3.5 (3.1, 3.8)	3.6 (3.2, 4.1)	3.6 (3.1, 4.0)	3.9 (3.3, 4.4)	3.9 (3.3, 4.4)	0.584	0.502	0.812
FMD,%	8.1 (5.4, 10.7)	7.8 (5.1, 10.6)	9.0 (6.4, 11.5)	9.2 (6.6, 11.8)	6.7 (3.3, 10.0)	7.7 (4.3, 11.1)	0.457	0.699	0.867
Normalised FMD, $x10^3$, %	26.1 (17.7, 34.5)	18.3 (9.5, 27.0)	21.4 (13.4, 29.5)	23.7 (15.4, 32.1)	17.3 (6.5, 28.1)	19.8 (9.0, 30.6)	0.854	0.917	0.977
Time to peak, seconds	47.0 (20.6, 73.3)	44.9 (17.5, 72.3)	44.4 (18.9, 69.9)	52.7 (26.4, 79.0)	31.0 (-2.6, 64.7)	34.4 (0.8, 68.1)	0.604	0.722	0.859
Femoral artery									
Baseline diameter, mm	4.8 (4.2, 5.4)	5.2 (4.6, 5.8)	5.7 (5.2, 6.2) *	5.8 (5.3, 6.3) *	6.0 (5.5, 6.6) *	5.8 (5.3, 6.4) *	**0.025**	0.258	0.054
Peak diameter, mm	5.5 (4.8, 6.2)	5.8 (5.1, 6.5)	6.4 (5.8, 7.0)	6.4 (5.8, 7.0)	6.4 (5.7, 7.1)	6.4 (5.7, 7.0)	0.441	0.809	0.240
FMD,%	13.3 (7.5, 19.2)	11.1 (5.5, 16.7)	12.7 (8.2, 17.3)	10.7 (5.8, 15.5)	6.3 (1.0, 11.6)	9.8 (4.5, 15.1)	0.319	0.829	0.183
Normalised FMD, $x10^3$, %	10.8 (1.4, 20.1)	15.0 (6.2, 23.8)	15.2 (8.1, 22.3)	20.1 (12.4, 27.8)	13.5 (5.1, 21.8)	17.8 (9.4, 26.1)	0.289	0.118	0.709
Time to peak, seconds	55.8 (12.7, 98.9)	94.3 (54.0, 134.5)	69.4 (37.1, 101.7)	63.1 (27.6, 98.7)	60.4 (22.2, 98.6)	123.7 (85.5, 161.9) ^	0.381	**0.049**	0.148

Data presented as mean with 95% upper and lower confidence intervals. Bold font: $p < 0.05$. Abbreviations: FMD, flow-mediated dilation; Normalised FMD, FMD normalised to shear rate; VC%, vasodilatory capacity. *significant difference compared to powerlifters $p < 0.05$, irrespective of hormone phase; ^significant difference (within-group) compared to Low Hormone phase $p < 0.05$.

Table 3 (Continued). Upper and lower limb vascular outcome measures, represented by athlete group and hormone phase (visit).

Variable	Powerlifters n=8		Cyclists n=9		Runners n=4		Group *p*	Visit *p*	Interaction *p*
	Low	High	Low	High	Low	High			
Brachial vasodilatory capacity									
Baseline diameter, mm	3.2 (2.8, 3.7)	3.4 (2.9, 3.8) ^	3.2 (2.8, 3.6)	3.3 (2.9, 3.7)	3.4 (2.9, 4.0)	3.5 (3.0, 4.0)	0.751	**0.003**	0.482
Peak diameter, mm	3.9 (3.4, 4.3)	3.8 (3.4, 4.2)	3.7 (3.3, 4,1)	3.8 (3.4, 4.2)	4.1 (3.5, 4.6)	4.1 (3.6, 4.7)	0.496	0.703	0.556
VC,%	20.1 (13.7, 26.5)	13.4 (6.8, 20.0)	16.5 (10.2, 22.7)	15.5 (9.0, 22.1)	17.9 (9.8, 26.0)	17.3 (8.4, 26.1)	0.927	0.178	0.307
Normalised VC, $x10^3$, %	3.7 (1.1, 6.2)	4.7 (2.1, 7.2)	3.2 (0.8, 5.5)	4.3 (1.7, 6.9)	3.6 (0.5, 6.8)	3.0 (-0.5, 6.6)	0.829	0.618	0.776
Time to peak, seconds	96.3 (60.5, 132.0)	71.5 (34.0, 109.0)	101.0 (66.6, 135)	91.6 (54.1, 129.0)	92.9 (46.1, 140)	93.1 (39.9, 146)	0.738	0.476	0.809

Data presented as mean with 95% upper and lower confidence intervals. Bold font: $p < 0.05$. Abbreviations: FMD, flow-mediated dilation; Normalised FMD, FMD normalised to shear rate; VC%, vasodilatory capacity. *significant difference compared to powerlifters $p < 0.05$, irrespective of hormone phase; ^significant difference (within-group) compared to Low Hormone phase $p < 0.05$.

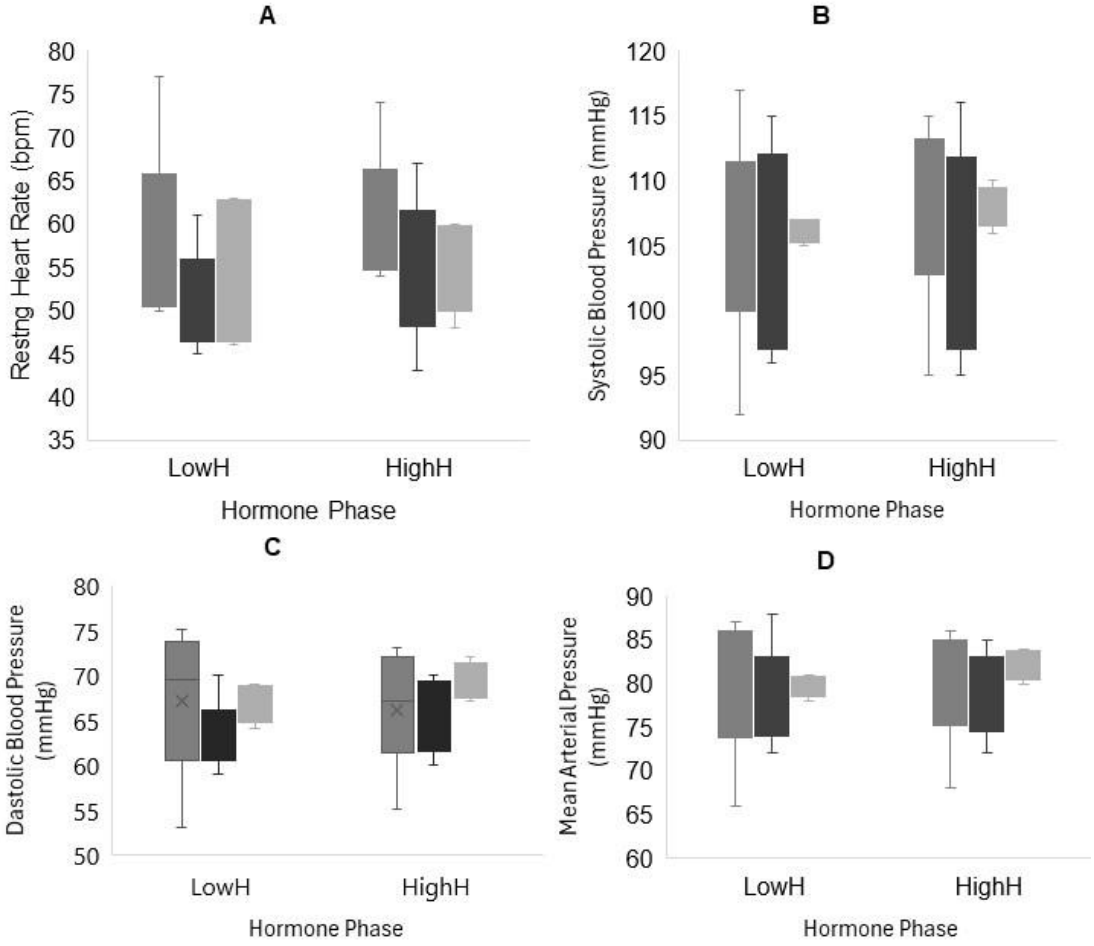

Figure 3. Cardiovascular measures at rest, by athlete group [powerlifters (POW), n = 8 black bars, cyclists (CYC), n = 8, light-grey bars, and runners (RUN), n = 4, mid-grey bars) and hormone phase (Low and High)]. Data presented as mean ± standard deviation. A: Resting heart rate (beats per minute); B: Systolic blood pressure (mmHg); C: Diastolic blood pressure (mmHg); D: Mean arterial pressure (mmHg). No significant differences between athlete group or hormone phase.

DISCUSSION

Main Findings

Endothelial function (FMD%) of the brachial and femoral arteries, and brachial artery structural remodelling (VC%), were equivalent between groups of divergent female athletes. We did not observe any differences between hormone phase for either FMD% or brachial artery VC%. Our findings agree with previous studies in non-athletic females assessing FMD% across the menstrual cycle (Williams et al., 2001; Adkisson et al., 2010; Shenouda et al., 2018) however only partially supports prior investigations in male athlete groups regarding FMD% (Naylor et al., 2021). In the present study, femoral artery diameter was largest in female endurance-based athletes (cyclists and runners) compared to powerlifters, irrespective of hormone phase, which supports previous meta-analytic data in male cyclists and triathletes (Black et al 2016). Interestingly, time to peak diameter of the femoral artery was significantly longer in the HighH phase, particularly in runners. In addition, we noted that baseline brachial artery diameter was larger during HighH, notably in the powerlifters, but this was only apparent during the assessment of VC%, not during measurement of brachial FMD%. Both oestrogen and progesterone increased significantly from LowH to HighH, indicating that athletes were indeed measured during two distinct hormone phases. The athlete groups in this study were well-matched for age, training experience and menstrual status. As expected, the endurance-based (high-dynamic) athletes had significantly greater $\dot{V}O_2$peak compared to powerlifters, while the powerlifters had greater lean body mass in the upper body when compared to the runners. Body mass index was greatest in the powerlifters when compared to the cyclists, despite no other anthropometric differences between the groups. To our knowledge, this study is the first to summarise the impacts of hormone phase and sporting modality on vascular morphology and function in a cohort of reproductive-aged female athletes participating in heterogenous sport categories.

Localised Adaptation of the Femoral Artery in Endurance Athletes

Studies in vascular morphology and function comparing between athletic groups has previously been reported in the brachial artery only (Lundgren et

al., 2015; da Silva et al., 2018), with most studies conducted in males (Agrotou et al., 2013, Smith et al., 2015; Naylor et al., 2021). Studies in reproductive-aged female athletes are limited to endurance-type sports (Moe et al., 2005, Kyte et al., 2022). In mixed-sex cohort studies, sex-disaggregated data is often not presented (Nualnim et al., 2011; DeVan et al., 2011; Das et al., 2018) however, a recent study compared lower limb macrovascular parameters between the sexes in chronically trained, lower-limb predominant participants (speedskaters) and untrained controls (Rasica et al., 2022). Authors reported larger femoral artery baseline diameter in athletes regardless of sex, however, did not find any training- or sex-related differences in femoral artery FMD%. These findings agree with the present study, where femoral artery diameter was largest in the lower-limb focused endurance athletes accompanied by no difference in FMD%, however Rasica and colleagues (2022) only measured a single athlete group compared to untrained individuals. In contrast, a male-only cohort study reported no differences in femoral artery diameter between runners, powerlifters, weightlifters and controls despite a larger FMD% in runners and weightlifters when compared to powerlifters and controls (Naylor et al., 2021). Yet, when diameter was indexed to body size, femoral artery diameter was greatest in the endurance athletes (runners) (Naylor et al., 2021). Meta-analytic data (Black et al., 2016) supports the notion that endurance athletes participating in predominantly lower-limb activities (cycling, running, triathlon) possess enlarged femoral arteries, which highlights the proposed mechanism for artery remodelling being localised to the exercising limb (Rowley et al., 2011).

The purported mechanism for arterial morphological adaptation in endurance-based athletes is related to repeated, episodic elevations in shear stress (Green et al., 2017; Churchill, 2020). Over time, this continual increase in arterial shear results in NO-dependent chronic arterial remodelling (Tinken et al., 2010; Green et al., 2023). The increase in artery diameter occurs with no concomitant increase in arterial function (FMD%), suggesting differences in the time-course of adaptation between morphology and function resulting in normalization of function (Spence et al., 2013; Green & Smith, 2018). Supra-normalization of artery function may be expected in athlete-models, an

observation which has been previously termed the ‘athlete paradox’ (Green et al., 2012; Green et al., 2013). While this paradox is not considered dogma, it is plausible that the enlarged femoral artery diameter measured in the female endurance athletes in our study contributed to the lack of any statistically meaningful FMD% differences between athlete groups. Likewise, arterial remodelling has implications for an increasing aerobic capacity in both athletic and non-athletic populations (Poole et al., 2021). Although FMD% and femoral artery diameter were not correlated in the presented study (results not presented), further investigation is warranted to appreciate vascular impacts on athletic training of differing exercise modalities, coupled with the potential influences of the menstrual cycle in females.

Upper Limb Adaptations in Powerlifting Athletes

It could be postulated that the powerlifters in our study would have larger upper limb vasculature owing to frequent barbell gripping resulting from their sport, as has been previously observed in resistance-trained males (Spence et al., 2013), yet this finding was not apparent. The proposed cardiovascular stimulus during resistance-type exercise is related to increased afterload, generating transient elevations in blood pressure rather than shear stress per se, resulting in little to no chronic adaptation to arterial diameter (Green et al., 2017; Churchill, 2020). These mechanisms may rationalise the lack of any training-induced adaptation in brachial or femoral artery size of female powerlifters observed in our study. A recent mixed-sex cohort training intervention reported increased brachial FMD% in females after a 3-month resistance-training program, however no accompanying artery diameter changes were reported (Green et al., 2023). These findings by Green and colleagues (2023) may represent the offset of time-course for functional compared to structural adaptation. With limited available sex-specific literature in chronically resistance-trained females, comparison of our data with male-only cohorts must suffice. Compared to runners, powerlifters and control participants, male weightlifters had the largest brachial artery diameters, yet FMD% between athlete groups remain unchanged (Naylor et al 2021), the latter finding echoed

in male powerlifters and long-distance runners (da Silva et al., 2018). Together, these results suggest that powerlifting may not be a potent stimulus for overt vascular morphological adaptation. Additional considerations for appreciating adaptive responses include training frequency and intensity (Lässing et al., 2023), however, we did not quantify training loads in the present study. Training age was reported, and albeit not statistically different, the relatively young training age of the powerlifters (~ 3 years) may have been insufficient to induce any measurable diameter differences compared to lower-limb predominant endurance athletes with more training experience (~ 4 - 6 years). There were no significant differences in brachial artery diameter or FMD%, suggesting a lack of brachial artery structural and functional changes in the powerlifting athletes recruited in this study. Possible reasons for this lack of adaptation relate to the potential for functional changes to occur prior to structural (Tinken et al., 2008), and the training age of the athletes being relatively low.

Impacts of Hormone Phase on Vascular Measures

While no apparent hormone phase-related changes in FMD% in the present study are supported by others investigating non-athletes (Williams et al., 2001; Adkisson et al., 2010; Shenouda et al., 2018), our study proposes that hormone phase may impact other aspects pertaining to vasculature function. First, the time to peak dilation during HighH in the femoral arteries of runners, was significantly longer (+ 63.27 s) than that measured during LowH. The relatively longer time to peak dilation may suggest changes to arterial wall elastic properties, namely increased stiffness (Black et al., 2007), whereby ovarian hormones may function as mediators (Laakkonen et al., 2021). While we did not directly measure indices of arterial stiffness in the present study, haemodynamic characteristics (SBP, DBP, MAP and RHR) did not differ between hormone phases. Previous studies report mixed results with some indicating that arterial stiffness and pulsewave velocity were no different during the luteal phase compared to other menstrual cycle phases (Adkisson et al., 2010; Priest et al., 2018), while others indicate reduced augmentation index

(reduced arterial stiffness) in the luteal phase (Robb et al., 2009). However, none of these studies included chronically trained female participants. Future studies should explore the potential relationship between hormone phase and arterial compliance in female-specific athletic cohorts. Secondly, our study showed that resting brachial artery diameter measured during VC% increased from LowH compared to HighH, notably in the powerlifting athletes. This unexpected finding may be due to the combination of methodological artefact and the hormonal milieu during HighH. The brachial artery was assessed twice in a single visit, first to determine FMD% and then VC%. Our protocol included a 20-minute wash-out period between successive brachial measurements, above the recommended 10 to 15-min suggested for repeated assessments (Corretti et al., 2002), but perhaps this timeframe was insufficient particularly during HighH. Higher circulating levels of both oestrogen and progesterone during the HighH phase may have contributed to a greater NO bioavailability and prolonged vasodilation from the preceding occlusion stimulus during the brachial FMD% (Serviente et al., 2016). Powerlifters who frequently engage in isometric hand-gripping during their sport may have been more susceptible to altered vascular tone in HighH. Methodological guidelines should be revisited in the context of repeated measures in females to mitigate any potential hormonal effects.

Methodological Quality in Females

Attempts to definitively answer the debate whether to control for menstrual cycle phase in vascular studies continues (Stanhewicz & Wong, 2020; Wenner & Stachenfeld, 2020), with meta-analytic evidence (k = 30, N = 1,363) describing menstrual cycle phase impacts on vascular function (Williams et al., 2020) reiterating an important point; study methodological quality is paramount. The authors report with a “very low” degree of certainty that endothelial function (measured using FMD) is unchanged in the luteal compared to follicular phases (k = 12, n = 183, I^2 = 84%), however, limited available data with high heterogeneity indicates that research employing best-practice methodological approaches for both vascular outcomes (Thijssen et

al., 2019; Corretti et al., 2002) and when assessing females as participants (Elliott-Sale et al., 2021) is lacking. This is further compounded when investigating vascular exercise responses in chronically trained female participants. In the present study, we followed consensus expert guidelines for FMD (Thijssen et al. 2019) when assessing reproductive-aged female participants within the resourcing constraints of the study. While we did not perform repeat DXA or $\dot{V}O_2$peak measures, nor did we collect duplicate assessments across two menstrual cycles as this impacted on both participant burden and study resources, this study has addressed all criteria for appropriate methodological grading (Smith et al., 2022).

Impact of Oral Contraception-Use

Direct comparisons of mOCP-users with non-users in the present study is not possible as only n = 3 mOCP-users were recruited. As such, mOCP-users were pooled with naturally menstruating participants and data were statistically analysed with mOCP as a covariate. As previously outlined, all mOCP formulations were different despite all being classified as monophasic. No statistically meaningful effects were observed for mOCP-use for any study outcome measures, however SHBG concentrations were notably higher in those using mOCP. This result is not unexpected as hormonal contraception downregulates endogenous hormones for fertility control and an increase in SHBG is a common observation (Glintborg et al., 2014). We noted however, that a single mOCP-user in the cycling group did not exhibit the same SHBG increase as the powerlifter or runner. Previous assessments of hormonal profiles in athletes (mixed sex cohort) participating in different sports did not observe any SHBG differences between athlete group which suggests that our findings may be reflective of the mOCP dose and/or formulation rather than the sport. This finding should be explored further, as well as effects of LARC use and its impacts on vascular function in the context of exercise.

Study Limitations

Study results must be interpreted with respect to several known limitations. First, the small sample size reflects the challenges faced in the recruitment process. The potential perceived study burden (i.e., multiple visits, invasive procedures, cycle tracking, at-home LH testing) may have dissuaded prospective participants. Compared to males, females reportedly differ in their motivations to participate in sport science research which may reflect a potential volunteer bias (Nuzzo, 2021; Nuzzo & Deaner, 2023). Future studies in females should incorporate designs which strike a balance to address both participant burden concerns and methodological requirements. Secondly, a non-athletic control group was not included in this study. As such, we were unable to compare athletes to non-athletes, however that was not the primary aim of this study. We aimed to identify whether a specific female athletic vascular phenotype was evident in athletes participating in diverse sports, each with a unique cardiovascular loading profile. To describe hormonal influences, a within-participant comparison was used where athletes acted as their own control. Also, adequate quality femoral data were unable to be obtained in several powerlifters owing to the athletes' relative larger upper thigh girth. Although we did not quantify thigh girth, this should be noted for future investigations when assessing femoral artery parameters.

CONCLUSION

In conclusion, chronic exercising of differing modalities resulted in no differences in brachial and femoral artery function between athlete groups. However, a distinct structural phenotype was established with endurance-based (high dynamic) athletes having a larger femoral artery diameter in comparison to powerlifting (high static) athletes which may reflect localised adaptation in these female athletes. Despite the distinct LowH and HighH phases there were no differences in brachial and femoral artery function between hormone phases. Although, brachial artery diameter in the powerlifting athletes may be more susceptive to changes in HighH conditions, however an upper-limb vascular phenotype was not clearly discernible.

Considering the methodological quality of vascular studies in female athletes will enable findings to be applied to the broader female athletic population.

As there are an increasing number of females partaking in sport, particularly at a higher level, there is a need to understand their physiology in response to exercise. This is the first study to investigate the potential effects of exercise modality and hormone phase on vascular morphology. The implications of these findings may have an impact on female athlete training, performance and health however further research is needed.

Chapter 4: Thesis Discussion

Main Findings

The results from this thesis have identified that females are underrepresented in vascular exercise studies with only 38% of studies included in our review of the literature conducted in female populations, despite females being included in over 50% of studies. After auditing the quality of studies in female populations using an existing audit framework (Smith et al., 2022), it was determined that there were no gold graded (best-practice) studies in naturally menstruating cohorts, females using HC, or females who had a menstrual cycle irregularity. Only a small number of studies in pregnant and menopausal females were deemed of a sufficiently high methodological quality to be awarded gold grading. In our experimental study, we employed the best-possible practice for methodologies (within the resource constraints of the study) to account for ovarian hormone effects which included cycle tracking, objective hormone measurement and ovulation confirmation. Our key findings suggest a morphological phenotype specific to the femoral arteries of endurance-based (high dynamic) lower limb athletes. We also noticed an upper limb morphological difference between hormone phase in powerlifting athletes, indicative of altered vasoreactivity when ovarian hormones were elevated. There was no apparent functional phenotype for either the upper or lower limb vasculature. Similarly, endothelial function remained unchanged with hormone phase in all athlete groups.

Influence of Exercise on Arterial Structure and Function

Systematic review of the literature revealed that only 25% of the total female population sampled were athletes. These athletes were classified as Tier 2 and Tier 3, with no studies including elite/international level females (Tier 4) or world class (Tier 5). In comparison, female athletes included in the experimental study were classified as Tier 2 and Tier 3 as well as n = 5 athletes classified as Tier 4. The proportion of female athletes at the 2024 Summer Olympic Games is set to reach 50% for the first time, yet the representation of elite female athletes in exercise physiology, particularly vascular studies, is still relatively lower than males despite sex-specific data continuing to emerge

(Cowley et al., 2021; Cowley et al., 2023). As the number of elite female athletes continues to grow, there is a need to understand how these athletes respond to exercise in terms of arterial size and function as this may impact athletic performance and health. Female athletes should be studied during their athletic careers as well as beyond their retirement to appreciate the long-term effects of exercise and the intersection with ageing on arterial parameters.

The primary exercise intervention type in the systematic review of the literature was acute, which was defined as a single session with measures of FMD taken prior to and immediately after the intervention. Acute interventions were used in 62% of studies. There is suggestion that acute exercise may produce an immediate increase in FMD following exercise before returning to normal levels in healthy participants termed the 'exercise paradox' (Dawson et al., 2013). While we did not assess the acute effects of exercise in our experimental study, we conducted at-rest assessments in chronically trained female athletes with a training age of more than two years, across repeated timepoints corresponding with distinct hormone phases. We observed a significant difference in femoral baseline diameter between powerlifters, cyclists and runners. The endurance athletes (cyclists and runners) had the largest femoral artery diameter of the three discrete sporting groups. These results are consistent with meta-analytic evidence of Black and colleagues (2016) which supports the notion of predominantly lower-limb endurance athletes (triathletes, runners) possessing structurally enlarged femoral arteries. This reiterates the proposed mechanism for arterial remodelling being localised to the exercising limb (Rowley et al., 2011). In the experimental study, athletes had two conduit artery beds assessed, each representing the upper and lower limb macrovasculature (brachial and femoral artery, respectively) whereas only 9% of studies in the systematic review assessed more than one artery bed. The purpose of our review was not to meta-analyse the results of the systematic review therefore we cannot draw any definitive conclusions as to whether potential localised changes had been missed in studies which only assessed one artery bed. Future studies should aim to include concurrent

assessment of multiple arterial beds, particularly in females to elucidate any localised adaptive responses.

Menstrual Cycle Influence on Arterial Structure and Function

The review identified 112 studies which assessed female populations, with 72 detailing the menstrual status of their participants. The majority (49%) of studies (including mixed menstrual status) assessed premenopausal females. A small proportion (n = 6) of these studies intentionally did not control for the menstrual cycle. This raises the existing argument as to whether the menstrual cycle needs to be controlled in vascular physiology studies. Wenner and Stachenfeld (2020) argued for the need to control for menstrual phase as there is potential to miss direct or indirect influences of ovarian hormones on endothelial function. Stanhewicz and Wong (2020) reasoned there is no need to control for menstrual phase unless the research question explicitly details the need to address ovarian hormones. Regardless of the debate, studies including female participants should, at the very least, aim to include sufficient methodological information regarding menstrual status to achieve a bronze grading, according to the best-practice methodologies for assessing females. For example, in studies with naturally menstruating populations, researchers should attempt to state i) whether participants experienced menstruation, ii) the average length of a typical menstrual cycle, that is whether the cycle length is between 21 and 35 days (inclusive), iii) whether ovulation was confirmed, iv) whether objective hormone analysis confirmed the cycle phase, v) which menstrual cycle phase participants were assessed using consistent terminology, v) whether participants experienced 9 or more consecutive periods in a year, and vi) state whether hormonal contraception was used in the 3 months prior to the study recruitment (Elliott-Sale et al., 2021).

Studies conducted in female populations predominantly assessed FMD in their participants during the early follicular phase of the menstrual cycle. This is aligned with the current consensus guidelines for best-practice FMD protocols, which ensures limited influences of ovarian hormones as oestrogen and

progesterone concentrations are at their lowest in the early follicular phase (Corretti et al., 2002; Harris et al., 2010; Thijssen et al., 2011; Thijssen et al., 2019). In the experimental study, we assessed female participants in Phase 1 (menses; LowH) and Phase 4 (mid-luteal; HighH) of the menstrual cycle. We can be confident that these were distinctly different phases as we objectively measured and reported serum hormone data and noted a significant difference in E2 and P4 levels between the two phases. Interestingly, we observed a significant difference between hormone phase in the resting brachial artery diameter during the VC% assessment in powerlifters. We did not anticipate this finding as others have reported no difference in arterial parameters between different hormone phases in non-athletic females (D'Urzo et al., 2018, Shenouda et al. 2018). We propose that the higher circulating levels of oestrogen and progesterone during the HighH phase may have contributed to a greater NO bioavailability and prolonged the preceding occlusion stimulus during the brachial FMD% assessment conducted 20-minutes prior (Serviente et al., 2016). It could be postulated that the powerlifters may have been more susceptible to the prior hyperaemic stimulus owing to the repetitive upper limb shear stimulus that powerlifters are exposed to from repeatedly gripping a barbell. It is prudent to acknowledge the methodological design decisions in the experimental study. A longer duration (> 30 minutes) between successive brachial artery assessments may have negated this difference. While our observations warrant further exploration with mechanistic studies, perhaps the recommendation for 10- to 15-minute timeframe between repeated FMD assessments (Corretti et al., 2002) should be re-evaluated with respect to female participants.

Methodologies Used in Studies with Female Participants

The audit framework proposed by Smith and colleagues (2022) derived from the guidelines of Elliott-Sale and colleagues (2021) was used to audit the study methodologies in female participants included in the review. Following the audit, it was determined there were no studies graded gold in naturally menstruating or HC-using female participants, or participants who had a

menstrual irregularity. This agrees with previous studies in performance nutrition literature which also determined there to be minimal studies with sufficient information to be given a gold grade (Smith et al., 2022b; Kuikman et al., 2023). In the experimental study, we utilised the highest-quality methodology possible, within the constraints of the study resourcing. A three-method validation process was used to confirm hormone phase during the participant testing cycle (Schaumberg et al., 2017), along with the methods reporting this would likely be awarded a silver grading. We were unable to track participants for two months prior to their testing cycle, which would otherwise have deemed our study eligible for gold grade. The study faced several challenges with regards to participant recruitment, thus including the additional tracking would have substantially increased the burden on both participants and the research team, as well as the time and cost to conduct the study.

The studies included in the review covered the reproductive lifespan of females including reproductive-aged, pregnant, and menopausal populations. In our review, we expanded the existing audit framework to enable auditing studies conducted in menopausal and pregnant cohorts, which we based on guidelines by Elliott-Sale and colleagues (2021). This revised audit methodology determined there to be 3.5 and 2 studies in postmenopausal and pregnant populations respectively. It may be possible that studies which are focussed on outcomes in specific female populations (pregnancy and menopause) may be more likely to adequately describe the status of participants. Studies in our systematic review which included both male and female participants (mixed cohort), were the least likely to report menstrual status of the female participants with 33 of 68 (48%) mixed cohort studies not providing sufficient information to categorise menstrual status, which perpetuates a male-norm for methods reporting (Williams et al., 2020). In addition, there were only two studies which specifically aimed to report sex-differences regarding vascular and exercise responses, which highlights a significant gap in the available literature.

Thesis Strengths, Limitations and Future Directions

This thesis expanded and applied an existing audit framework used to assess both menstrual status and best-practice methodologies used to assess the impact of ovarian hormones on endothelial function. The audit framework was developed from specific guidelines related to research studies in females (Elliott-Sale et al., 2021, Smith et al., 2022). We included additional categories in the audit framework to include menopause and pregnancy which enabled a lifespan approach to understanding FMD responses to exercise in females, both athletic and non-athletic. The specific guidelines for conducting research in females (Elliott-Sale et al., 2021; Smith et al., 2022) were followed in the experimental study when accounting for the menstrual cycle to ensure we achieved the highest possible methodological quality.

While the review is limited to studies which assessed endothelial function using best-practice FMD guidelines (Corretti et al., 2002; Harris et al., 2010; Thijssen et al., 2011 and Thijssen et al., 2019), further research needs to take into consideration the other protocols used to measure endothelial function via the use of FMD, particularly as FMD methodological differences may be more discriminatory than those relating to menstrual cycle phase (Wilson et al., 2015). The small sample size in the experimental study reflects the challenges faced with recruitment and thus results should be interpreted accordingly. Females have been reported to differ compared to men in their motivations to participate in sport science research which may reflect a potential volunteer bias (Nuzzo, 2021; Nuzzo & Deaner, 2023). This is also reflected in the total female population cohort in the systematic review, with females making up 38% of the total population sampled.

Ensuring best-practice approaches in female athletes to account for potential ovarian hormone influences are time consuming, costly and demand a substantial impost on participants. Developing lower cost and less invasive approaches to blood sampling for serum hormone analysis could be explored further such as existing DBS technology, which is minimally invasive, requires less researcher-training and offers stable storage (Trifonova et al., 2019).

Applying this process to hormonal profiling could considerably reduce the cost and burden on participants hence future research should investigate the validity and reproducibility of alternate technologies. It is also recommended that studies should include diverse contraceptive options, to appreciate the potential impacts of LARC devices and systems on endothelial function as typically only OCP-users are studied, as is the case in both the review and experimental studies presented in this thesis.

CONCLUSION

In conclusion, we have presented evidence which supports the call to include more female participants in exercise and sport science research, particularly in vascular physiology. In female athletes, we observed a lower limb arterial morphological phenotype, specifically in endurance athletes, with no apparent functional phenotype in either the upper or lower limb vasculature. Endothelial function was unchanged with disparate hormones phases, which was confirmed using objective serum hormone analysis. Upper limb female athletes (powerlifters) may have enhanced vasoreactivity when serum hormone analysis is high, however this may reflect methodological design features rather than hormone effects as such. There is a considerable burden in both time and cost associated with conducting vascular research in female populations to adequately account for ovarian hormones. This is reflected with very few high-quality studies available in the current body of literature. To reach equitable female participant representation, a combination of identifying sex-specific barriers to participation as well as technological advancements to make objective hormone analysis more affordable, less time-consuming and less invasive, may entice more females to participate in research studies. To enhance the overall quality of vascular studies in female participants, researchers should aim to adequately describe menstrual status of participants to achieve minimal best-practice reporting guidelines.

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Appendices

Appendix 1 – Ethics Approval Letter

Research Office at Curtin

GPO Box U1987
Perth Western Australia 6845

Telephone +61 8 9266 7863
Facsimile +61 8 9266 3793
Web research.curtin.edu.au

12-May-2021

Name: Angela Spence
Department/School: School of Physiotherapy and Exercise Science
Email: Angela.Spence@curtin.edu.au

Dear Angela Spence

RE: Ethics Office approval
Approval number: HRE2021-0245

Thank you for submitting your application to the Human Research Ethics Office for the project **Is there a female 'athlete's artery'? Characterising arterial phenotype across distinct exercise modalities in the female athlete**.

Your application was reviewed through the Curtin University Low risk review process.

The review outcome is: **Approved**.

Your proposal meets the requirements described in the National Health and Medical Research Council's (NHMRC) *National Statement on Ethical Conduct in Human Research (2007)*.

Approval is granted for a period of one year from **12-May-2021** to **11-May-2022**. Continuation of approval will be granted on an annual basis following submission of an annual report.

Personnel authorised to work on this project:

Name	Role
Spence, Angela	CI
Naylor, Louise	Co-Inv
Brade, Carly	Co-Inv
Thompson, Sarah	Student
Henley-Martin, Sarah	Co-Inv

Approved documents:

Document

Standard conditions of approval

1. Research must be conducted according to the approved proposal
2. Report in a timely manner anything that might warrant review of ethical approval of the project including:
   - proposed changes to the approved proposal or conduct of the study
   - unanticipated problems that might affect continued ethical acceptability of the project
   - major deviations from the approved proposal and/or regulatory guidelines
   - serious adverse events

3. Amendments to the proposal must be approved by the Human Research Ethics Office before they are implemented (except where an amendment is undertaken to eliminate an immediate risk to participants)
4. An annual progress report must be submitted to the Human Research Ethics Office on or before the anniversary of approval and a completion report submitted on completion of the project
5. Personnel working on this project must be adequately qualified by education, training and experience for their role, or supervised
6. Personnel must disclose any actual or potential conflicts of interest, including any financial or other interest or affiliation, that bears on this project
7. Changes to personnel working on this project must be reported to the Human Research Ethics Office
8. Data and primary materials must be retained and stored in accordance with the Western Australian University Sector Disposal Authority (WAUSDA) and the Curtin University Research Data and Primary Materials policy
9. Where practicable, results of the research should be made available to the research participants in a timely and clear manner
10. Unless prohibited by contractual obligations, results of the research should be disseminated in a manner that will allow public scrutiny; the Human Research Ethics Office must be informed of any constraints on publication
11. Approval is dependent upon ongoing compliance of the research with the Australian Code for the Responsible Conduct of Research, the National Statement on Ethical Conduct in Human Research, applicable legal requirements, and with Curtin University policies, procedures and governance requirements
12. The Human Research Ethics Office may conduct audits on a portion of approved projects.

Special Conditions of Approval

It is the responsibility of the Chief Investigator to ensure that any activity undertaken under this project adheres to the latest available advice from the Government or the University regarding COVID-19

This letter constitutes low risk/negligible risk approval only. This project may not proceed until you have met all of the Curtin University research governance requirements.

Should you have any queries regarding consideration of your project, please contact the Ethics Support Officer for your faculty or the Ethics Office at hrec@curtin.edu.au or on 9266 2784.

Yours sincerely

Amy Bowater
Ethics, Team Lead

Appendix 2 – Data Management Plan

Curtin University

Research Data Management Plan

Is there a female 'athlete's artery'? Characterising arterial phenotype across distinct exercise modalities in the female athlete

Principal Investigator	Angela Spence
Data Management Plan Edited by	Angela Spence
Modified Date	13/02/2021
Data Management Plan ID	SPENCA-HS08982
Faculty	Health Sciences

1 Research Project Details

1.1 Research project title

Is there a female 'athlete's artery'? Characterising arterial phenotype across distinct exercise modalities in the female athlete

1.2 Research project summary

It is well established that cardiovascular adaptation is observed following long-term exercise exposure, which is commonly referred to as the 'athlete's heart'. More recently, the question whether an arterial phenotype is apparent in conduit artery adaptation has been raised, specifically, increased lumen diameter, arterial wall thickness and functional adaptation, or the 'athlete's artery'. While there is a suggestion that conduit artery remodelling may be localised to the predominant exercising muscle vascular beds, this has not yet been exclusively studied in female athletes, accounting for menstrual cycle phase, contraception-use, and exercise modality i.e. static versus dynamic sport categorisation. The aim of this study is to cross-sectionally evaluate arterial structure and function in female athletes participating in divergent sporting/exercise modes. We hypothesis that, compared to apparently healthy female controls, athletes will have structurally larger arteries and enhanced functional capacity, due to their athletic status. Furthermore, we expect distinct patterns of adaptation will be evident in female athletes representing specific exercise modalities (i.e. high-static, low-dynamic vs. high-dynamic, low-static, vs. high-static, high-dynamic). Lastly, it is hypothesised that hormonal status (high vs. low hormone concentration; active vs. non-active pill phase) will not impact the degree of observed adaptation. In this cross-sectional study, we will recruit reproductive-aged female athletes (18 - 35 years) with a substantial training history (> 2 years) and level (state/national sport representation) from a range of sporting domains that represent sporting categorisations according to static and dynamic criteria. Participants that are both naturally-cycling or using monophasic oral contraception (OC) will be included, and menstrual cycle tracking including urinary ovulation testing (luteinising hormone, LH) will occur over the study duration (~2 natural/pill cycles). We will also recruit apparently healthy controls, matched for age and OC-use. All participants will undergo the following procedures: non-invasive assessment of arterial structure and function, using high-resolution ultrasound of the brachial and femoral arteries as our primary outcome measure. Specifically, we will use the flow-mediated dilation (FMD) protocol to determine arterial size and function, represented as the change in diameter following 5-min of occlusion using an inflated cuff around the forearm (FMD%). We will also quantify arterial wall thickness (intima-media thickness; IMT) using ultrasound. To determine whether hormone concentration influences the primary outcome measures, these measures will be repeated during the low-(early follicular) and high-hormone (mid-luteal) phases for naturally cycling individuals, and the inactive and active pill phases for OC-users. Serum samples will be collected from participants at both visits and will be analysed for oestrogen and progesterone to confirm hormone phase. For participant characterisation, we will also measure anthropometrics including height, mass and body composition using DXA; blood pressure and peak oxygen consumption using a graded exercise test protocol. Data will undergo statistical analysis using a 4 x 2 (group x time) mixed-model analysis of variance, with significant differences or main effects using appropriate post-hoc testing. The results of this study will describe, in detail, arterial structural and functional differences in female athletes specifically, across a range of sporting domains. This data has implications not only for athletic performance enhancement, but also the overall impact of exercise on cardiovascular health of females in their reproductive-aged years.

1.3 Keywords
Endothelial function, female physiology, female athlete, menstrual cycle

2 Research Project Data Details

2.1 Research project data summary

Not Applicable

2.2 Will the data be identifiable

- Re-identifiable — identifiers have been removed and replaced by a code, but it is possible to re-identify an individual

2.3 Will biospecimens or human participant information be sent overseas?

No

2.4 Will novel information about controlled goods or technologies on the Defence and Strategic Goods List (DSGL) be sent overseas?

No

2.5 Data organisation and structure

Not Applicable

3 Research Project Data Storage, Retention and Dissemination Details

3.1 Storage arrangements

Not Applicable

3.2 Estimated data storage volume

Not Applicable

3.3 Safeguarding measures

Not Applicable

3.4 Retention requirements

7 years (All other research with outcomes that are classed as Minor)

3.5 Collaboration

Not Applicable

3.6 Data dissemination

Not Applicable

3.7 Embargo period

Not Applicable

Appendix 3 – Radiation Safety Approval

Radiation Project Approval

Curtin University

Appendix 5 – Radiation Safety Approval

23 Mar 2021

Project details

Project number	RSC2021-05
Project Title	Characterising arterial phenotype across distinct exercise modalities in the female athlete
Chief Investigator	Angela Spence
School	Curtin School of Allied Health

I am pleased to inform you that your application to conduct this radiation project has been approved. Your application was reviewed against requirements of the Radiation Safety Act 1975 and the Nuclear Non-Proliferation Act 1987 using the Radiation Safety Committee's low risk assessment process.

Approval is granted subject to these conditions:

- Work is conducted as described in the protocol.
- An amendment request must be submitted and approved before implementing any proposed changes to the protocol.
- Self-audit reports are submitted in a timely manner when requested (once a year from the above date).
- A completion report is submitted when the radiation aspects of the project come to an end.
- If a licence holder is no longer able to take responsibility for the radiation producing goods referred to in the protocol then use of the goods must cease until a replacement licence holder is found.

Please note that this approval only relates to the radiation-related aspects of the project. All of Curtin University's research governance requirements must be met for the project to proceed.

Please do not hesitate to contact the Radiation Safety Officer (tim.finney@curtin.edu.au, 9266 1708) if you have any queries.

Best regards,

Tim Finney
Radiation Safety Officer

Health and Safety Department | Version1.0 | safe today >>> safer tomorrow

Appendix 4 – Online Menstrual Cycle Diary

Please complete the following diary each day, starting on the **FIRST DAY** of your period/bleed and ending on the **FIRST DAY** of your next period/bleed. Please bring the completed diary to your First Testing Session at Curtin University, Bentley.

Cycle Day	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**	**22**	**23**	**24**	**25**	**26**	**27**	**28**	**29**	**30**	**31**	**32**
Date																																
No of tampons/ pads/ cups																																
For each the following, record 0 if NONE; 1 if MINIMAL; 2 if MODERATE; 3 if MODERATELY-INTENSE; 4 if VERY INTENSE																																
Cramps																																
Stomach pain																																
Back pain																																
Breast tenderness																																
Fluid retention																																
Constipation																																
Bloating																																
Headaches																																
Sleep disturbances																																
Feeling depressed																																
Feeling anxious																																
Feeling angry/ upset																																
Use descriptors (see below) for:																																
Cervical mucus																																
Indicate positive or negative for:																																
Ovulation test results																																

Cervical mucus descriptors:
(D) Dry: discharge is absent, or there is a slight dampness present
(S) Sticky: discharge is opaque and sticky/rubbery/crumbly
(C) Creamy: discharge is opaque and creamy/lotion-like/milky
(EW) Egg white: discharge is slippery/stretchy and streaked/clear, consistency of raw eggwhite

Appendix 5 – Participant Pre-Screening Form

PARTICIPANT SCREENING FORM

HREC Project Number:	TBC
Project Title:	*Is there a female "athlete's artery'? Characterising arterial phenotype across distinct exercise modalities in the female athlete.*
Chief Investigator:	*Dr Angela Spence, PhD*
Student researchers:	*Sarah Thompson, BSc*
Version Number:	*V1*
Version Date:	15/03/2021

The purpose of this questionnaire is to confirm your eligibility for participation in this study. Please read each question and follow its instructions carefully. You may find some of the details required hard to recall – if so, please make estimates as accurately as possible. Thank you for assisting us by answering these questions.

Section to be completed by participants:

Name:	
Today's Date:	
Date of Birth:	
Age:	
Sex:	

1. Have you or anyone in your household been diagnosed with COVID-19?	Yes / No
2. Are you or anyone in your household unwell and/or have symptoms related to COVID-19? This includes fever, coughing, sore throat or sneezing?	Yes / No

3. Are you or anyone in your household self-isolating, for example, because of any recent travel?	Yes / No
4. Are you a current smoker? Males → **Skip to question 13**	Yes / No
5. Are you currently pregnant?	Yes / No
6. Are you currently trying to conceive?	Yes / No
7. Have you been pregnant in the past six months?	Yes / No
8. Are you currently taking an oral contraceptive pill? If *yes*, what is the name (Brand) of the pill? _____ If yes, do you take the inactive pills? If *no*, have you taken an oral contraceptive pill in the past six months?	Yes / No Yes / No Yes / No
9. Are you currently using any other form of hormonal contraception? If *yes*, please specify in the space below.	Yes / No
10. Do you intend on beginning any form of hormonal contraception within the next three (3) months? If *yes*, please specify in the space below.	Yes / No
11. How long is your menstrual cycle on average (i.e. the number of days between the start of two consecutive periods)?	

_____ days	
12. How many days is you period on average (i.e. how many days do you experience active bleeding)? _____ days	
13. Have you been diagnosed with or previously had any of the following conditions: Cardiovascular disease Polycystic ovarian syndrome Endometriosis Cancer If *yes*, please specify. _____ Stroke Myocardial infarction (heart attack) Congenital heart disease Hypertension Diabetes Dyslipidaemia Metabolic syndrome Renal/kidney disease Rheumatoid arthritis Systemic lupus erythematosus Systemic vasculitis Respiratory diseases, e.g., asthma/cystic fibrosis If *yes*, please specify. _____	 Yes / No Yes / No Yes / No Yes / No Yes / No Yes / No Yes / No Yes / No Yes / No Yes / No Yes / No Yes / No Yes / No Yes / No Yes / No Yes / No
14. Are you currently taking any prescribed medications? If yes, please specify which medication(s) in the space below.	Yes / No

_____	
15. Are you an athlete? ☐ If no → **Skip to question 19**	Yes / No
16. What is your primary sport/activity? _____	
17. What is the highest level you have achieved in your sport? (Select all that apply) ☐ State level representation ☐ National level representation ☐ International/World Championship/Commonwealth Games/Olympic Games level representation	
18. For how long have your participated at this level in your sport? _____ years	
The following questions (19 – 24) are about all the physical activities you did in the **last 7 days** solely for **recreation, sport, exercise or leisure**:	
19. During the **last 7 days**, on how many days did you **walk** for at least 10 minutes at a time **in your leisure time?** **____ days per week** ☐ No walking in leisure time → **Skip to question 21**	
20. How much time did you usually spend on one of those days **walking** in your leisure time? **_____ hours per day** **_____ minutes per day**	
21. Think about only those physical activities that you did for at least 10 minutes at a time. During the **last 7 days**, on how many days did you do **vigorous** physical activities like aerobics, running, fast bicycling, or fast swimming **in your leisure time**? **_____ days per week** ☐ No vigorous activity in leisure time → **Skip to question 23**	
22. How much time did you usually spend on one of those days doing **vigorous** physical activities in your leisure time?	

_____ **hours per day** _____ **minutes per day**	
23. Again, think about only those physical activities that you did for at least 10 minutes at a time. During the **last 7 days**, on how many days did you do **moderate** physical activities like bicycling at a regular pace, swimming at a regular pace, and doubles tennis **in your leisure time**? _____ **days per week** ☐ No moderate activity in leisure time → **Skip to question 25**	
24. How much time did you usually spend on one of those days doing **moderate** physical activities in your leisure time? _____ **hours per day** _____ **minutes per day**	
25. What is your approximate Height _____ cm Body mass _____ kg Note: We will use these to calculate your BMI.	

END OF QUESTIONNAIRE – THANK YOU FOR YOUR ASSISTANCE.