The impact of 17beta-estradiol treatment on insulin sensitivity, immune function, inflammation and skeletal muscle metabolism following exercise training in sedentary, overweight males

Protocol Number: University of Waterloo REB#46726

Principal Investigator: Michaela Devries-Aboud

Sponsor: Michaela Devries-Aboud and University of Waterloo

Funded by: Internal Funding by University of Waterloo

Version Date: July 31, 2026

TABLE OF CONTENTS

1.0 STATEMENT OF COMPLIANCE 4

2.0 PROTOCOL SUMMARY 5

2.1Synopsis 5

2.4 Schema of 16 Day Drug Administration Period 7

2.3Schema of Entire study and visits 7

2.4 Schema of Lab Interventions (Visits 2, 3 and Visits 4-9) 8

3.0 INTRODUCTION 10

3.1 Background & Study Rationale 10

3.2 History of Medication Use 11

3.4 Risk/Benefit Assessment 11

4.0 OBJECTIVES & ENDPOINTS 12

5.0 STUDY DESIGN 14

5.1 Overall Design 14

5.2 Scientific Rationale for Study Design 15

5.3 Completion of the study 16

6.0 STUDY POPULATION 16

6.1 Inclusion Criteria 16

6.3 Lifestyle 17

6.4 Screening Failures 17

6.5 Strategies for Recruitment and Retention 18

7.0 STUDY INTERVENTION 18

7.1 Study Intervention and Administration 18

7.2 Preparation/Handling/Storage/Accountability 26

7.3 Measures to Minimize Bias 27

7.4 Study Intervention Compliance 27

7.5 Concomitant Theory 27

8.0 DISCONTINUATION AND WITHDRAWAL 27

8.1 Discontinuation of Study Intervention 27
8.2 Participant Discontinuation/Withdrawal from the Study 27
8.3 Lost to Follow-Up 28
9.0 STUDY ASSESSMENTS AND PROCEDURES 29
9.1 Overview and Assessments 29
9.2 Adverse Events and Serious Adverse Events 30
10.0 STATISTICAL CONSIDERATIONS 34
10.1 Statistical Hypothesis 34
10.2 Sample Size Determination 34
10.*3* Statistical Analysis 35
11.0 SUPPORTING DOCUMENTATION AND OPERATIONAL CONSIDERATIONS 36
11.1 Regulatory, Ethical, and Study Oversight Considerations 36
11.1.8 Data Handling & Record Keeping 38
12.0 LIST OF ABBREVIATIONS 39
13.0 References: 39

1.0 STATEMENT OF COMPLIANCE

The trial (REB#46726) will be conducted in accordance with this protocol with applicable regulatory requirements. The trial will also be conducted in compliance with the principles of Good Clinical Practice (GCP) and all applicable Health Canada regulatory requirements. The principal investigator (PI) will ensure that no deviations from, or changes to, the study protocol will occur without documented approval from the Research Ethics Board (REB) at the University of Waterloo, except where necessary to eliminate any immediate hazard to trial participants.

The protocol, informed consent form(s), recruitment materials, and all participant materials will be submitted to the REB for review and approval. Approval of both the protocol and the consent form(s) must be obtained before any participant is enrolled. Any amendment to the protocol will require review and approval by the REB, which will be in consultation with Health Canada's Guidance Document for Clinical Trial Sponsors: Clinical Trial Applications before the changes are implemented to the study. All changes to the consent form will be REB approved; a determination will be made regarding whether a new consent needs to be obtained from participants who provided consent, using a previously approved consent form.

Name of Principal Investigator (Print): Michaela Devries-Aboud

Signature of Principal Investigator: Date:

Site Address: 200 University Ave W, Waterloo, ON N2L 3G1

2.0 PROTOCOL SUMMARY

2.1SYNOPSIS

Title:	The impact of 17beta-estradiol treatment on the effects of exercise training on insulin sensitivity, immune function, inflammation and skeletal muscle metabolism in sedentary, overweight males
Study Description:	A repeated measures analysis with a within-between subject interaction design will be used to study the effects of two-weeks of low-volume, high-intensity, interval training (HIIT) between participants supplementing 17β-estradiol and placebo to determine its impact on insulin sensitivity, glycemic control, leukocyte proliferation and function, and inflammatory markers. This study will use a randomized, double-blind, placebo-controlled protocol. Participants will undergo pre-exercise testing (anthropometric assessments, VO2peak testing, oral glucose tolerance test and muscle biopsies), two-weeks of training while taking 17β-estradiol (1 mg/d for 2 days, 2 mg/d for 14 days) or placebo (polycose), followed by post-testing (using the same protocols as pre-testing). This study is expected to take approximately one year to complete. The initial months will involve participant recruitment and randomization into either the 17β-estradiol or placebo group. Each participant will be enrolled in the study for approximately four weeks, including screening, pre-testing, the two-week training and supplementation period, and post-testing. Data collection will be staggered so a new participant begins the protocol every 7-10 days. Such staggering will be implemented to ensure control over lab resources (e.g., equipment availability, staffing, and biopsy scheduling). This approach allows for overlap between participants during the middle and later stages of testing but ensures sufficient time for individualized supervision during testing days and exercise sessions. Based on

	this pacing, and accounting for occasional gaps due to participant compliance, scheduling conflicts, or holidays, data collection is expected to take 10-12 months.
Objectives:	Primary Objective: To observe if 17-β estradiol blunts the effects of HIIT on glycemic control and insulin sensitivity in overweight/obese males? Secondary Objectives: To determine whether supplementation with 17-β estradiol, compared with placebo, attenuates the effects of high-intensity interval training (HIIT) on skeletal muscle insulin signaling in overweight/obese males. To examine how 17-β estradiol, compared with placebo, influences the effect of HIIT on immune cell count in overweight/obese males. To investigate how 17-β estradiol, compared with placebo, alters the effect of HIIT on immune cell function in peripheral blood mononuclear cells from overweight/obese males. To assess how 17-β estradiol, compared with placebo, modifies the effect of HIIT on inflammatory cytokine concentrations in overweight/obese males. To evaluate how 17-β estradiol, compared with placebo, influences the effect of HIIT on maximal oxygen consumption (VO_2max) in overweight/obese males. To determine how 17-β estradiol, compared with placebo, affects the impact of HIIT on mitochondrial content, function, and respiration in overweight/obese males. To assess how 17-β estradiol, compared with placebo, influences the effect of HIIT on intramyocellular lipid content and lipid metabolism capacity in overweight/obese males.

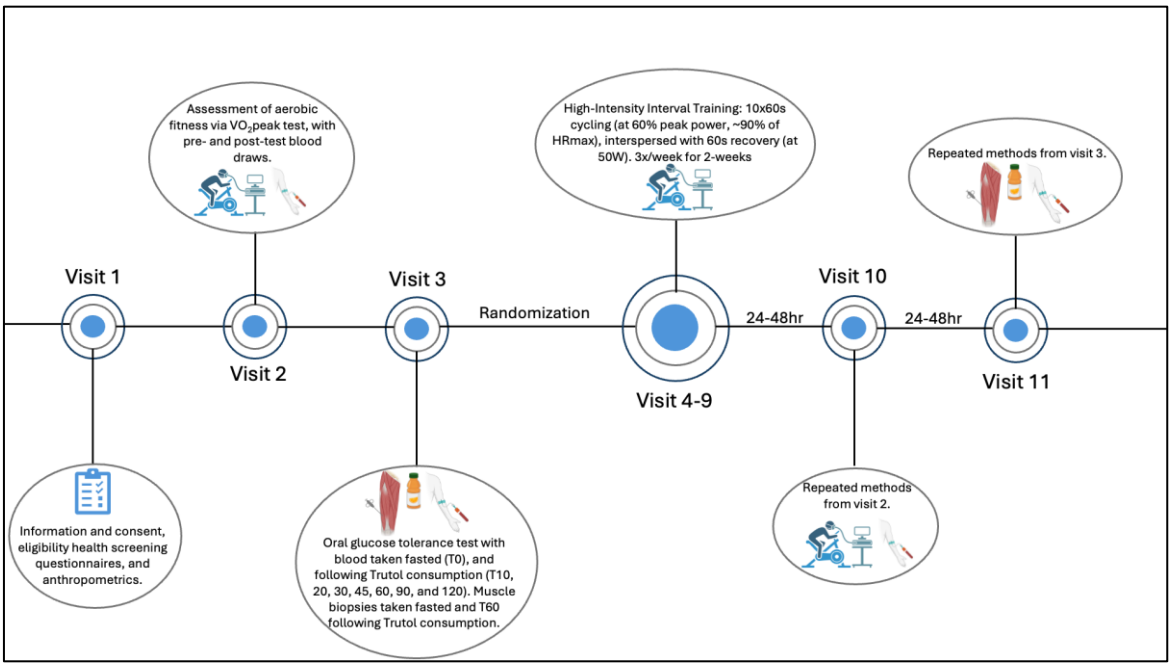
	To determine if estrogen influences post-exercise hormone responses.
Endpoints:	Primary Endpoint: Completion of the pre- and post-exercise testing, completion of the 6 bouts of HIIT, and complying to full supplementation period.
Study Population:	Total sample size = 24. Healthy biological males between 18 and 35 years old with no previous hormonal supplementation). Participants must be sedentary individuals (i.e., engaging in intentional moderate-to-vigorous physical activity <2x/week), with a body mass index 27-32 kg/m^2, and a body fat % > 22%.
Phase:	N/A, other
Description of Study Intervention	Drug Intervention: Placebo: Participants will receive 400 mg per day of glucose polymer for 16 days total. E_2: Participants will receive 1 mg per day for 2 days and 2 mg per day of E_2 (17beta-estradiol) for 14 days.
Study Duration:	2 years
Participant Duration:	11 days (over the span of 1 month)

2.4 SCHEMA OF 16 DAY DRUG ADMINISTRATION PERIOD

Treatment groups and associated doses. Note: PLA=placebo, E=E_2 only group.

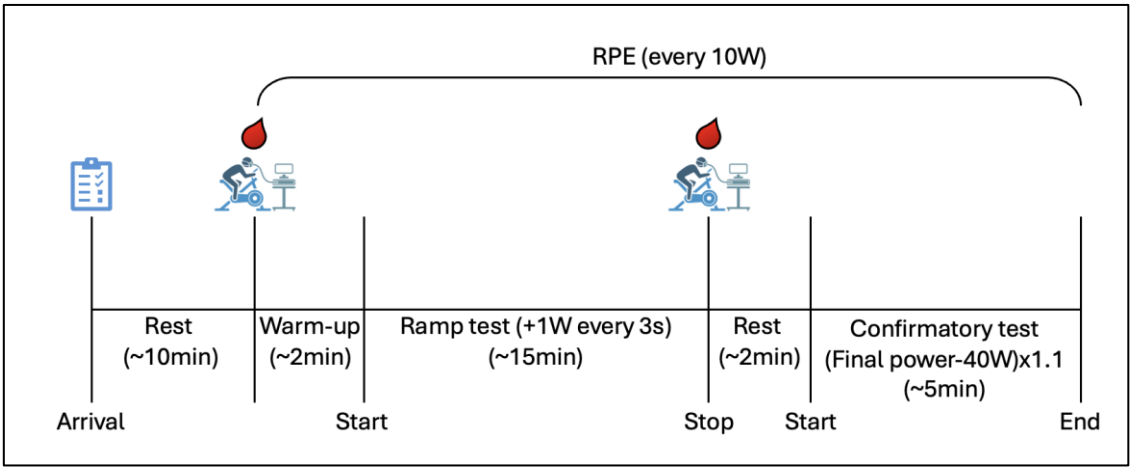
Group	**Type of Treatment Received**
PLA	400 $mg \cdot d^{-1}$ Polycose
E	E_2; 1 $mg \cdot d^{-1}$ for 2 d & 2 $mg \cdot d^{-1}$ of E_2 for 14 d (DIN: 02451255)

2.3SCHEMA OF ENTIRE STUDY AND VISITS



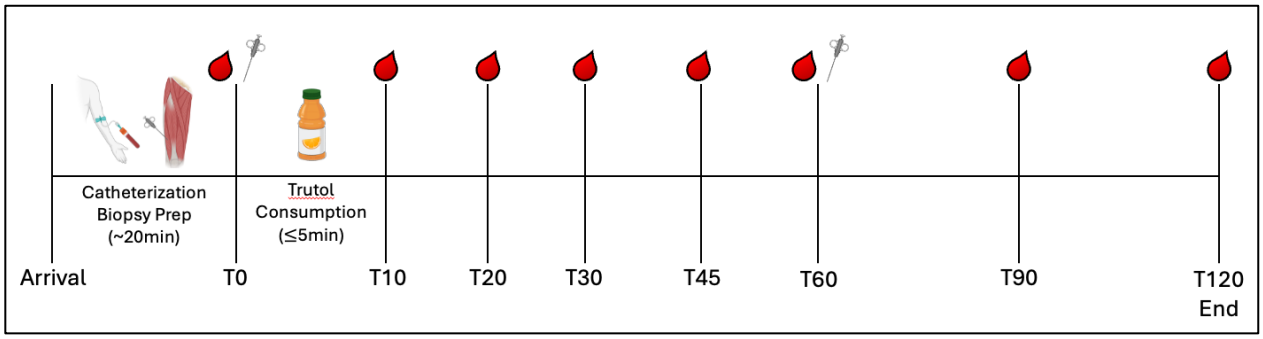
Study design overview. Participants will be randomized into their treatment groups following pre-training testing (i.e., visit 3) and will return to the lab for six high-intensity interval sessions (~30 min each). Upon completing training, participants will complete post-training testing (visit 10 and 11).

2.4 SCHEMA OF LAB INTERVENTIONS (VISITS 2, 3 AND VISITS 4-9)

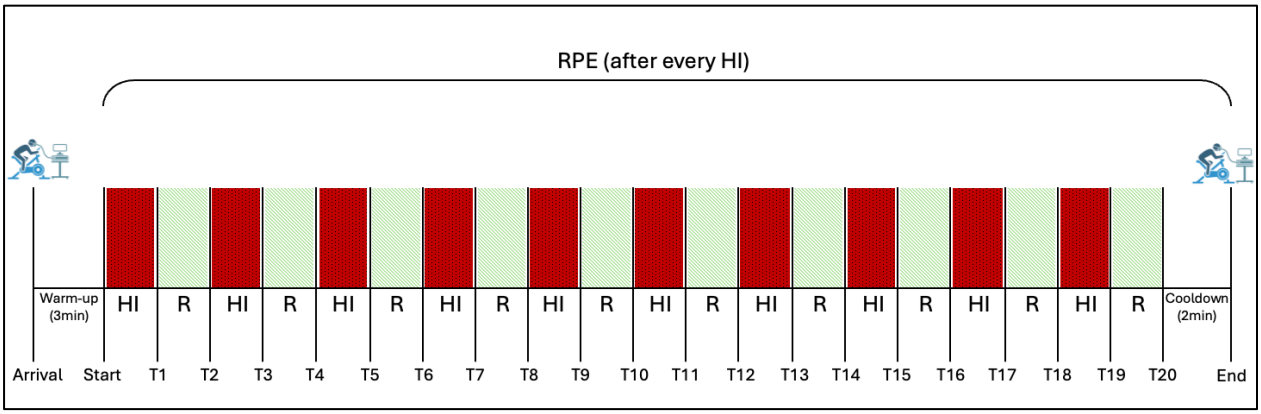


Visit 2 will allow for assessment of their current aerobic fitness. Participants will be asked to complete the immunological, and sleep screening questionnaire as these are all variables that could mitigate some of the desired responses viewed in the immunological parameters (depicted by clipboard in schema). They will complete an assessment of maximal aerobic capacity (VO2peak).

The VO2peak test will be used to determine the loads used for high-intensity training. Prior to commencing VO2peak test, they will have a catheter inserted into a forearm. Participants will have a catheter inserted into the antecubital and a pre-exercise testing blood sample will be taken (depicted by blood droplet in schema). The VO2peak test will start with a warm up on a bicycle at a low intensity. During the test the intensity will continue to increase until they reach a point where they can no longer maintain the exercise. At this point the test will be stopped and they will cool down on the bike at a low intensity (not depicted in schema). Participants will have a post-exercise testing blood sample taken immediately following the exercise test (depicted in schema by the blood droplet), but before the cool-down, and the catheter will be removed. Throughout the test, they will have a mouthpiece in their mouth that will collect the air that they breathe. Rating of perceived exertion (RPE) will be measured following every 10W increase. NOTE (not depicted in schema): following the VO2peak test, participants will rest for one hour and venous blood samples (6 mL, EDTA) will be collected at 30 and 60mins post-VO2peak.



Visit 3 and 11 overview occurring onsite at University of Waterloo, where participants will report to the lab following an overnight fast. Participants will need to have refrained from from moderate-to-vigorous physical activity for 72 h and alcohol for 48 hours prior to this visit. During this visit, participants will complete a baseline OGTT and muscle biopsy. The OGTT and muscle biopsy procedure is as follows. Participants will have a catheter placed in an antecubital vein and a fasted blood sample will be taken. A muscle biopsy (~100-150mg) will then be taken from the vastus lateralis (depicted by the biopsy probe in schema) ~20cm proximal to the knee under local anaesthetic (1% lidocaine) using a custom-suction-modified Bergstrom needle (5mm diameter), as previously described (Tarnopolsky et al., 2011). Following the muscle biopsy, participants will consume a 75g glucose beverage (we will use one of two OGTT products based on availability. Either Trutol beverage which has an NPN 01932179 or Azer Scientific Glucose Drink with NPN 800036990) and blood samples will be taken at 10, 20, 30, 45, 60, 90, and 120-minutes following glucose consumption (depicted by blood droplet in schema) and a muscle and fat biopsy will be taken at 60 minutes following glucose consumption (depicted by the biopsy probe in schema).



High-intensity interval training overview (visits 4 to 9) occurring onsite at University of Waterloo.
Regardless of group, after completing pre-testing, participants will be asked to return to the lab for training (3 sessions/week for 2 weeks). Participants will attend the BMH 2407 lab wearing exercise attire (e.g., shorts, running shoes, t-shirt, etc.). Participants will also be asked to wear a Polar telemetric heart rate monitor (Polar, Canada) for the measurement HR) throughout the training session. The exercise training protocol will be completed, whereby the participant will begin cycling with a warm-up at 50 watts (W) for a total of 3-mins. The high-intensity interval training (HIIT) protocol will immediately follow the warm-up and will consist of 10 x 60 second cycling intervals [at 60% peak power, ~90%HRmax (depicted with 'HI' in schema)] interspersed with 60 second recovery (depicted with 'R' in schema) (at 50W). Upon completion of the exercise protocol, the participants will be asked to complete a cool- down at 50W for a total of 2-mins. The entire duration of the exercise session will be 25-mins. RPE will be measured following each high-intensity interval. Urine will be collected at visits 5, 7 and 9 to monitor estrogen fluctuations.

3.0 INTRODUCTION

3.1 BACKGROUND & STUDY RATIONALE

According to Diabetes Canada (2025), approximately 1 in 10 Canadians (over 4 million people), are living with diabetes, and 90% of these cases (3.6 million) are type 2 diabetic, underscoring an urgent public health challenge. The number of diagnoses are expected to increase 26% by 2033. Physical inactivity is associated with higher prevalence of type 2 diabetes (T2D) (Hosseini et al., 2019), and exercise is known to improve insulin sensitivity and prevent T2D (Metcalfe et al., 2012).

Biological sex influences the prevalence of T2D, with males being diagnosed more than females (De Paoli et al., 2014). However, recently it's become apparent that the response to exercise training is also mitigated by biological sex where females have a blunted/absent improvement in glycemic control and insulin sensitivity following exercise training (Gillen et al., 2014; Metcalfe et al., 2012; Richards et al., 2010; Whyte et al., 2010). Reasons for this difference in prevalence and response to training are not understood, but may stem from sex differences in inflammation, which is likely mediated by estrogen (Nowak et al., 2019). Females have higher circulating white blood cells,

which promotes anti-inflammatory cytokine release and reduces the risk of developing type 2 diabetes.

Estrogen induces an anti-inflammatory effect by binding to its receptors on white blood cells, which enhances white blood cell function (Gillum et al., 2011; Oertel-Prigione, S., 2011). Supporting estrogen's protective role in inflammation and insulin sensitivity, sex differences in insulin sensitivity are absent in post-menopausal females and age-matched males, who are both more prone to impaired insulin signaling (De Paoli et al., 2014). While a chronic pro-inflammatory state increases the risk of insulin resistance and type 2 diabetes, acute increases in inflammation induced by exercise are important for inducing adaptations (Ashcroft et al., 2024; Egan and Sharples, 2023). Importantly, the inflammatory response to an acute bout of exercise is blunted in females (Tiidus, 2001; Gillum, 2001) and thus may explain the blunted improvement in glycemic control and insulin sensitivity post-training.

Therefore, we hypothesize that estrogen-driven anti-inflammatory cytokines protect females from glucose homeostasis impairments, while males depend more on external stressors, such as exercise, to achieve this. However, the mechanisms supporting this hypothesis are poorly understood. Thus, the purpose of this study will be to examine whether 17-beta estradiol blunts the effects of training on improvements in glycemic control and insulin sensitivity. We will also examine whether 17-beta estradiol blunts the effects of an acute bout of exercise on immune cell function and inflammatory markers. Lastly, we will also examine the effects of 17-beta estradiol on other training adaptations, including mitochondrial content and function, lipid metabolism and lipid storage.

3.2 HISTORY OF MEDICATION USE

17β-Estradiol has been widely studied for its role in physiological adaptations to exercise, particularly in the context of sex-based differences in metabolism, cardiovascular health, and musculoskeletal function. It has been shown to influence substrate utilization by enhancing lipid oxidation and modifying glucose kinetics at rest and during exercise. Additionally, 17β-estradiol modulates vascular function, improves endothelial responsiveness, and affects thermoregulation – all key factors in exercise performance and recovery. In postmenopausal women, hormone replacement therapy containing 17β-estradiol has been associated with the preservation of lean mass, maintenance of bone mineral density, and reduced exercise-induced inflammation. These findings support the inclusion of 17β-estradiol in exercise physiology research examining hormonal influences on performance, adaptation, and recovery. However, its role in modulating the exercise-insulin signalling pathway, and immune function remains poorly understood, despite emerging evidence suggesting it may impact glycemic control and immune function.

3.4 RISK/BENEFIT ASSESSMENT

The dosing protocols for these hormones have previously shown no adverse effects (in previous studies conducted by Dr. Michaela Devries-Aboud). Under the guidance of our qualified investigator and on-staff physician, Dr. Sean Gartner (gartners@rogers.com), the short-term use in this study is expected to pose minimal to no risk. Any concerning symptoms will prompt consultation between the principal investigator, Dr. Michaela Devries-Aboud, and Dr. Gartner. While generally well tolerated, mild side effects such as bloating, headaches, and migraines may occur, with rare reports of nausea, vomiting, diarrhea. Certain laboratory values may be mitigated

by estrogen supplementation, however, in healthy young individuals, minor symptoms and laboratory values are typically mild and resolve within 24 hours of stopping the medication. There are also very small risks pertaining to fertility, decreased libido erectile changes, stroke, venous thromboembolism, nipple tenderness, breast tenderness, mood and/or psychiatric changes, fluid retention, hepatic/biliary/pancreatic effects. However, given the small dose and duration of supplementation the likelihood of the risks mentioned above are unlikely in healthy individuals. There are no direct benefits to participants from the drug administration.

4.0 OBJECTIVES & ENDPOINTS

Objectives	Endpoints	Justification for Endpoints
Primary		
To observe if 17-β estradiol blunts the effects of HIIT on glycemic control and insulin sensitivity in overweight and obese males?	Completion of the pre-training tests, HIIT as well as the post-training tests.	Exercise improves insulin sensitivity and helps prevent T2D, but recent studies show females may experience blunted benefits compared to males. Estrogen likely contributes to this difference, as it influences fuel use, lipid storage, mitochondrial enzymes, and promotes antioxidant and anti-inflammatory effects. Since acute ROS and inflammation are important for exercise adaptation, estrogen may dampen these signals, potentially explaining reduced training responses in females.
Secondary		
To determine whether supplementation with 17-β estradiol, compared with placebo, attenuates the effects of high-intensity interval training (HIIT) on skeletal muscle insulin signaling in overweight/obese males. To examine how 17-β estradiol, compared with placebo, influences the effect	Secondary Endpoint: Completion of the entire 11 visits.	The secondary endpoint aims to provide additional insight into potential mechanisms that may help explain the previously observed sexual dimorphism.

of HIIT on immune cell count in overweight/obese males. To investigate how 17-β estradiol, compared with placebo, alters the effect of HIIT on immune cell function in peripheral blood mononuclear cells from overweight/obese males. To assess how 17-β estradiol, compared with placebo, modifies the effect of HIIT on inflammatory cytokine concentrations in overweight/obese males. To evaluate how 17-β estradiol, compared with placebo, influences the effect of HIIT on maximal oxygen consumption (VO_2max) in overweight/obese males. To determine how 17-β estradiol, compared with placebo, affects the impact of HIIT on mitochondrial content, function, and respiration in overweight/obese males. To assess how 17-β estradiol, compared with placebo, influences the effect of HIIT on intramyocellular lipid content and lipid metabolism capacity in overweight/obese males. To determine if estrogen influences post-exercise hormone responses. To assess how subcutaneous fat cells impact cytokine and adipokine secretion		

5.0 STUDY DESIGN

5.1 OVERALL DESIGN

A repeated measures analysis with a within-between subject interaction design will be used to study the effects of two-weeks of low-volume, high-intensity, interval training (HIIT) between participants supplementing 17β-estradiol and placebo to determine its impact on insulin sensitivity, glycemic control, leukocyte proliferation and function, and inflammatory markers. This study will use a randomized, double-blind, placebo-controlled protocol. Participants will undergo pre-exercise testing (anthropometric assessments, VO_2peak testing, oral glucose tolerance test and muscle biopsies), two-weeks of training while taking 17β-estradiol (1 mg/d for 2 days, 2 mg/d for 14 days) or placebo (polycose), followed by post-testing (using the same protocols as pre-testing). This study is expected to take approximately one year to complete. The initial months will involve participant recruitment and randomization into either the 17β-estradiol or placebo group. Each participant will be enrolled in the study for approximately four weeks, including screening, pre-testing, the two-week training and supplementation period, and post-testing. Data collection willbe staggered so a new participant begins the protocol every 7-10 days. Such staggering will be implemented to ensure control over lab resources (e.g., equipment availability, staffing, and biopsy scheduling). This approach allows for overlap between participants during the middle and later stages of testing but ensures sufficient time for individualized supervision during testing days and exercise sessions. Based on this pacing, and accounting for occasional gaps due to participant compliance, scheduling conflicts, or holidays, data collection is expected to take 10-12 months.

Primary objective:

1. Does 17-β estradiol blunt the effects of HIIT on glycemic control and insulin sensitivity in overweight/obese males?
    a. We hypothesize improvements in glycemic control and insulin sensitivity induced by HIIT will be attenuated in overweight/obese males supplemented with 17-β estradiol

Secondary objectives:

1. Does supplementation with 17-β estradiol compared with placebo attenuate the effects of high-intensity interval training (HIIT) on skeletal muscle insulin signaling in overweight/obese males?
    a. We hypothesize improvements in skeletal muscle insulin signaling induced by HIIT will be attenuated in overweight/obese males supplemented with 17-β estradiol
2. How does 17-β estradiol compared with placebo influence the effect of HIIT on immune cell count in overweight/obese males?

    a. We hypothesize immune cell count will increase to a greater extent during HIIT with 17-β estradiol compared to placebo in overweight/obese males
3. How does 17-β estradiol compared with placebo influence the effect of HIIT on immune cell function in peripheral blood mononuclear cells taken from overweight/obese males?
    a. We hypothesize immune cell function will increase to a greater extent during HIIT with 17-β estradiol compared to placebo in overweight/obese males
4. How does 17-β estradiol compared with placebo influence the effect of HIIT on inflammatory cytokine concentration in overweight/obese males?
    a. We hypothesize 17-β estradiol will increase inflammatory cytokine concentration to a greater extent during HIIT compared to placebo
5. How does 17-β estradiol compared with placebo influence the effect of HIIT on maximal oxygen consumption (VO2max) in overweight/obese males?
    a. We hypothesize there will be no difference in the effect of HIIT on VO2max between males supplemented with 17-β estradiol and males supplemented with placebo
6. How does 17-β estradiol compared with placebo influence the effect of HIIT on mitochondrial content, function and respiration in overweight/obese males?
    a. We hypothesize there will be no difference in the effect of HIIT on mitochondrial content, function or respiration between males supplemented with 17-β estradiol and males supplemented with placebo
7. How does 17-β estradiol compared with placebo influence the effect of HIIT on intramyocellular lipid content and lipid metabolism capacity in overweight/obese males?
    a. We hypothesize intramyocellular lipid content and lipid metabolism capacity will increase to a greater extent in males supplemented with 17-β estradiol compared with placebo
8. How does 17-β estradiol influence post-exercise hormone responses?
    a. We hypothesize that 2 weeks of 17-β estradiol supplementation in males will blunt post-exercise growth hormones and epinephrine concentrations.
9. How does 17-β estradiol and training mitigate adipokine and cytokine secretion in subcutaneous adipocytes compared to placebo?
    a. We hypothesize that 17-β estradiol supplementation will increase adipokine and cytokine secretion compared to females.

5.2 SCIENTIFIC RATIONALE FOR STUDY DESIGN

We employed a randomized controlled trial (RCT) with a double-blind, repeated-measures design to ensure the highest level of internal validity and control over confounding variables. Randomization minimizes selection bias by equally distributing both known and unknown confounders across the estradiol and placebo groups. A double-blind approach prevents performance and measurement bias by ensuring that neither participants nor researchers are aware of group allocation, which is particularly important when assessing physiological and immunological outcomes susceptible to expectation effects. The repeated-measures component allows each participant to serve as their own control across timepoints, reducing inter-individual variability and enhancing statistical power to detect meaningful within-subject changes in response to the intervention. This design is especially well-suited to evaluating the physiological effects of 17-β estradiol supplementation in combination with HIIT.

5.3 COMPLETION OF THE STUDY

A participant is considered to have completed the study if he or she has completed all phases of the study including the last visit or the last scheduled post-exercise test taking place on visit 11 (see schematic overview above). The duration of participation for each individual participant who completes all study visits will be approximately 11 visits over the span of 4-weeks. Data collection willbe staggered so a new participant begins the protocol every 7-10 days. Such staggering will be implemented to ensure control over lab resources (e.g., equipment availability, staffing, and biopsy scheduling). This approach allows for overlap between participants during the middle and later stages of testing but ensures sufficient time for individualized supervision during testing days and exercise sessions. Based on this pacing, and accounting for occasional gaps due to participant compliance, scheduling conflicts, or holidays, data collection is expected to take 10-12 months.

6.0 STUDY POPULATION

6.1 INCLUSION CRITERIA

2. Biological males (with no previous hormonal supplementation).
3. Between the ages of 18-35 years old
4. Non-smokers
5. Sedentary individuals (i.e., engaging in intentional moderate-to-vigorous physical activity <2x/week)
6. Body mass index 27-32 kg/m^2
7. Body fat % > 22%

Exclusion Criteria

1. Any state that can impact the hormonal milieu (e.g., drug/hormonal supplementation related to health).
2. Usage of anti-inflammatory medications, >1 glucose lowering medications, insulin, platelet inhibitors, anti-coagulant medications or simvastatin, beta-blockers, weight loss medications (Orlistat, Saxenda, Contrave, Ozempic) or any medications known to affect protein metabolism (i.e., corticosteroids).
3. Presence of cardiovascular, respiratory, metabolic, autoimmune or renal disease.
4. Presence of any condition that makes the participant unable to participate in physical activity as determined using the CSEP Get Active Questionnaire.
5. Presence of injury that restricts exercise performance.
6. VO2max > 50th percentile for age.
7. Previous (within the last 2 months) or current use of ergogenic aids (i.e. creatine).
8. Previous or current use of weight loss medications, including liraglutide, semaglutide, orlistat, ozempic, etc.
9. History and/or diagnosis of blood clots or family history of blood clot disorders.
10. History of allergies or sensitivities to local anesthetic
11. Ophthalmic vascular disease
12. Classical migraines
13. History of thromboembolic disease

14. Consume 15 or more alcoholic drinks/week or 3 drinks per day
15. Bleeding disorders
16. Severe peripheral neuropathy
17. Muscular dystrophy
18. Prior bariatric surgery
19. Osteoporosis/osteopenia
20. Orthopaedic issues
21. Presence of GI disorders (i.e., Colitis, Crohns disease, Celiac disease)
22. Presence of liver disease or dysfunction (i.e., diagnosed NAFLD, NASH, cirrhosis, previous liver transplant)
23. History of cancer
24. Type 1 or 2 diabetes
25. Presence of renal disorders (creatinine > 140)
26. Uncontrolled hypertension (>140/90 mmHg)
27. History of allergies or sensitivities to estradiol or to any ingredient in the formulation or component of the container
28. History and/or diagnosis of blood clots or family history of blood clot disorders
29. Have an implantable electronic device
30. Medical psychiatric history of depression, bipolar, anxiety, suicidal ideation, etc.
31. Inherited clotting disorders
32. Chest pain or shortness of breath
33. Inflammatory disorders (e.g., inflammatory bowel disorder)
34. Significant weight loss in the 3-month period prior to the study (10% of total body weight)
35. Any kind of major surgery recently
36. Bleeding disorders
37. Active or history of thromboembolic disease (including confirmed venous thromboembolism)
38. Ophthalmic vascular disease
39. Classical migraines

6.3 LIFESTYLE

Throughout participation for this study, participants will be asked to maintain regular daily activities (i.e., typical physical activity and eating behaviours), however will be asked the day before both oral glucose tolerance tests for the study (visit 3 and 11) to:

1. Refrain from consuming anything but water (including caffeine) at a minimum of 6 hours prior to their scheduled visit (or be fasted overnight and attend to the lab in a fasted state).
2. Refrain from moderate-vigorous physical activity 72 hours before the oral glucose tolerance test scheduled on visit 3.
3. Refrain from alcohol consumption 48 hours before visit 3.

6.4 SCREENING FAILURES

Screen failures refer to individuals who consent to participate in the clinical trial but are not subsequently randomized or enrolled in the study. To ensure transparent reporting, a minimal dataset – including demographic information, reason for screen failure, and relevant eligibility

criteria – will be collected. Individuals who are deemed ineligible for any reason will not be re-screened and will not be permitted to participate in the trial.

6.5 STRATEGIES FOR RECRUITMENT AND RETENTION

Participants will be recruited through multiple channels to ensure broad reach across the University of Waterloo and the surrounding community. Email invitations will be distributed to students, postdocs, faculty, and staff, accompanied by a recruitment script. Additionally, a recruitment notice will be included in the University of Waterloo's daily bulletin. Posters will be displayed at approved locations on campus and throughout the Kitchener-Waterloo area. Recruitment will also be promoted on social media using a post that includes both an image of the recruitment poster and a standardized caption.

7.0 STUDY INTERVENTION

7.1 STUDY INTERVENTION AND ADMINISTRATION

7.1.1 STUDY INTERVENTION DESCRIPTION

The study intervention involves the oral administration of either 17-β estradiol (E2) or a placebo. While E2 is naturally present in both males and females, it is predominantly found at higher levels in females. Administering the hormone or placebo for approximately 16-days is intended to elevate circulating E2 concentrations to levels more comparable to those observed in females. The medication will be prepared and encapsulated in gel capsules by a licensed Canadian pharmacy, then packaged into blister packs for distribution.

All participants, regardless of assigned treatment arm (E2 or placebo), will enter a follow-up period of two weeks after completion of the 15-day supplementation period or after early discontinuation of the investigational product. The follow-up period will be identical for both treatment groups.

7.1.2 DOSING AND ADMINISTRATION

The dosing schedule for both the estradiol and placebo groups follows the protocol established in previous studies examining the effects of E2 on glucose turnover, muscle glycogen utilization, and overall carbohydrate metabolism in both males and females (e.g., Devries et al., 2005). Each participant will be assigned to a single study arm and will not complete more than one condition.

E2: Oral, participants will receive 1 mg per day for 2 days and 2 mg per day of E2 for 14 days. The supplementation will be consumed orally at the same time every day for 16 days total.

Placebo: Oral, participants will receive 400 mg per day of glucose polymer for 16 days total. The supplementation will be consumed orally at the same time every day for 16 days total.

Follow-Up Period (All Arms): Following the final dose of investigational product (Day 23) or upon early withdrawal, participants in both the E2 and placebo groups will be followed for a period of two weeks for safety monitoring. During this follow-up period, no additional investigational product will be administered. Safety follow-up will include documentation of any adverse events

and follow-up contact from the study team within two weeks of treatment completion and provide an opportunity to report any concerns.

7.1.3 DOSE ESCALATION OR MODIFICATIONS

There will be no dose escalations or modifications throughout the study.

7.1.4 SCHEDULE OF ACTIVITIES

Day (s)	Visit	Drug (dose)	Intervention	Location
1	1	N/A	Information and consent letter health screening eligibility questionnaires body composition measures (fat mass, body fat %, lean mass, lean mass %, height/weight)	BMH 2407, University of Waterloo
2	2	N/A	Pre-exercise blood sample VO2peak test Post-exercise hormone concentrations Post-exercise blood sample	BMH 2407, University of Waterloo
5	3	N/A	Oral glucose tolerance test Blood sample taken fasted, followed by 7 time points following Trutol ingestion Muscle sample taken fasted and 60-minutes following Trutol ingestion	BMH 2407, University of Waterloo

8	4	1mg take in morning	HIIE session	BMH 2407, University of Waterloo
9	N/A	1mg take in morning	N/A	N/A
10	5	2mg take in morning Urine sample collected via Mira	HIIE session	BMH 2407, University of Waterloo
11	N/A	2mg take in morning	N/A	N/A
12	6	2mg take in morning	HIIE session	BMH 2407, University of Waterloo
13	N/A	2mg take in morning	N/A	N/A
14	N/A	2mg take in morning	N/A	N/A
15	7	2mg take in morning Urine sample collected via Mira	HIIE session	BMH 2407, University of Waterloo
16	N/A	2mg take in morning	N/A	N/A
17	8	2mg take in morning	HIIE session	BMH 2407, University of Waterloo
18	N/A	2mg take in morning	N/A	N/A
19	9	2mg take in morning	HIIE session	BMH 2407, University of Waterloo

		Urine sample collected via Mira		
20	N/A	2mg take in morning	N/A	N/A
21	N/A	2mg take in morning	N/A	N/A
22	10	2mg take in morning	Pre-exercise blood sample VO2peak test Post-exercise hormone concentrations Post-exercise blood sample	BMH 2407, University of Waterloo
23	N/A	2mg take in morning	Last supplementation of estrogen	N/A
24	11	N/A	Oral glucose tolerance test Blood sample taken fasted, followed by 7 time points following Trutol ingestion Muscle sample taken fasted and 60-minutes following Trutol ingestion	BMH 2407, University of Waterloo
25-54	N/A	N/A	Continuous (daily reporting) follow-ups for potential symptoms or adverse effects.	N/A

Analysis of the following measures will be completed either *post hoc* or immediately following collection. Analysis of the the desired measures will take approximately 1-2 years for full completion.

Measure	**Scientific method to measure**	**Collection Visit**	**Assessment Timeline**
Glucose	Hexokinase-based assay	Blood samples will be collected during the oral glucose tolerance test day.	Analytes will be assessed following completion of participant recruitment and sample collection, with all samples analyzed in batches within ~6 months of the final participant visit.
Insulin	Enzyme-linked immunoassay (ELISA)/radioimmunoassay (RIA)	Blood samples will be collected during the oral glucose tolerance test day.	Analytes will be assessed following completion of participant recruitment and sample collection, with all samples analyzed in batches within ~6 months of the final participant visit.
Insulin sensitivity	Using standardized calculations	Calculations derived from oral glucose tolerance test data.	Insulin sensitivity analyses will be conducted following completion of participant recruitment and data collection, with calculations completed within ~6 months of the final participant visit.

Insulin resistance	Using standardized calculations	Calculations derived from oral glucose tolerance test data	Insulin resistance analyses will be conducted following completion of participant recruitment and data collection, with calculations completed within ~6 months of the final participant visit.
Markers of insulin signalling	Western blot of the following markers: AMPKα Thr^{172} & total AMPK α ; phosphorylated Akt Ser^{437} & total Akt; phosphorylated TBC1D4 Thr^{642} & total TBC1D4; phosphorylated TBC1D1 Ser^{237} & total TBC1D; total GLUT4	Muscle samples will be collected during the oral glucose tolerance test day.	Analytes from muscle will be collected on the oral glucose tolerance day and will be assessed following completion of participant recruitment and sample collection, with all samples analyzed in batches within ~6 months of the final participant visit.
Membrane bound GLUT4	Stained via immunofluorescence and measured via microscopy	Muscle samples derived from the oral glucose tolerance test day.	Samples will be embedded in appropriate mounting medium and stored at -80°C until future cryosectioning and analysis. Assessements will follow completion of participant recruitment and sample collection, with all samples analyzed in batches within ~6 months of

			the final participant visit.
Peripheral blood mononuclear cells	Isolated using Ficoll-Paque density gradient and measured via flow cytometry	PBMCs will be processed on the same day as the VO2peak blood collection.	Subsequent analyses will be completed within 48 hours of sample collection.
Cytokine	Will be determined using a semi-quantitative 40-plex cytokine array commercially available kits. Based on the results from the array, quantitative measurements using ELLA will be completed	Blood, muscle, and fat samples from the VO2peak test, and oral glucose tolerance tests.	Cytokines will be collected from the supernatant of isolated PBMCs and stored at -80°C until analysis. Analyses will be conducted in batches following completion of participant recruitment and sample collection, with all samples analyzed within ~6 months of the final participant visit. Additionally, cytokines from skeletal muscle and adipose tissue will be obtained from biopsies collected during the oral glucose tolerance test visit. These samples will be homogenized and stored at -80°C until analysis, which will be conducted in batches following completion of participant recruitment and sample collection, within ~6 months of

			the final participant visit.
Mitochondrial content	Will be measured via electron microscopy of the muscle samples	Muscle samples derived from the oral glucose tolerance test day.	Samples will be embedded in appropriate mounting medium and stored at -80°C until future cryosectioning and analysis, in accordance with standardized protocols to ensure structural preservation and data quality.
Mitochondrial function	Will be measured in muscle samples using high-resolution respirometry (HRR), using an Oroboros	Muscle samples derived from the oral glucose tolerance test day.	Samples will be processed immediately following biopsy, and analyses will be completed on the same day to ensure sample integrity.
IMCL content and metabolism	Will be measured via electron microscopy and western blotting of muscle biopsies.	Muscle samples derived from the oral glucose tolerance test day.	Samples will be embedded in appropriate mounting medium or homogenized and stored at -80°C until future cryosectioning and analysis, in accordance with standardized protocols to ensure structural preservation and data quality. The stored samples will be assessed in batches following completion of participant

			recruitment and sample collection, within ~6 months of the final participant visit.
VO2peak	Will be measured using a graded exercise test and a metabolic cart	VO2peak test day.	Will be collected during the study on the VO2peak testing day.
Hormones	Estradiol, progesterone, growth hormone and glucagon, epinephrine will be measured using commercially available kits and Mira.	Blood samples from the VO2peak test, and/or oral glucose tolerance tests.	These samples will be stored at -80°C until analysis, which will be conducted in batches following completion of participant recruitment and sample collection, within ~6 months of the final participant visit. Urine will be collected at visits 5, 7 and 9 to monitor estrogen fluctuations throughout the trial.

7.2 PREPARATION/HANDLING/STORAGE/ACCOUNTABILITY

7.2.1 ACQUISITION AND ACCOUNTABILITY

The drugs and placebo will be acquired at a Canadian pharmacy, independent from the PI or any study team member, that will package all pills in custom made blister packs. Randomization will be performed by a researcher in the department of Kinesiology at the University of Waterloo.
The blister packs will be acquired by the qualified investigator who will (or a delegate will) distribute them to the participants. Any unused pills will be brought to a pharmacy for disposal.
Drug accountability record will be used to record drug information, including date received, quantity received, expiry date, lot number, quantity dispensed, and quantity returned.

7.2.2 FORMULATION, APPEARANCE, PACKAGING AND LABELLING

E_2: White, round engraved pills which will be packaged into gel capsules and made into blister packs.

Placebo: Glucose polymer prepared by the pharmacy to be into identical gel capsules and into custom made blister packs.

7.2.3 PRODUCT STORAGE AND STABILITY

E_2: 15-30°C in well-sealed containers.

7.3 MEASURES TO MINIMIZE BIAS

Randomization will be conducted by an independent researcher at the University of Waterloo, who will assign each participant a pre-randomized subject code. The randomization code will be provided to the Qualified Investigator in a sealed envelope. All investigators involved in data collection or analysis will remain blinded to group allocation until the completion of all data collection and statistical analysis. Unblinding will only occur in the event of a medically significant adverse event requiring treatment.

7.4 STUDY INTERVENTION COMPLIANCE

Participants will return their custom-made blister packs to verify adherence to the dosing schedule. Upon arrival, each participant will also confirm whether they successfully took all required doses.

7.5 CONCOMITANT THEORY

Participants will be instructed not to take any medications that were not approved during the initial screening. If additional medication becomes medically necessary during the study, the participant will be withdrawn from the trial.

8.0 DISCONTINUATION AND WITHDRAWAL

8.1 DISCONTINUATION OF STUDY INTERVENTION

Discontinuation of E2 or the placebo by a participant will result in their withdrawal from the study. Any newly identified clinically relevant findings will be documented and reported as an Adverse Event (AE).

8.2 PARTICIPANT DISCONTINUATION/WITHDRAWAL FROM THE STUDY

The participants participation in this study is voluntary. They may decide to leave the study at any time by communicating this to any of the study investigators. They may exercise the option of removing themselves or their data from the study at any time if they wish. The investigators reserve the right to withdraw them from the study if they believe that circumstances have arisen that

warrant doing so (i.e. failing to comply with the study protocol or for the safety of participants). They will need to notify the study investigators prior to the results being published if they do not want their data to be published, this will likely occur by March 2027.

An Investigator may discontinue or withdraw a participant from the study for the following reasons:

- Withdrawal of informed consent (participant chooses to withdraw for any reason)
- Recurrent adverse events (AEs)
- Occurrence of a serious adverse event (AE)
- Significant non-compliance with the study intervention
- Identification of an exclusion criterion (newly developed or previously unrecognized) that precludes continued participation

The reason for each participant's withdrawal or discontinuation will be documented in the study file. Participants who provide informed consent and are randomized but do not receive the study intervention may be replaced. Similarly, participants who are randomized, receive the intervention, and are later withdrawn or discontinued may also be replaced. Data from withdrawn or discontinued participants will be included in the analysis unless they request otherwise. The type of data to be collected will include, where feasible, safety assessments (e.g., adverse events), primary and/or secondary outcome measures obtained up to the point of withdrawal, and the reason for withdrawal.

Participants withdrawn from investigational product/trial treatment will be followed for safety, including monitoring and documentation of any ongoing or unresolved adverse events until resolution, stabilization, or return to baseline, as appropriate. Withdrawal from investigational product/trial treatment does not automatically constitute withdrawal from study follow-up unless the participant explicitly withdraws consent for further participation or data collection. As the study is blinded and investigators will not know which product the participant received, frequent follow-up notices will be sent within two weeks (or unresolved adverse events until resolution, stabilization, or return to baseline, as appropriate) following withdrawal to encourage participants to report any concerns or adverse events and to contact the study team if anything arises.

8.3 LOST TO FOLLOW-UP

A participant will be considered lost to follow-up if they miss all scheduled visits and cannot be reached by study staff. If a participant fails to attend a required visit, the following steps will be taken:

- Study staff will attempt to contact the participant, reschedule the missed visit within one day, emphasize the importance of adhering to the visit schedule, and determine whether the participant is willing and able to continue in the study.

- Before declaring a participant lost to follow-up, the Principal Investigator or their delegate will make multiple efforts to re-establish contact via phone or email. All contact attempts will be documented in the participant's study file or medical record.
- If the participant remains unreachable after these efforts, they will be considered withdrawn from the study, with the reason noted as lost to follow-up.

9.0 STUDY ASSESSMENTS AND PROCEDURES

9.1 OVERVIEW AND ASSESSMENTS

This study was submitted to the University of Waterloo Research Ethics Board (REB) prior to receiving the No Objection Letter (NOL) from Health Canada. The study will not begin until the NOL has been obtained and full REB approval has been granted. All experiments and procedures will take place in the B.C. Matthews Hall (BMH 2407; 20908 Ring Rd, Waterloo, ON) under supervision of trained personnel. 24 healthy, overweight/obese sedentary males between the ages of 18 to 35 will be conveniently recruited using posters, University of Waterloo's Daily bulletin, social media, and word of mouth.

All participants will be randomized into one of two possible groups: placebo or E_2 and will be asked to complete 11 visits outlined below:

Visit 1: At this visit participants will be provided with the consent form and will be informed with all components required to participate in this study, and will have the opportunity to pose questions. Upon providing informed consent, participants will be provided with the health screening questionnaire and CSEP Get Active Questionnaire to confirm eligibility for the study. Upon confirmation of eligibility, participants will undergo assessment of anthropometric measures (weight, height, blood pressure, waist circumference, bioelectrical impedance) and will be given instructions on how to complete a 3-day food log and 7-day physical activity log and complete the IPAQ physical activity questionnaire. They will be sent home with log sheets to record food intake and physical activity along with a pedometer to determine habitual daily activity level.

Visit 2: The second study visit will allow for assessment of their current aerobic fitness. Participants will be asked to complete the immunological, and sleep screening questionnaire as these are all variables that could mitigate some of the desired responses viewed in the immunological parameters. They will complete an assessment of maximal aerobic capacity (VO2peak, using a metabolic cart [Canadian license number: 88254]). The VO2peak test will be used to determine the loads used for high-intensity training. Prior to commencing VO2peak test, they will have a catheter inserted into a forearm. Participants will have a catheter inserted into the antecubital and a pre-exercise testing blood sample (~16mL of blood) will be taken. The blood draw will consist of approximately 16mL of blood in two separate tubes (1x 10mL EDTA, and 1x 6mL EDTA) for immediate macrophage and monocyte (determined via peripheral blood mononuclear [PBMC] isolation), total white blood cell count (determined via Neubauer hemocytometer), granulocyte count (determined via Wright-Giemsa stains), inflammatory cytokine production (e.g., IL-1β, IL-6, IL-10, TNF-α, TGF-β etc.) from plasma and stimulated (via lipopolysaccharide) and unstimulated PBMCs. The VO2peak test will start with a warm up on a bicycle at a low intensity. During the test the intensity will continue to increase until they reach

a point where they can no longer maintain the exercise. At this point the test will be stopped and they will cool down on the bike at a low intensity. Participants will have a post-exercise testing blood sample (~16mL of blood) will be taken, and the catheter will be removed. Throughout the test, they will have a mouthpiece in their mouth that will collect the air that they breathe. Following the VO2peak test, participants will rest for one hour and venous blood samples (6 mL, EDTA) will be collected at 30 and 60mins post-VO2peak. These measurements will be used to assess post-exercise metabolic substrate utilization and to determine the influence of estrogen and exercise training on these responses.

Visit 3: For the third visit, you will report to the lab following an overnight fast. Additionally, you will need to have refrained from moderate-to-vigorous physical activity for 72 h and alcohol for 48 hours prior to this visit. During this visit, you will complete a baseline oral glucose tolerance test (OGTT) and muscle and fat biopsies. The OGTT and biopsy procedures are as follows. A muscle and fat biopsy (~100-150mg) will then be taken ~20cm above the knee from the *vastus lateralis* and surround fat tissue under local anaesthetic (1% lidocaine) using a custom-suction-modified Bergstrom needle (5mm diameter). Following the muscle and fat biopsy, you will have a catheter placed in an antecubital vein and a fasted blood sample will be taken. You will then consume a 75g glucose beverage (we will use one of two OGTT products based on availability. Either Trutol beverage which has an NPN 01932179 or Azer Scientific Glucose Drink with NPN 800036990) and blood samples will be taken at 10, 20, 30, 45, 60, 90, and 120-minutes following glucose consumption and a muscle and fat biopsy will be taken at 60 minutes following glucose consumption. The total amount of blood drawn throughout the oral glucose tolerance test will be approximately 110mL (~7 tablespoons). While resting during the OGTT you will be asked to complete the following exercise questionnaire: International Physical Activity Questionnaire (IPAQ).

Visit 4-9: After completing pre-testing, participants will be asked to return to the lab for training (3 sessions/week for 2 weeks). Participants will attend the BMH 2407 lab wearing exercise attire (e.g., shorts, running shoes, t-shirt, etc.). Participants will also be asked to wear a Polar telemetric heart rate monitor (Polar, Canada) for the measurement HR) throughout the training session. The exercise training protocol will be completed, whereby the participant will begin cycling with a warm-up at 50 watts (W) for a total of 3-mins. The high-intensity interval training (HIIT) protocol will immediately follow the warm-up and will consist of 10 x 60 second cycling intervals (at 60% peak power, ~90%HRmax) interspersed with 60 second recovery (at 50W). Upon completion of the exercise protocol, the participants will be asked to complete a cool- down at 50W for a total of 2-mins. The entire duration of the exercise session will be 25-mins. Urine will be collected at visits 5, 7 and 9 to monitor estrogen fluctuations.

Visit 10: This visit will occur 24-48h after visit 10 and will be a repeat of what occurred at visit #1 and 2.

Visit 11: This visit will occur 24-48 h after visit 11 and will be a repeat of what occurred at visit #3.

9.2 ADVERSE EVENTS AND SERIOUS ADVERSE EVENTS

Dr. Sean Gartner has reviewed and approved the use of E2 for this study, based on prior research demonstrating its safe use without reports of serious adverse effects. Known serious reactions and contraindications are well documented and thoroughly screened for during participant eligibility assessment. The doses administered in this study are considered safe and not expected to pose any health risks.

9.2.1 DEFINITION OF ADVERSE EVENTS (AE)

An Adverse Event (AE) can be defined as any unexpected medical occurrence in a study participant associated with the use of an intervention, regardless of whether a causal relationship with the intervention is established. This may include any unfavorable or unintended sign (such as an abnormal lab result), symptom, or illness that arises during the study period. All participants will be screened using the inclusion and exclusion criteria, and those identified as being at risk for an AE will be excluded from participation.

9.2.2 DEFINITION OF SERIOUS ADVERSE EVENTS (SAE)

Adverse Events (AEs) are categorized as either serious or non-serious. A Serious Adverse Event (SAE) is defined as any AE that:

- Results in death
- Is life-threatening
- Requires inpatient hospitalization or prolongation of existing hospitalization
- Causes significant or lasting disability or incapacity
- Involves a congenital anomaly or birth defect

The term “life-threatening” refers to an AE where the participant was at immediate risk of death at the time of the event, not one that could have become fatal if it had worsened.

9.2.3 CLASSIFICATION OF AN ADVERSE EVENT

The severity of an AE is assessed by a qualified physician (Dr. Sean Gartner) who is a part of the study team, who should use the following definitions when assessing the intensity of an AE:

1. Mild – Events require minimal or no treatment and do not interfere with the participant’s daily activities.
2. Moderate – Events that result in a low level of inconvenience or concern with the therapeutic measures. Moderate events may cause some interference with activities of daily living.
3. Severe – Event interrupts daily, usual activity and may require another form of treatment. Severe events can be life-threatening or incapacitating.

In previous use of E2 (Devries et al. 2005) no adverse events were reported.

9.2.4 RELATIONSHIP TO STUDY INTERVENTION

All AEs must have their relationship to the study intervention assessed by a qualified physician who is part of the study team based on temporal relationships and clinical judgement. The degree of certainty about causality will be graded using the following categories:

1. Definitely Related – There is clear evidence to suggest a causal relationship, and other possible contributing factors can be ruled out. The clinical event, including an abnormal laboratory test result, occurs in a plausible time relationship to study intervention administration and cannot be explained by concurrent disease or other drugs or chemicals. The response to withdrawal of the study intervention should be clinically plausible.
2. Probably Related – There is evidence to suggest a causal relationship, and the influence of other factors is unlikely. The clinical event, including an abnormal laboratory test result, occurs within a reasonable time after administration of the study intervention, is unlikely to be attributed to concurrent disease or other drugs or chemicals, and follows a clinically reasonable response on withdrawal.
3. Possibly Related – There is some evidence to suggest a causal relationship (e.g., the event occurred within a reasonable time after administration of the study intervention). However, other factors may have contributed to the event (e.g., the participant's clinical condition, other concomitant events).
4. Unlikely to be related – A clinical event, including an abnormal laboratory test result, whose temporal relationship to study intervention administration makes a causal relationship improbable (e.g., the event did not occur within a reasonable time after administration of the study intervention) and in which other drugs or chemicals or underlying disease provides plausible explanations (e.g., the participant's clinical condition, other concomitant treatments).
5. Unrelated – The AE is completely independent of study intervention administration, and/or evidence exists that the event is definitely related to another etiology.

9.2.5 EXPECTEDNESS

A qualified physician (Dr. Sean Gartner) who is part of the study team will be responsible for determining whether an Adverse Event (AE) is expected or unexpected. An AE will be considered unexpected if the nature, severity, or frequency of the event is not consistent with the risk information previously described for the study intervention.

9.2.6 TIME PERIOD AND FREQUENCY FOR EVENT ASSESSMENT AND FOLLOW-UP

All Adverse Events (AEs) and Serious Adverse Events (SAEs) occurring after administration of the study intervention will be documented through 7 days post-intervention for non-serious AEs and 30 days for SAEs. Participants will be contacted by phone at 7 days and again at 30 days after their final study visit to inquire about any adverse events, health changes, or medical concerns that may have arisen. Participants will also be encouraged to contact the research team at any time during this period should any symptoms or concerns arise. AEs and SAEs may be identified through clinical assessments (e.g., abnormal lab results), participant self-reporting, responses to

questions during study visits, or telephone follow-up calls. At each visit, participants will be asked about any changes in health status and updates to previously reported AEs or SAEs. If no SAE is reported within 30 days post-intervention, participants will receive a follow-up call to confirm.

All AEs and SAEs must be documented regardless of their relationship to the study intervention. Collected information will include a description of the event, date and time of onset and resolution (if possible), outcome, and an assessment of seriousness, expectedness, relationship to the study intervention, and severity, as determined by a qualified physician delegate. Any pre-existing condition noted at baseline that worsens during the study will be reported as an AE or SAE.

Changes in severity will also be documented to track the duration of the event at each severity level. Events will be followed until they are resolved, the participant is deemed stable with no further follow-up required, or the participant is lost to follow-up, as determined by the Principal Investigator or a qualified physician delegate.

9.2.7 ADVERSE EVENT REPORTING

AE will be reported to University of Waterloo's Research Ethics Board (REB) according to reporting requirements.

9.2.8 SERIOUS ADVERSE EVENT REPORTING

All Serious Adverse Events (SAE) must be reported to Dr. Michaela Devries-Aboud within 24 hours of becoming aware of the SAE. The initial report must be emailed to michaela.devries-aboud@uwaterloo.ca and should contain as much information as available, at a minimum, the report must contain:

- Name of Site and Principal Investigator,
- Participant Identification Code,
- Adverse Event Term,
- Study Drug Dose and Start/Stop Dates

On the next working day, email completed trial-specific Serious Adverse Event form or Council for International Organizations of Medical Sciences (CIOMS) form. Only adverse drug reactions that are both serious and unexpected are subject to expedited reporting to Health Canada. Expedited reporting of reactions which are serious but expected is not required. Expedited reporting is also inappropriate for serious events from clinical investigations that are considered unrelated to the study product, whether or not the event is expected. During a clinical trial, the PI is required to inform Health Canada of any serious, unexpected adverse drug reaction (SUADR) that has occurred:

1. Where it is neither fatal nor life-threatening, within 15 days after becoming aware of the information;
2. Where it is fatal or life-threatening, immediately where possible and, within 7 days after becoming aware of the information; and
3. Within 8 days after having informed Health Canada of the SUADR, submit as complete a report as possible which includes an assessment of the importance and implication of any findings. Final reports of fatal or life-threatening reactions must include an assessment of the importance and implication of the findings, including relevant previous experience with the same or similar drugs.

Each SUADR which is subject to expedited reporting should be reported individually in accordance with the data element(s) specified in the Health Canada / ICH Guidance Document E2A: Clinical Safety Data Management: Definitions and Standards for Expedited Reporting. In situations when causality assessment and determination of expectedness is not straightforward, the report should be submitted in the expedited manner and the relevant issues addressed in a cover letter.
There are situations in addition to the above that may necessitate rapid communication to Health Canada, and appropriate scientific and medical judgment should be applied to each situation. For example, information that might influence the risk-benefit assessment of a drug, or that would be sufficient to consider changes in drug administration, or in the overall conduct of a clinical trial, represent such situations, including:

1. For an "expected" serious Adverse Drug Reaction (ADR), an increase in the rate of occurrence which is judged clinically important;
2. A significant hazard to the patient population, such as lack of efficacy with a drug used in treating a life-threatening disease; and
3. A major safety finding from a newly completed animal study.

9.2.9 REPORTING EVENTS TO PARTICIPANTS

Participants will be promptly informed of any new information, including safety-related updates, that may affect their decision to continue in the study. When applicable, this information will be communicated through an updated, REB-approved Informed Consent Form, and the communication will be documented accordingly, considering the timeliness of the update.

9.2.9 EVENTS OF SPECIAL INTEREST

No events of special interest are expected.

10.0 STATISTICAL CONSIDERATIONS

10.1 STATISTICAL HYPOTHESIS

Primary Endpoint: Glycemic control; insulin sensitivity

Secondary Endpoints & Hypotheses: Skeletal muscle insulin signaling; Immune cell count; Immune cell function (PBMCs); Inflammatory cytokines; Mitochondrial content, function, and respiration; Intramyocellular lipid content and metabolism

10.2 SAMPLE SIZE DETERMINATION

Twenty-four participants will be recruited for this study. Using an *a priori* calculation of sample size (G*Power, version 3.1.9.6, Germany (Faul *et al.*, 2007; Kang, 2021)), an effect size of

0.3865557 was initially derived from unpublished data from our laboratory, which demonstrated sex-related differences in glucose AUC after adjusting for lean body mass, corresponding to a partial eta squared ($\eta 2$) of 0.18. However, for the *a priori* calculation, we selected a more conservative $\eta 2$ of 0.13 to reduce risk of Type I error and ensure robust sample size estimation. The calculation was based on an α error probability of 0.05, power of 0.9, two groups (17β-estradiol versus placebo), two measurements (17β-estradiol or placebo, pre- versus post-training), and 0.5 correlation among repeated measures was used to calculate a total sample size of 20. Accounting for an anticipated 20% dropout rate, the final target sample size was set at 24.

10.3 STATISTICAL ANALYSIS

10.3.1 GENERAL APPROACH TO ANALYSIS

All data will be presented as mean±standard deviation and will be analysed by R using R Studio 2025.05.0+496. Significance will be set at $p<0.05$.

10.3.2 ANALYSIS OF PRIMARY ENDPOINTS

Two factor (group x time) repeated measures analysis of variance (RM ANOVA) will be used to detect differences in glycemic control and insulin signalling pathways (glucose, insulin, c-peptide, insulin receptor, insulin receptor substrate-1, protein kinase B, TBC1D1/TBC1D4, GLUT4, white blood cells (total, granulocyte, and PBMCs)). Three-day food logs will be collected weekly and anlysed using the same previously mentioned approach. Area under the curve (AUC) for some of the blood-related parameters, glucose, insulin and c-peptide. All AUC values will be compared via two-factor ANOVAs (treatment x session). E_2 will be analysed in the blood via two-factor ANOVAs (group x time). Bonferroni post-hoc analysis will be used where main effects and interactions are found. Partial eta-squared (η_p^2) values will be used to estimate the effect sizes (small: 0.01, medium: 0.06, and large: 0.14) for main effects and interactions where necessary. Cohen's d will be used to estimate effect size (small 0.2, medium 0.5, large 0.8, very large 1.3) for post-hoc comparisons.

10.3.3 ANALYSIS OF SECONDARY ENDPOINTS

Three-way ANOVA will be used while investigating the difference in PBMC function, because ex vivo PBMCs will be treated with lipopolysaccharide (an immune cell stimulus) or vehicle to determine whether E2 promotes PBMC function and production of inflammatory cytokines (group, time, treatment). All remaining secondary endpoints will be analysed via two-way ANOVA or three-way ANOVA wherever applicable. Bonferroni post-hoc analysis will be used where main effects and interactions are found. Partial eta-squared (η_p^2) values will be used to estimate the effect sizes (small: 0.01, medium: 0.06, and large: 0.14) for main effects and interactions where necessary. Cohen's d will be used to estimate effect size (small 0.2, medium 0.5, large 0.8, very large 1.3) for post-hoc comparisons.

10.3.4 SAFETY ANALYSIS

Safety endpoints will be analyzed with categorical analysis based on the frequency of occurrence.

10.3.5 BASELINE DESCRIPTIVE STATISTICS

Baseline participant characteristics will be presented as mean ± standard deviation.

10.3.6 PLANNED INTERIM ANALYSIS

No interim analysis planned.

10.3.7 SUB GROUP ANALYSIS

A subgroup analysis will be conducted to explore whether treatment effects differ based on key variables such as age, baseline hormone levels, physical fitness or body composition.

10.3.8 EXPLORATORY ANALYSIS

Currently there are no exploratory analysis planned.

11.0 SUPPORTING DOCUMENTATION AND OPERATIONAL CONSIDERATIONS

11.1 REGULATORY, ETHICAL, AND STUDY OVERSIGHT CONSIDERATIONS

11.1.1 STUDY DISCONTINUATION AND CLOSURE

This study may be temporarily suspended or prematurely terminated if there is sufficient reasonable cause. Written notification, documenting the reason for study suspension or termination, will be provided by the suspending or terminating party to study participants, investigators, funding agency, PI and regulatory authorities. If the study is prematurely terminated or suspended, the PI will promptly inform study participants, the REB and PI and will provide the reason(s) for the termination or suspension. Study participants will be contacted, as applicable, and be informed of changes to study visit schedule.

Circumstances that may warrant termination or suspension include, but are not limited to:

- Determination of unexpected, significant, or unacceptable risk to participants
- Data that are not sufficiently complete and/or evaluable

Study may resume once concerns about safety, protocol compliance, and data quality are addressed, and satisfy the PI, REB, DSMB, and/or regulatory agency.

11.1.2 CONFIDENTIALITY AND PRIVACY

Participant confidentiality and privacy is strictly held in trust by the participating investigators, their staff, and (if applicable) the Sponsor(s). This confidentiality is extended to cover testing of biological samples and genetic tests in addition to the clinical information relating to participants. Therefore, the study protocol, documentation, data, and all other information generated will be held in strict confidence.
Any research information obtained about the patient in this study will be kept confidential. A patient will not be identified by name, only by unique study ID number. The patient's name or any identifying information will not appear in any reports published as a result of this study. All identifying information will be kept as per equivalent institutional policy at University of Waterloo, under the supervision of the study/site PI and will not be transferred outside of the institution. These processes have been approved by the REB.

The study monitor, auditor and other authorized representatives of the PI, representatives of the Research Ethics Board (REB), regulatory agencies supplying study product may inspect all documents and records required to be maintained by the Investigator, including but not limited to, medical records and pharmacy records for the participants in this study. The clinical study site will permit access to such records.
Study participant research data, which is for purposes of statistical analysis and scientific reporting, will be transmitted to and stored at University of Waterloo. This will not include the participant's contact or identifying information. Rather, individual participants and their research data will be identified by a unique study identification number. The study data entry and study management systems used by clinical sites and by research staff will be secured and password protected.

11.1.3 FUTURE USE OF STORED PARTICIPANT DATA

Data collected for this study will be analyzed and stored at the University of Waterloo. Any biosamples will be stored in a -80° freezer, abiding by institutional policies as stated by the REB. At any point in study participation, participants will be able to choose to withdraw samples from further analysis.

11.1.4 KEY ROLE AND STUDY GOVERNANCE

Principal Investigator	**Medical Monitor & Qualified Investigator**
Michaela Devries-Aboud, PhD, Professor	Sean Gartner, MD
University of Waterloo	University of Waterloo
200 University Ave West Waterloo, ON NSL 3G1	200 University Ave West Waterloo, ON NSL 3G1
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The Medical Monitor has been actively engaged in the development of the protocol—including trial design, inclusion and exclusion criteria, safety assessments and reporting, and discontinuation

criteria—and will continue to be involved in the trial's closeout and reporting.

11.1.5 SAFETY OVERSIGHT

The study safety will be monitored by the study investigators. Safety will be monitored by the Qualified Investigator and the research team. The Qualified investigator will verify each participant is suitable and safe to participate. Any unanticipated events will be monitored by the Qualified investigator and reported accordingly.

11.1.6 SAFETY MONITORING

The study safety will be monitored by the study investigators. Safety will be monitored by the Qualified Investigator and the research team. The Qualified investigator will verify each participant is suitable and safe to participate. Any unanticipated events will be monitored by the Qualified investigator and reported accordingly.

11.1.7 QUALITY ASSURANCE & QUALITY CONTROL

The clinical site will perform internal quality management of study conduct, data and biological specimen collection, documentation and completion. The PI will be responsible for all auditing activities. Any trial-related duty or function transferred to and assumed by a third party, including auditing, will be specified in a clinical trial agreement and oversight provided by the PI.

11.1.8 DATA HANDLING & RECORD KEEPING

The PI, QI and staff will have access to the data.

11.1.8.1 DATA COLLECTION & MANAGEMENT RESPONSIBILITIES

Data collection is the responsibility of the clinical trial staff at the site under the supervision of the site Investigator. The Investigator is responsible for ensuring the accuracy, completeness, legibility, and timeliness of the data reported.

All source documents should be completed in a neat, legible manner to ensure accurate interpretation of data.

Data recorded in the case report form (CRF) derived from source documents should be consistent with the data recorded on the source documents.

11.1.8.2 STUDY RECORDS RETENTION

To enable evaluations and/or audits from Health Canada and/or the PI, the Principal Investigator agrees to keep all records pertaining to the clinical trial, including the identity of all participating patients (sufficient information to link records, CRFs and hospital records), all

original signed informed consent forms, copies of all CRFs, source documents, and detailed records of treatment disposition in a secure location for a minimum of 15 years.

11.1.9 PROTOCOL DEVIATIONS

A protocol deviation is any noncompliance with the clinical trial protocol or Manual of Procedures (MOP) requirements, if applicable. The Principal Investigator will assure that no deviation from, or changes to the protocol will take place without prior agreement from the PI and documented approval from the Research Ethics Board (REB), except where necessary to eliminate an immediate hazard(s) to the trial participants. The noncompliance may be either on the part of the participant, the Investigator, or the study site staff. As a result of deviations, corrective actions are to be developed by the site and implemented promptly. All protocol deviations will be documented; the Principal Investigator will assess each protocol deviation to determine the impact to the patient's rights, safety or welfare, study efficacy and data integrity. If there is any uncertainty regarding the impact of the protocol deviation, the Principal Investigator will consult with the Medical Monitor.

It is the responsibility of the Principal Investigator to use continuous vigilance to identify and report deviations within 5 working days of identification of the protocol deviation, or within 7 working days of the scheduled protocol-required activity. All deviations must be addressed in study source documents and reported to the PI. Protocol deviations must be sent to the reviewing REB in accordance with their policies. The Principal Investigator is responsible for knowing and adhering to the reviewing REB requirements.

11.1.10 CONFLICT OF INTEREST POLICY

The independence of this study from any actual or perceived influence, such as by the pharmaceutical industry, is critical. Therefore, any actual conflict of interest of persons who have a role in the design, conduct, analysis, publication, or any aspect of this trial will be disclosed and managed. Furthermore, persons who have a perceived conflict of interest will be required to have such conflicts managed in a way that is appropriate to their participation in the design and conduct of this trial.

12.0 LIST OF ABBREVIATIONS

ADR = Adverse Drug Reaction, AE = Adverse Event, ANOVA = Analysis of variance, cm = centimetres, CRF = case report form, °C = degrees Celsius, E_2 =17B- Estradiol, GAQ = Get Active Questionnaire, GCP = Good Clinical Practice, HIIT = High-intensity interval training, HR = heart rate, IL = interleukin, mg = milligram, mL = millilitre, kg/m^2 = kilograms/metre squared, NOL = no objection letter, OGTT = Oral glucose tolerance test, PBMC = Peripheral blood mononuclear cells, PI = principal investigator, QI = qualified investigator, RCT = randomized control trial, RM ANOVA = Repeated measures analysis of variance, REB = Research Ethics Board, RPM = revolutions per minute, SAE = Severe Adverse Event, TGF = transforming growth factor, TNF = tissue necrosis factor, $\dot{V}O_{2max}$ = Maximal oxygen consumption, W = Watts.

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