DOES AN ACUTE BOUT OF EXERCISE PERFORMED AT THE MAXIMAL FAT OXIDATION RATE HAVE AN IMPACT ON HUMAN ADIPOSE TISSUE MICROVASCULAR ENDOTHELIAL CELLS?

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ABSTRACT

The adipose tissue (AT) vasculature ensures healthy tissue function, supporting whole-body homeostasis. Endothelial cells (EC) line vascular capillaries and control their growth (i.e. angiogenesis). Exercising at an intensity eliciting maximal fat oxidation rate (maxFOR) increases AT blood flow and might support vascular remodelling. Yet, the impact of maxFOR on AT EC remains unknown. The workload required to achieve maxFOR was determined for 11 healthy females. Blood samples were collected pre-, immediately post- and 3h post- a 45-minute bout of aerobic exercise performed at maxFOR. Serum was isolated to treat Human Adipose Microvascular EC (HAMEC) to assess cellular behavior and gene expression. Serum free fatty acid (FFA) concentration increased post-exercise for all participants. HAMEC had reduced migratory capacity and increased Fatty Acid Binding Protein 4 (*FABP4)* gene expression when treated with serum collected 3h post-exercise. Exercise at maxFOR may stabilize EC and prime AT capillaries for efficient FFA transport and uptake.

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LIST OF ABBREVIATIONS

%BF: Percent Body Fat

ANG-1: Angiopoietign-1

ANG-2: Angiopoietign-2

ANGPTL4: Angiopoietin-Like Protein 4

AT: Adipose Tissue

ATBF: Adipose Tissue Blood Flow

ATP: Adenosine Triphosphate

BAT: Brown Adipose Tissue

BMI: Body Mass Index

BP: Blood Pressure

CaO_2: Arterial Oxygen Content

CD36: Cluster of Differentiation 36

CHD5: Chromodomain-helicase-DNA-binding protein 5

CLDN5: Claudin-5

CO_2: Carbon Dioxide

CPT1A: Carnitine Palmitoyl Transferase I

CRF: Cardiorespiratory Fitness

CVD: Cardiovascular Disease

CvO_2: Venous Oxygen Content

DLL4: Delta-Like-Ligand 4

DMSO: Dimethyl Sulfoxide

dNTP: Deoxyribonucleotides

EC: Endothelial Cells

ECM: Extracellular Matric

EE: Energy Expenditure

EGR3: Early Growth Response 3

FA: Fatty Acids

FABP: Fatty Acid Binding Protein

FFA: Free Fatty Acids

FFM: Fat-Free Body Mass

FGF: Fibroblast Growth Factor

FGFR: Fibroblast Growth Factor Receptor

FOXO1: Forkhead Box O1

FOR: Fat Oxidation Rate

FRIEND: Fitness Registry and the Importance of Exercise Database

GLUT: Glucose Transporter

HAMEC: Human Adipose Microvascular Endothelial Cells

HB: Hemoglobin

HFD: High-Fat-Diet

HGF: Hepatocyte Growth Factor

HIF: Hypoxia-Inducible Factors

HPRT1: Hypoxanthine-Guanine Phosphoribosyl Transferase 1

HR: Heart Rate

HSL: Hormone-Sensitive Lipase

HUVEC: Human Vein Umbilical Cord Endothelial Cells

IMTG: Intramuscular Triglycerides

LDH: Lactate Dehydrogenase

LPL: Lipoprotein Lipase

MaxFOR : Maximal Fat Oxidation Rate

MMP9: Matrix Metalloproteinase 9

MPH: Miles Per Hour

MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

MUFA: Monounsaturated Fatty Acids

NIH: National Institutes of Health

NO: Nitric Oxide

O_2: Oxygen

PA: Physical Activity

PALM: Physical Activity and Lifestyle "R" Medicine

PAR-Q: Physical Activity Readiness Questionnaire

PBS: Phosphate Buffered Saline

PF4: Platelet-Factor 4

PFK-1: Phosphofructokinase-1

PFKFB3: 6-Phosphofructo-2-Kinase/Fructose-2.6-Biphosphatase 3

PHD: Prolyl Hydroxylase Domain Proteins

PIK3: Phosphatidyl Inositol Kinase 3

PPARy: Peroxisome Proliferator-Activated Receptor

PUFA: Polyunsaturated Fatty Acids

qPCR: Real-Time Polymerase Chain Reaction

RER: Respiratory Exchange Ratio

ROS: Reactive Oxygen Species

RQ: Respiratory Quotient

SAT: Subcutaneous Adipose Tissue

SFA: Saturated Fatty Acids

SMI: Skeletal Muscle Index

SMM: Skeletal Muscle Mass

SO5S: Sum of 5 Skinfolds

TIE2: Tyrosine-Protein Kinase Receptor

TSP-1: Thrombospondin-1

VAT: Visceral Adipose Tissue

VCO_2: Ventilatory Carbon Dioxide

V_E: Minute Ventilation

VEGF: Vascular Endothelial Growth Factor

VEGFR: Vascular Endothelial Growth Factor Receptor

VO_2: Ventilatory Oxygen

VO_2 Max: Maximal Oxygen Uptake

WAT: White Adipose Tissue

WC: Waist Circumference

Xe: Xenon

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1.0.LITERATURE REVIEW

1.1. OVERVIEW

Physical activity (PA) is defined as "any bodily movement produced by skeletal muscles that results in energy expenditure" (Caspersen et al., 1985; Khan et al., 2012). PA encompasses exercise, sport, unstructured recreation, active transport and many activities of daily living (Khan et al., 2012). Specifically, exercise is planned, threshold intensity-based, structured and repetitive bodily movement that brings about changes in physical and physiological fitness. For instance, an individual may participate in unstructured recreation and therefore is physically active, without participating in exercise (Khan et al., 2012). All PA contributes to health in part through their effects on cardiorespiratory fitness (CRF) and musculoskeletal fitness which includes: muscular strength, endurance, power, flexibility, agility and mobility (D. Lee et al., 2011). Interestingly, low CRF and musculoskeletal fitness are better predictors of mortality than obesity or hypertension and low PA levels are a major risk factor for cardiovascular diseases (CVDs) (Luo et al., 2024). Today, physical inactivity is recognized as the fourth leading cause of mortality worldwide (WHO, 2010) with an increase in global trends in CVD death associated with poor PA (Luo et al., 2024). Females show a greater steady rise in these patterns compared to their male counterparts, suggesting that women engage less in PA than men (Luo et al., 2024). Yet, Ji and colleagues revealed, through a prospective study of 412,412 U.S. adults, that women have greater gain in all-cause and cardiovascular mortality risk reduction from equivalent doses of PA than men (Ji et al., 2024). These results underscore the importance of regular PA participation, especially for women. Exercise is a well-described stimulus in the promotion of blood vessel growth in skeletal muscle (Andersen & Henriksson, 1997; Bloor, 2005; Carrow et al., 1967; Egginton, 2009; Gustafsson & Kraus, 2001). Recently, the endothelium has also gained attention as a central regulator of

metabolism, specifically in the adipose tissue (AT). For instance, AT lipolysis is controlled by endothelial cells (EC), helping to maintain metabolic health in the mouse model of obesity (Rudnicki & Haas, 2022). The transcription factor Forkhead Box O1 (FoxO1) is a key regulator in EC behaviour. Using an EC-specific FoxO1 knock-out mice model, Rudnicki and colleagues showed that mice lacking the FoxO1 protein had more blood vessels in their fat tissue, healthier looking fat cells and normal blood sugar levels when placed on a high-fat-diet (HFD) (Rudnicki, Abdifarkosh, Nwadozi, et al., 2018). Karki and colleagues observed endothelial insulin resistance in the AT of individuals with obesity, which improved with the inhibition of FoxO1 (Karki et al., 2015). These results suggest the integral role of EC in the maintenance of AT health, especially in the context of obesity. Conversely, obesity has been closely linked to endothelial dysfunction (Crewe et al., 2017; Honkala et al., 2020). Inducing apoptosis in adipose EC, via the subcutaneous injection of a ligand-directed peptidomimetic, reduced both obesity and insulin resistance in monkeys with obesity (Barnhart et al., 2011). This effect was not observed in lean monkeys who received the injection, suggesting a potential obesity-specific effect of the peptide. These contradictory findings suggest that the role of EC in AT metabolism and health may be context specific, with a potential dysregulation in the context of obesity.

In this regard, it is important to further investigate the impact of exercise on the endothelium, specifically in the context of free fatty acid (FFA) release and transport, as it can greatly impact individuals with obesity and improve overall cardiovascular health. Furthermore, identifying the mode of exercise that can best elucidate these benefits is crucial. This should be investigated in women, to address the sex-specific differences in response to engaging in PA and the increased benefit observed in PA participation in this population.

1.2. PART 1: EXERCISE PHYSIOLOGY, MaxFOR CHARACTERIZATION AND THE ADIPOSE TISSUE

1.2a. Overview of Whole-Body Dynamic Exercise

Exercise improves whole-body function, longevity, and health (Bouchard et al., 2012). Whole-body dynamic activity, commonly referred to as aerobic exercise, can be performed continuously, as intervals or intermittently. Performance in this type of exercise is dependent on an individual's functional capacity and maximal oxygen uptake (VO_2 max). Aerobic exercise can be characterized as light-to-moderate intensity, about 50-75% of an individual's age-predicted maximum heart rate (HR; **Equation 1**: *age predicted HR = 220 – age*) or up to 65% of their VO_2 max. Conversely, moderate-to-vigorous intensity aerobic exercise can be described as over 75% of an individual's age-predicted maximum HR and over 65% of their VO_2 max (Åstrand, 2003). The current PA recommendation for adults is 150-300 minutes of moderate-intensity exercise or 75-150 minutes of vigorous-intensity exercise per week while concurrently reducing sedentary time (Piercy et al., 2018). There are numerous health benefits involved with regular intensity-based aerobic exercise, including a decreased risk in chronic diseases such as diabetes, cancer, obesity and osteoporosis (Ruegsegger & Booth, 2018; Warburton, 2006).

When an individual begins a bout of intensity-based aerobic exercise, there is a great increase in the body's energy demand, challenging systemic metabolic homeostasis to meet those needs (Ashcroft et al., 2024). The interplay and integration of the anaerobic and aerobic systems is essential to meet the 100-fold increase in the demands for Adenosine Triphosphate (ATP) (Parolin et al., 1999, Ashcroft et al., 2024). In the early stages of a bout of aerobic exercise, ATP is mainly supplied by phosphocreatine hydrolysis and glycolysis, both anaerobic processes (Gaitanos et al., 1993; Parolin et al., 1999). As the bout of exercise continues, there is an increase

in oxidative phosphorylation, further increasing the ATP supply. The longer the bout of exercise, the greater the increase in oxygen (O_2) consumption, cardiac output and redistribution of blood flow to the working skeletal muscle allowing the contribution of oxidative phosphorylation (commonly referred to as aerobic metabolism) as the main source of ATP re-synthesis (De Cort et al., 1991).

1.2b. Acute Aerobic Exercise Requires the Coordination of Multiple Tissues and Impacts Whole-Body Metabolism and Blood Flow

The shift observed in whole-body metabolism and substrate use during an acute bout of aerobic exercise requires the coordination of multiple tissues and other internal factors. Carbohydrates and lipids are the energy sources used during exercise and their relative utilization depends on the intensity of the exercise performed (Hargreaves & Spriet, 2020). Generally, lipids are used as the main substrate source at lower exercise intensities. Fat oxidation rates increase as exercise intensity increases, eventually hitting a peak and markedly decreasing as exercise intensity continues to increase. At exercise intensities > 85% maximum HR, carbohydrates are the primary substrate used as fuel (Hargreaves & Spriet, 2020; Jeukendrup & Wallis, 2005). In the early stages of a bout of moderate-intensity exercise, energy demands are met by an increase in both carbohydrate and fat oxidation rates (van Loon et al., 2001). FFA are released into circulation through AT lipolysis stimulated by the ß-adrenergic system and oxidation of intramuscular triglycerides (IMTG) droplets is observed (Ashcroft et al., 2024; Romijn et al., 1993). The working skeletal muscle increases glucose uptake and blood glucose levels are maintained via hepatic glycogenolysis or glucose that has been ingested prior to the exercise bout (Ahlborg et al., 1974). Hepatic glucose production is further stimulated by the change in ratio of insulin to glucagon, where a decrease in the release of insulin is seen due to α-adrenergic inhibition of pancreatic ß-

cells during exercise (Ashcroft et al., 2024). As the bout of moderate-intensity exercise continues, there is a decrease in carbohydrate oxidation as fat oxidation takes over (Watt et al., 2002). At this stage, the increase in fat oxidation is predominantly due to the delivery of plasma FFA, rather than an increase in oxidation of IMTG stores (Watt et al., 2002). A continuous rise of plasma glucagon levels stimulates hepatic glucose output through the uptake of precursors such as lactate and pyruvate. Blood glucose levels are maintained through hepatic glycogenolysis (Ahlborg et al., 1974). As the exercise bout continues, fatigue will occur as hepatic glucose outputs will fail to meet the energy demands (Watt et al., 2002). If exercise intensity increases, intramuscular glycogen will be utilized for glycogenolysis, becoming the main source of fuel. At higher exercise intensities, there is a decrease in AT blood flow (ATBF), causing reduced oxidation of plasma FFA and IMTG (Ashcroft et al., 2024; Romijn et al., 1993). There are multiple factors that impact the shift from FFA oxidation to glycolysis as the main source of fuel during higher exercise intensities. These include reduced intracellular availability of FFA, reduced transport of FFA to the mitochondria and mitochondrial substrate interference (Sahlin et al., 2008).

It has been suggested that women may have a greater reliance on the use of FFA for fuel during moderate-intensity aerobic exercise (Devries, 2016). This is reflected in the higher rate of appearance of glycerol in women during exercise (Carter et al., 2001). It has been reported that women have greater IMTG content than men (Devries et al., 2007; Roepstorff et al., 2002), although there are contradictory findings when it comes to differences in utilization of these stores during exercise between sexes (Devries et al., 2007; Steffensen et al., 2002; Zehnder et al., 2005). Furthermore, Tarnopolsky and colleagues found greater muscle glycogen stores and enhanced performance in men, and not women, when using a 75% carbohydrate-loading regimen,

elucidating the difference in metabolism between sexes and the greater reliance on FFA as fuel in women (Devries, 2016; M. Tarnopolsky et al., 1995).

During exercise, smooth muscle surrounding arterioles (upstream of capillaries) control blood flow to various tissue. At rest, sympathetic innervation contributes to modest vasoconstriction and is referred to as vascular tone. Exercise increases smooth muscle cell contraction, increasing sympathetic vasoconstriction and vascular tone. Conversely, metabolites produced by exercising muscle reduces vascular smooth muscle contraction and tone (Andersen & Saltin, 1985; Joyner & Casey, 2015). This pattern of vasoconstriction and vasodilation leads to a redistribution of blood flow during exercise (McAllister, 1998). The redistribution of blood flow has been well described in healthy and untrained young men. At rest, total cardiac output is 5L/min, where 20% of this output is blood flow to the skeletal muscle. Blood flow to other organs, including the brain, kidneys, liver, gut and non-working muscle represent the remaining 80% of the cardiac output. At high-intensity exercise, cardiac output increases to 20L/min and blood flow increases to the working skeletal muscle (and slightly in the brain), up to 80% of the cardiac output, and decreases in all other organs (20% cardiac output) (Joyner & Casey, 2015). The increase of blood flow to working skeletal muscle allows the increase delivery of O_2 and nutrients and efficient removal of metabolic waste products (Joyner & Casey, 2015). In a competitive athlete, the overall cardiac output during exercise is almost double that of an untrained person (40mL/min), allowing a greater volume of blood to be redistributed to the working skeletal muscle during exercise (Joyner & Casey, 2015).

1.2c. Determining Substrate Use During Exercise

The Respiratory Quotient (RQ) is a ratio that indicates which substrate is being used for energy and is obtained via indirect calorimetry, which measures the volume of O_2 and carbon

dioxide (CO_2) consumption corresponding to the cellular respiration and allows the calculation of energy expenditure using Weir's equation (EE; **Equation 2**: *EE (kcal) = (3.815 + (1.232*RQ)*VO_2)*duration of exercise (min)*) (Mehta et al., 2015; Mtaweh et al., 2018). RQ numbers vary from 0.7-1.0 and is calculated as follows: **Equation 3:** *RQ = Volume CO_2 (VCO_2) / Volume O_2 (VO_2).* An RQ between 0.7 and 0.85 indicates the dominant use of lipids, whereas an RQ of 0.85 and 1.0 indicates the dominant use of carbohydrates as the primary source of energy (Patel et al., 2023). The CO_2 and O_2 ratios are derived using the equations for glucose (**Equation 4**) and FFA oxidation (**Equation 5**).

Equation 4 - *Glucose Oxidation:* $C_6H_{12}O_6 + 6\ O_2 \rightarrow 6\ CO_2 + 6\ H_2O$

Equation 5 - *FFA Oxidation (palmitate):* $C_{16}H_{32}O_6 + 23\ O_2 \rightarrow 16\ CO_2 + 16\ H_2O$

When glycolysis takes place (**Equation 3**) the RQ = VCO_2 / VO_2 = 6 / 6 = 1. When ß-oxidation takes place (**Equation 4**) the RQ = VCO_2 / VO_2 = 16 / 23 = 0.7 (Mtaweh et al., 2018; Patel et al., 2023).

The RQ ratio only considers CO_2 and O_2 as a product of metabolism. In some cases, this ratio can be greater than 1.0 and is thus invalid in determining the estimated substrate use during exercise. This occurs due to extra CO_2 produced from the buffering of lactic acid and an increase in hyperventilation during strenuous exercise. Instead, the change in respiratory exchange is represented by the Respiratory Exchange Ratio (RER), a tool that reflects high ventilation rates and blood lactate levels (Deuster & Heled, 2008). Interestingly, women have been shown to have lower RQ during moderate-intensity aerobic exercise, indicating less reliance on carbohydrates as a fuel for exercise (Carter et al., 2001; Romijn et al., 2000; L. Tarnopolsky et al., 1990).

1.2d. Measuring Maximal Oxygen Consumption (VO_2 Max)

The best measure of CRF comes from an individual's VO_2 max (Kodama et al., 2009; Nauman et al., 2017). VO_2 max depicts the maximum ability to take up, deliver and utilize O_2 during exercise and can be measured directly using the FICK equation (**Equation 6**): $VO_2 = Cardiac\ Output\ (L/min) \bullet (CaO_2 - CvO_2)\ (mL/100\ mL);$ where CaO_2 and CvO_2 are O_2 content in arterial and venous blood, respectively (Huckabee & Judson, 1958). Cardiac output is defined as the volume of blood pumped by the heart in one minute and it is determined by multiplying heart rate by stroke volume (mL/kg/beat) (Kenney et al., 2012). The major determinants of CaO_2 are hemoglobin (Hb) concentration (g/dL), Hb saturation and dissolved O_2 present in arterial blood (Guyton & Hall, 2006). Primarily, Hb content can impact O_2 carrying capacity, as Hb is an iron-rich protein found in red blood cells and is required to bind O_2 to transport it to our tissues (Webb et al., 2023). In healthy individuals, approximately 97% of O_2 transported from the lungs to the tissues is carried through Hb (Kenney et al., 2012; Webb et al., 2023).

During incremental to maximal exercise, there are several physiological factors that can affect an individual's VO_2 max. The two main determinants of O_2 consumption are: 1) Central- the amount of blood supplied to the skeletal muscle via cardiac output; 2) Peripheral- the ability of the skeletal muscle to extract and utilize the transported O_2. The O_2 consumption by the skeletal muscle accounts for most of the increase in the arterio-venous O_2 difference observed (Kenney et al., 2012; Raghuveer et al., 2020). Furthermore, individuals with greater muscle mass will have greater extraction and utilization of O_2, causing an increase in VO_2 max (Shah et al., 2022). Females have VO_2 max values 10-20% lower than males due decreased Hb content, less muscle mass, smaller blood volume, cardiac output and stroke volume. Despite these physiological

factors, individuals can train to improve their VO_2 max, with higher VO_2 max values associated with greater health benefits and quality of life (Carnethon et al., 2005; Sui et al., 2007).

VO_2 max is commonly determined via indirect calorimetry during an exercise graded test. The Fitness Registry and the Importance of Exercise Database (FRIEND) was developed to establish reference values for CRF derived from studies using proper cardiopulmonary exercise testing (Kaminsky et al., 2015). Data from laboratories with experience conducting exercise tests were included in the database. The laboratories were also well established, indicated valid and reliable calibration and testing procedures and used qualified personnel to conduct exercise tests, consistent with American Heart Association guidelines (Myers et al., 2014; Peterman et al., 2020). Most laboratories use an adapted version of the Astrand (Astrand et al., 1964) graded exercise protocol to determine VO_2 max, where a systemic and linear increase in exercise intensity overtime is used until the individual is no longer able to maintain or tolerate the workload (Beltz et al., 2016). There are criteria that a participant must meet to determine if VO_2 max has been reached (Keiller & Gordon, 2018): **1)** no further increase in VO_2 with increasing workloads, **2)** participant was unable to continue , **3)** RER > 1.0, **4)** HR > 90% age predicted HR**)** (Hancock et al., 2023; Howley et al., 1995).

1.2e. Exercising at Maximal Fat Oxidation Rate: An Example of Low-to-Moderate Intensity Aerobic Exercise

The highest rates of fat oxidation are found at low-to-moderate exercise intensities (35-65% VO_2 max). The exercise intensity at which the highest rate of fat oxidation is observed is referred to as maxFOR exercise (**Figure 1**) (Achten et al., 2002). MaxFOR is generally identified as ~50% of an individual's VO_2 max. This percentage can greatly differ between individuals, ranging between 40-60% of a person's VO_2 max (Achten et al., 2002). Fat oxidation rate (FOR)

can be calculated using Frayn's equation (**Equation 7**) as follows: $Fat\ (g \cdot min^{-1}) = 1.67 * VO_2\ (L \cdot min^{-1}) - 1.67 * VCO_2\ (L \cdot min^{-1})$.

During a bout of exercise performed at maxFOR, there are several steps involved in the efficient oxidization of fat in the skeletal muscle: lipid supply via lipolysis, entrance of lipids into the muscle and then into the mitochondria and mitochondrial FFA processing (Brun et al., 2011). FFA are supplied to the muscle through the blood stream, released from the AT via lipolysis, either bound to plasma albumin or glycerol (Van Der Vusse et al., 1998). FFA are released from their circulating proteins and cross over the endothelium, via diffusion or facilitated by transporters (i.e. Fatty Acid Binding Proteins - FABP), into the skeletal muscle (Van Der Vusse et al., 1998). Oxidative conversion of FFA occurs in the mitochondria of the muscle. Specifically, FFA undergo ß-oxidation. Once FFA enter the outer mitochondrial membrane, they form a thieoster bond with Coenzyme A (CoA), dependent on the enzyme acyl-CoA synthetase (Alves-Bezerra & Cohen, 2017). The capacity of the muscle to uptake FFA and the number of mitochondria in the muscle impact the rate of fat oxidation observed in an individual (Van Der Vusse et al., 1998). It has been demonstrated that lipid supply is not a limiting factor during maxFOR, as sedentary individuals with obesity (high fat depots) have significantly lower rates of fat oxidation compared to their lean counterparts (Brun et al., 2011; Lanzi et al., 2014). The limiting factor in this process is dependent on the enzyme Carnitine Palmitoyl Transferase I (CPT1A), that allows the transport of FFA across the outer mitochondrial membrane of skeletal muscle (Brun et al., 2011; Starritt et al., 2000). CPT1A simultaneously converts the FFA-CoA moiety to fatty acylcarnitine, which is then transported into the mitochondrial matrix where it can undergo β-oxidation (Longo et al., 2016).

Several circulating hormones influence fat oxidation and the balance of substrates during exercise (Brun et al., 2011). For instance, the release of epinephrine and norepinephrine increase

lipolysis and muscle fat oxidation, respectively (Glisezinski et al., 2009; Oberhaensli et al., 1985). Growth hormone increases whole body fat oxidation, helping to increase maxFOR (Vijayakumar et al., 2010). Interleukin-6 (IL-6) acts as an energy sensor and enhances both lipolysis and fat oxidation in the AT (Hoene & Weigert, 2008).

The maxFOR workload (expressed as a %VO_2 max) and the maximal amount of fat oxidized by an individual is affected by sex, training status and diet (Brun et al., 2022; Maunder et al., 2018). Overall, being female, higher VO_2 max and a fat-rich diet lead to greater maxFOR rates (Brun et al., 2022; Lima-Silva et al., 2010; Maunder et al., 2018; Venables et al., 2005).

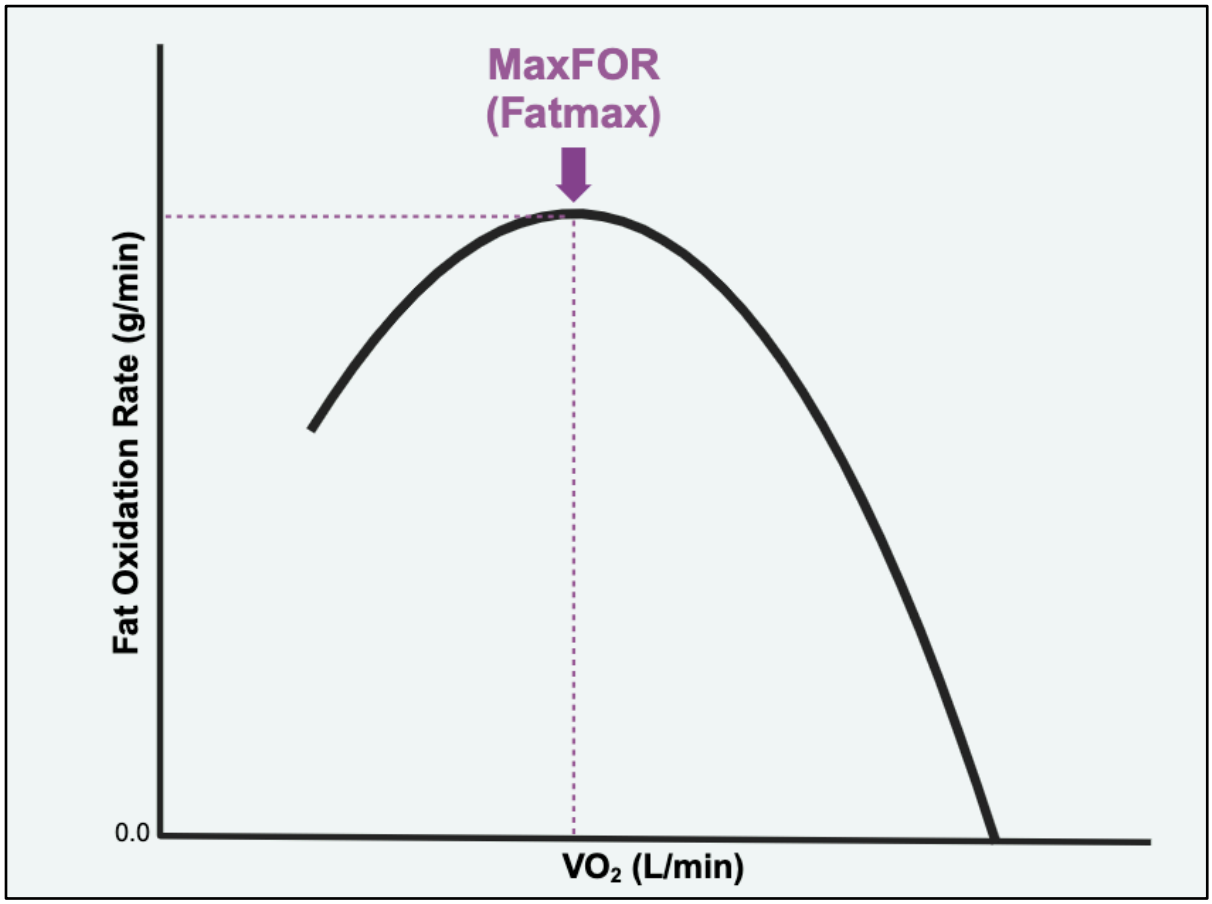


Figure 1. Exercise Intensity That Elicits Maximal Fat Oxidation Rate
(Adapted from Achten et al., 2002).

1.2f. Functions of Adipose Tissue

The AT is a highly metabolic and active endocrine organ. There are two main types of AT that exist: white AT (WAT) and brown AT (BAT) (Y.-H. Lee et al., 2014; Zhang et al., 2018; Ziqubu et al., 2023). WAT is unilocular and is responsible for energy storage, insulation, and adipokine secretion and its expansion is observed in instances of weight gain. WAT is distributed viscerally (VAT) and subcutaneously (SAT). VAT is located between the internal organs in the abdominal cavity (liver, kidneys, intestine) and around the heart whereas SAT is found throughout the whole body in the spaces between the skin and underlying muscles (Ibrahim, 2010). Waist circumference (WC) is used as a surrogate for visceral (or central) adiposity (Gadekar et al., 2020), whereas skinfolds may be used as a measure of subcutaneous fat (Vaquero-Cristóbal et al., 2020). Conversely, BAT is multilocular and is a mitochondria-dense thermogenic tissue responsible for heat generation and maintaining our body's core temperature. BAT is mainly active in young infants and regresses with age. In adults, remaining BAT is located surrounding the vertebrae, above the clavicles, in the upper back and in the central compartment of the thoracic cavity (Ibrahim, 2010; Ziqubu et al., 2023).

Adipocytes are the main cellular constituents of AT. The number of triglycerides stored within adipocytes reflects the imbalance between energy expenditure and consumption. Adipocytes can expand, to accommodate for excess energy storage (via lipogenesis) or can shrink (via lipolysis) to meet the body's energy demand (Ailhaud et al., 1992). This is illustrated in rapidly enlarged mice adipocytes fed a HFD, where AT weight can double in as little as one week. Notably, there is a reduction of this weight within 24 hours of fasting (Crewe et al., 2017). The ductility of this tissue has made it an essential player in maintaining homeostasis in response to times when food is either abundant or limited.

1.2g. The Adipose Tissue and Exercise Performed at MaxFOR

The greatest absolute change in fat mass in response to repeated bouts of exercise comes from the SAT, as it is usually the largest fat depot (Chávez-Guevara et al., 2020). Dietary manipulation is also required to create an energy deficit and greater weight loss, as changes in fat mass from exercise alone are minimal (Wilmore et al., 1999). Albeit exercise presents other metabolic benefits such as: preservation of skeletal muscle, reduction in the amount of lipids in circulation and reduction in overall and ectopic fat distribution (Johnson et al., 2009). Specifically in the AT in humans, exercise training can reduce adipocyte size and number (Ahn et al., 2022; Ashcroft et al., 2024; Thompson et al., 2012), improving AT inflammatory profile (Weisberg et al., 2003) and insulin sensitivity (Olefsky, 1977).

The SAT has been estimated to contribute the greatest portion of FFA oxidized during low to moderate exercise intensities (~40-70% VO_2 max) (Thompson et al., 2012). The release of FFA from the AT during exercise is influenced by the rate of AT lipolysis, FFA re-esterification and ATBF (Frayn & Karpe, 2014; Pistor et al., 2015; Thompson et al., 2012). FFA oxidized during maxFOR serve as fuel in the working skeletal muscle. Measurements done using micro dialysis and arteriovenous difference demonstrate clear mobilization of FFA from the SAT to the working muscle during exercise (Moro et al., 2004; Stallknecht et al., 2001, 2007; Stich et al., 2000; Thompson et al., 2012). For instance, Moro and colleagues utilized local dialysis during exercise to determine lipid mobilization in overweight men and found an increase in dialysate glycerol concentration during exercise (Moro et al., 2004). Similarly, Stich and colleagues reported higher AT lipolysis during a bout of aerobic moderate-intensity exercise, assessed via glycerol dialysate leaving the AT (Stich et al., 2000).

Evidence suggests that exercising at maxFOR, ATBF is at its peak and the body reaches its highest ability to break down and release fat (Jeukendrup & Achten, 2001; Özgünen et al., 2019; Thompson et al., 2012). Both micro dialysis and ^{133}Xenon (Xe) washout have been used to determine ATBF, although ^{133}Xe is a more sensitive measure due to the larger area of tissues covered compared to the localized micro dialysis technique (Karpe et al., 2002). Using ^{133}Xe, Bulow and Madsen assessed ATBF before, during and after exercise in both abdominal and perirenal fat depots in young lean men. The authors reported a 4-fold and 7-fold increase in blood flow during exercise at 50% VO_2 max for 50 minutes to the abdominal and perirenal depots, respectively (Bulow & Madsen, 1978). Enevoldsen and colleagues reported a 3-fold increase in blood flow to the abdomen in young lean men during cycling exercise at 50% VO_2 max for 60 minutes (Enevoldsen et al., 2001). Similarly, Lyngso and colleagues observed a 2-fold increase and a 1.5-fold increase of blood flow to the AT in the abdominal region during and after cycling exercise at 60% VO_2 max for 60 minutes in young lean men, respectively (Lyngsø et al., 2002). This phenomenon remains consistent in individuals with obesity. A 20% increase in blood flow to abdominal AT has been reported in older overweight men and women with type 2 diabetes when exercising at 60% VO_2 max for 60 minutes (Bulow et al., 2004). Intriguingly, moderate-intensity exercise may cause an increase in blood flow to AT distant from the working skeletal muscle, although there is no significant change in AT lipolysis reported (Stallknecht et al., 2007).

The delivery of biologically relevant mediators (i.e., hormones, angiogenic factors) to the AT is a function of both concentration and blood flow (delivery = concentration x blood flow). It has been hypothesized that vigorous intensity exercise induces vasoconstriction of AT via released catecholamines, decreasing ATBF (Thompson et al., 2012). Exercising at maxFOR increases blood flow to the AT facilitating the crosstalk between adipocytes and circulating factors released

in the blood during exercise. He and colleagues have demonstrated the release of several biomarkers (Interleukin-15, Follistatin, Myostatin, Irsin, Resistin and Omentin) in healthy untrained men in response to a single bout (45 min) of exercising at maxFOR, immediately and 3h post-exercise (He et al., 2019).

1.2h. Gaps of Knowledge

Most studies investigating the effects of exercising at maxFOR use the same workload at a fixed percentage of VO_2 max for every subject in the study, despite known interindividual differences in exercise intensities that elicit maxFOR. Furthermore, most studies were completed in men. Due to known differences in metabolism and response to exercise between men and women, results from these studies cannot be generalized to both sexes.

1.3. PART 2: MOLECULAR AND CELLULAR ASPECTS OF ANGIOGENESIS

1.3a. The Angio-Adaptive Response and Mechanisms of Angiogenesis

Angio-adaptation refers to the dynamic processes that occur at molecular, and cellular levels, which regulate the tissular adaptations of the growth, stabilization, or regression of capillaries. At the molecular level, angio-adaptation is regulated by a balance of pro- and anti-angiogenic factors (Christiaens & Lijnen, 2010; Corvera & Gealekman, 2014; Hellsten & Hoier, 2014; Lemieux & Birot, 2021; Olfert & Birot, 2011). At the cellular level, capillaries are lined with a monolayer of EC and angio-adaptation can require the ability of these cells to proliferate, migrate, and assemble into vascular tubes in the context of angiogenesis. Conversely, angio-adaptation can also involve EC apoptosis in the context of capillary regression (Hellsten & Hoier, 2014; Hudlicka et al., 1992). An overview of the angio-adaptive response is illustrated in **Figure 2**.

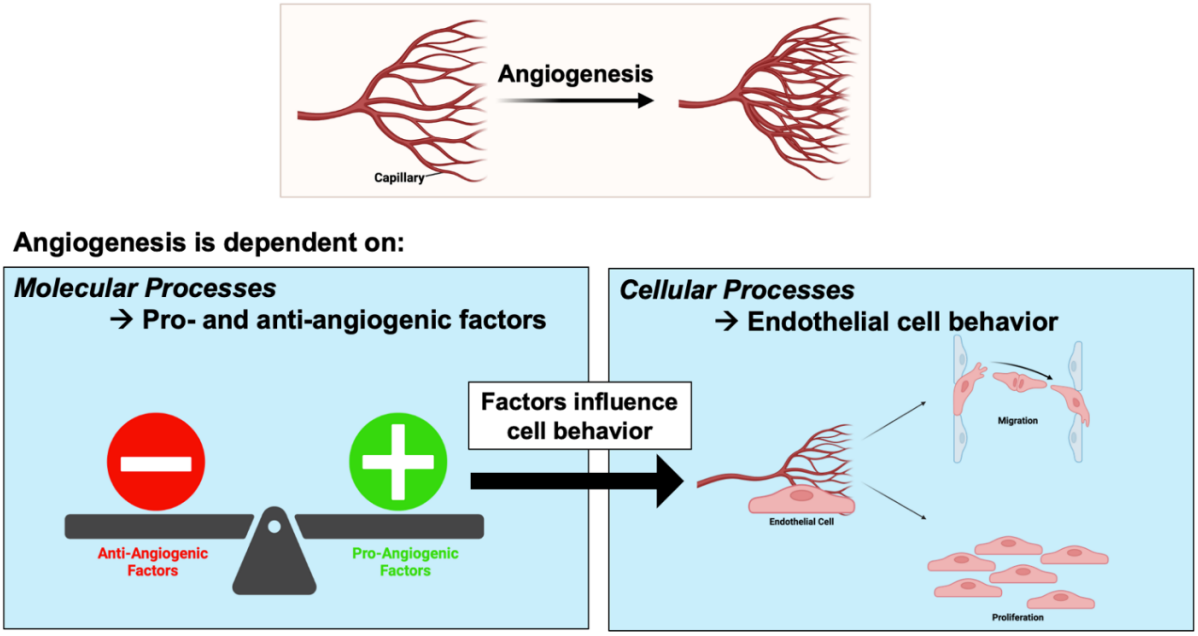


Figure 2. The Angio-Adaptive Response

There are two main modes of vessel formation in normal growing tissues: sprouting and non-sprouting angiogenesis. During sprouting, the major developmental form of vessel growth, extracellular matrix (ECM) proteolytic degradation is followed by chemotactic migration and proliferation of EC. This is a dynamic process controlled by pro-angiogenic signals, activating EC motility (Potente et al., 2011). These motile EC, or so-called tip cells, extend long filopodia and migrate in response to chemotactic and pro-angiogenic factors, such as Vascular Endothelial Growth Factor (VEGF), but rarely proliferate (Carmeliet, 2005; Carmeliet & Jain, 2011; Potente et al., 2011). Conversely, stalk cells proliferate to elongate the branch and establish a lumen. Notch signaling largely controls tip and stalk EC identity, where stalk cells show high Notch activity, whereas tip cells show low Notch activity. VEGF enhances expression of the Notch ligand Delta-Like-Ligand 4 (DLL4) in tip cells, which in turn activates Notch in neighbouring EC and inhibits tip cell behaviour (Akil et al., 2021). This interplay allows for lateral inhibition and suppression of the tip cell phenotype in stalk cells. Tip cells of newly formed vascular tubes interact with the existing vasculature to fuse and expand the capillary network (Potente et al., 2011). The sprouting

process is terminated when pro-angiogenic signals subside and supporting transcriptional networks stabilize newly formed vessels. Tube perfusion allows EC to become quiescent phalanx cells, but their ability to rapidly switch and allow for vessel sprouting is maintained. Autocrine and paracrine factors act as survival signals allowing the maintenance of vessel integrity and protection from environmental stressors. These include VEGF, Fibroblast Growth Factor (FGF) and Angiopoietin-1 (Ang-1), which can prevent EC non-pathological apoptosis, strengthening of adherent junctions and promote deposition of bone marrow components for vascular wall stability (Potente et al., 2011; Udan et al., 2013). Both adherent and tight junctions are important in mediating cell-cell interactions. Specifically, the assembly of adherent junctions precedes that of tight junctions and is greatly involved in the establishment of cell adhesion in EC (Harris & Nelson, 2010). VE-cadherin is the transmembrane component responsible in endothelial adherent junctions and is an important player in overall EC integrity and vascular formation (Nan et al., 2023). VE-cadherin promotes vascular lumen formation during angiogenesis and its inhibition is linked to EC dysfunction (Lampugnani, 2012; Nan et al., 2023).

The main driver of sprouting angiogenesis is the lack of O_2 (i.e., hypoxia). Pro-angiogenic signals increase in instances of hypoxia caused by expanded tissue mass, vessel dysfunction or occlusion (Carmeliet & Jain, 2011; Potente et al., 2011; Shweiki et al., 1992). Under normoxic conditions, Prolyl Hydroxylase Domain Proteins (PHDs) use O_2 to hydroxylate Hypoxia-Inducible Factors (HIFs), targeting them for proteasomal degradation. Under hypoxic conditions, PHDs become inactive, and HIFs are no longer degraded, activating the expression of pro-angiogenic genes, VEGF and FGF, stimulating angiogenesis via binding of their tyrosine kinase receptors VEGFR2 (i.e., FLK-1) and FGFR1, respectively. The activation of these signaling pathways support key angiogenic processes (migration and proliferation) and allow an increase in the O_2

supply of the working tissue (Carmeliet & Jain, 2011; Gustafsson & Kraus, 2001). Concurrently, the role of anti-angiogenic factors must not be undermined. Endogenous negative regulators allow for controlled vessel growth and regression- an adaptation required in instances of physical deconditioning or pathology (Olfert, 2016; Olfert & Birot, 2011; Roudier et al., 2010). Examples include Thrombospondin-1 (TSP-1), Interferons, and Platelet-Factor 4 (PF4). For example, TSP-1 is a matrix-derived endogenous inhibitor of cell migration and induces EC apoptosis (Olfert & Birot, 2011). In the AT, inhibition of anti-angiogenic pathways, such as FoxO1 (Rudnicki, Abdifarkosh, Nwadozi, et al., 2018) and pharmacological inhibition of the VEGF receptor and VEGFR1, (Seki et al., 2018) can support greater AT vascularization. Overall, the angio-adaptive response is composed of pro- and anti-angiogenic signals, and the balance between the two, shifting towards the pro-angiogenic profile during times of vessel formation.

The second mode of angiogenesis, non-sprouting angiogenesis or intussusception, is the splitting of vessels from pre-existing ones (Potente et al., 2011; Skalak & Price, 1996). This mode of angiogenesis can be initiated by mechanical forces, such as fluid shear stress, to the vessel wall (Chlench et al., 2007; Egginton, 2001; Skalak & Price, 1996). VEGF signaling has been found to be necessary for shear-stress dependent splitting of capillaries in skeletal muscle (Gianni-Barrera et al., 2011, 2018). EC can convert mechanical stimuli to intracellular signals that affect cellular behaviours including proliferation, migration, and gene expression (Dixit et al., 2007; Goettsch et al., 2008; Li et al., 2005; Tinken et al., 2010). There are multiple sensing mechanisms used by EC to detect changes in mechanical forces that can activate a plethora of signaling cascades (Tinken et al., 2010). A primary regulator of the effects of shear stress is the serine/threonine kinase Akt. Phosphatidyl Inositol 3 Kinase (PIK3) activates Akt which leads to the phosphorylation of transcription factor FoxO1, excluding it from the nucleus. When FoxO1 is dephosphorylated, it

can translocate to the nucleus to induce the transcription of genes related to apoptosis. Notably, the angiopoietins Ang-1 and Angiopoietin -2 (Ang-2) play key roles in regulating vessel stabilization and maturation (Akwii et al., 2019). Ang-1 activates its receptor Tyrosine-Protein Kinase Receptor (Tie2) to stabilize the vessel wall, whereas Ang-2 antagonizes Ang-1 by binding to Tie2 to destabilize the vessel wall. The transcription factor FoxO1 induces the expression of Ang-2. Interestingly, Ang-1 inhibits FoxO1, further inhibiting its antagonist Ang-2 (Chlench et al., 2007; Dixit et al., 2007; Goettsch et al., 2008; Li et al., 2005; Tinken et al., 2010). It has been reported that FoxO1 and its target Ang-2 expression were decreased under shear stress (6 dyne/cm^2 for 24h) in Human Vein Umbilical Cord Endothelial Cells (HUVEC) (Chlench et al., 2007). Furthermore, a study by Bongrazio and colleagues reported a reduced expression in the anti-angiogenic factor TSP-1 when HUVEC were exposed to shear stress (6 dyne/cm^2 for 72h) (Bongrazio et al., 2006). These findings support the notion that shear stress may improve vessel maturity and stabilization.

1.3b. The Role of Endothelial Cell Metabolism in Angiogenesis

EC metabolism has been shown to be a significant co-determinant of vessel sprouting (Cruys et al., 2016; Draoui et al., 2017; Yu et al., 2017; Zecchin et al., 2017). Upon VEGF stimulation, EC rewire their metabolism to meet the elevated energetic demands needed for the formation of new vessels. Both tip and stalk EC rely heavily on glycolysis to yield sufficient ATP. The use of glucose as the main substrate source provides many advantages: greater ATP re-synthesis in a shorter time, sparing O_2 for other cells and the reduction of generated reactive O_2 species (ROS) (De Bock et al., 2013; Zecchin et al., 2017). VEGF stimulation also increases the expression of Glucose Transporter 1 (GLUT1), the glycolytic enzyme Lactate Dehydrogenase (LDH-A) and the enzyme 6-Phosphofructo-2-Kinase/Fructose-2.6-Biphosphatase 3 (PFKFB3).

The latter enzyme generates fructose-2,6-bisphosphate, activating the rate-limiting glycolytic enzyme Phosphofructokinase-1 (PFK-1) (De Bock et al., 2013; Draoui et al., 2017).

Despite the importance of glycolysis in supporting angiogenic processes, it has been demonstrated that stalk cells also depend on the fatty acid oxidation (FAO) pathway (Schoors et al., 2015). This is a multi-step process that allows the transport of FFA into the cell, where they can then be transported to the mitochondria, via the enzyme CPT1A, after the addition of an acetyl-CoA molecule. These FFA then undergo β-oxidation, further producing acetyl-CoA to allow the cycle to continue. The entry of FFA to the mitochondria allows the continuous production of aspartate, used for deoxyribonucleotides (dNTPs) synthesis essential for DNA replication in proliferating EC. The rate-limiting step in this process is dependent on the activity of the CPT1A enzyme (Draoui et al., 2017; Schoors et al., 2015).

During quiescent physiological conditions, EC have high glycolytic activity. Interestingly, Kalucka and colleagues have shown that quiescent EC also upregulate FFA β-oxidation to prevent EC dysfunction. FoxO1 is an important player in promoting EC quiescence (Kalucka et al., 2018; Rudnicki, Abdifarkosh, Nwadozi, et al., 2018). This transcription factor is described as a gatekeeper of quiescence via the inhibition of Myc, as FoxO1 deletion in EC causes severe vascular defects mediated by dysregulated EC metabolism (Draoui et al., 2017).

1.3c. Adequate Capillarization of the Adipose Tissue is Essential for its Proper Function

Capillaries, our smallest blood vessels, are responsible for the delivery of substrates and O_2 to our bodies' tissues and the removal of metabolic waste. The capillary network is plastic and can expand (Carmeliet & Jain, 2011; Risau, 1997; Udan et al., 2013). In many contexts, angiogenesis seems to be linked with adipogenesis (expansion of AT) (Christiaens & Lijnen, 2010). During overnutrition, adipocytes expand in size and eventually reach their O_2 diffusion

limit. This mild hypoxia activates angiogenesis to facilitate AT expansion and reduce the lack of O_2 available to the tissue (Ambrosini et al., 2002; Grosfeld et al., 2002; Hodson et al., 2013). When there is adequate O_2 supply to meet the growing metabolic demands, this is termed healthy AT expansion (Meister et al., 2022) (**Figure 3**, left panel). In some instances, as seen in obesity, fat depots grow beyond the tissue's capacity for adequate angiogenesis, causing unhealthy AT expansion that is accompanied by chronic and persistent hypoxia, fibrosis, cellular senescence, low-grade inflammation and necrotic adipocyte death (Crewe et al., 2017; Herold & Kalucka, 2021; Pasarica et al., 2009) (**Figure 3**, right panel).

The AT is highly vascularized, with each adipocyte lying adjacent to at least one micro vessel in healthy tissue (Y. Cao et al., 2018). This proximity allows for facilitated crosstalk between both cell types, where the endothelium can influence AT lipid dynamics and remodelling (Ioannidou et al., 2022). A healthy AT vasculature contains continuous capillaries, where EC are tightly aligned and impermeable to free and unregulated movement of macromolecules (Krüger-Genge et al., 2019). Instead, the transport of molecules is tightly regulated by different transport systems (Hasan & Fischer, 2021; Komarova & Malik, 2010). The non-leaky nature of the AT capillaries highlights the importance of regulating the type of molecules taken up or released by the tissue, affecting AT function (Ioannidou et al., 2022). The continuity of the endothelium is achieved via the formation of tight junctions between adjacent EC alongside the basement membrane of the vessel. Tight junctions are a multiprotein complex consisting of transmembrane and cytosolic proteins, composed of claudin building blocks. The claudin family is made up of 27 members, with claudin-5 being highly expressed in EC, enhancing the endothelial barrier function (Gonçalves et al., 2013; Ling et al., 2024).

As previously described, VEGFs and their receptors (VEGFRs) control growth and remodelling of the vasculature. The activation of angiogenesis in the AT through the VEGF/VEGFR2 axis has been shown to protect mice from obesity and associated metabolic complications (Elias et al., 2012; Sun et al., 2012; Sung et al., 2013). Robciuc and colleagues reported improved glucose metabolism, insulin sensitivity and reduced inflammation in the AT of mice with increased *VEGFB* expression in the AT. In obese mice, the induction of vascular remodelling by VEGFB alleviated metabolic complications of obesity by improving the delivery and function of insulin (Robciuc et al., 2016).

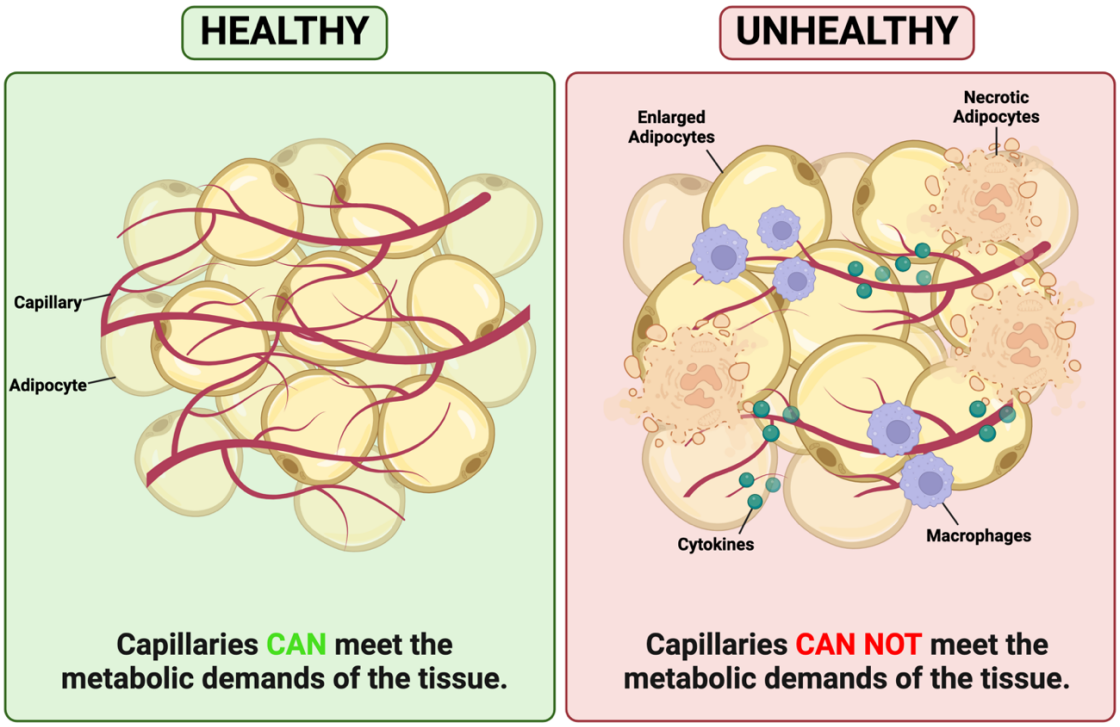


Figure 3. Healthy vs Unhealthy Adipose Tissue Expansion

1.3d. Endothelial Cell / Adipose Tissue Crosstalk and Adipose Tissue Angiogenesis

EC are heterogenous throughout the body and contain specific transcriptional profiles, as they support organ-specific functions (The Tabula Sapiens Consortium* et al., 2022). Even within

a group of specialized EC, heterogeneity exists due to EC adaptability and specific responses to their immediate microenvironment. Specially, AT EC contain an upregulation of genes involved in lipid metabolism: Fatty Acid Binding Protein 4 (FABP4) and Cluster of Differentiation 36 (CD36). The presence of these genes supports the role of EC in the mediation of AT metabolism and function. For instance, the presence of CD36 receptors on EC membranes optimize tissue FFA uptake, where EC-*CD36* knock-out mice showed reduced uptake of radio labelled long-chain FFA into the heart, brain and BAT (Son et al., 2018). Furthermore, uptake of FFA by the AT is facilitated by the presence of Lipoprotein Lipase (LPL), anchored to the membrane of the endothelium, aiding in the breakdown of triglycerides. Intriguingly, Gogg and colleagues reported the role of microvascular EC isolated from human SAT biopsies in regulating FFA transport to the AT as well as adipose cell Peroxisome Proliferator-Activated Receptor (PPARy) – a key regulator of adipose cell differentiation- activation, elucidating the crosstalk between EC and adipose cells (Gogg et al., 2019). Monelli and colleagues have also demonstrated the role of EC polyamines in stimulating lipolysis in adipocytes and WAT homeostasis in mice, further validating the crosstalk between EC and the AT (Monelli et al., 2022).

A reciprocal relationship occurs where FFA released by the AT can be used by EC to increase FFA oxidation, increasing EC proliferation and growth (AlZaim et al., 2023). Furthermore, the AT is a key endocrine organ. Adipokines, such as adiponectin or leptin, secreted by the AT are key modulators of vascular function. Adiponectin increases the production of endothelial nitric oxide (NO) and leptin promotes vascular permeability (AlZaim et al., 2023) (**Figure 4**). Both WAT and BAT produce and secrete many different types of pro- and anti-angiogenic factors that act in an endocrine and paracrine manner on neighbouring EC. The main

factors produced by adipocytes are VEGF, leptin, Hepatocyte Growth Factor (HGF) and TSP-1 (Christiaens & Lijnen, 2010).

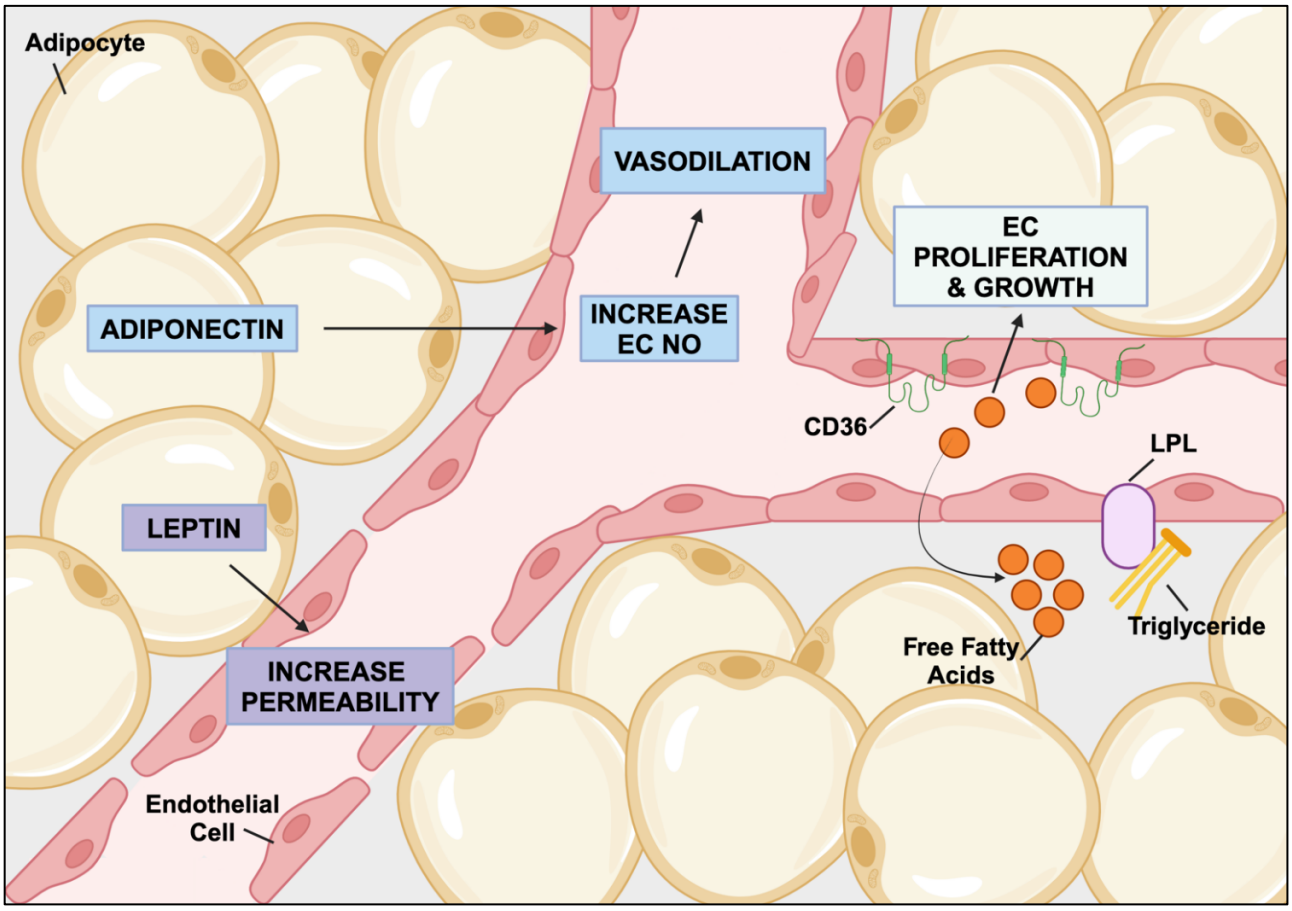


Figure 4. Adipocyte and Endothelial Cell Crosstalk. Adapted from (AlZaim et al., 2023).

There are a multitude of studies exploring the difference in angiogenic capacity and capillary density between AT depots. For instance, Song and colleagues reported higher expression of angiogenesis-related genes and increased angiogenic capacity in VAT (epididymal fat depot) of diet-induced obese mice compared to SAT (inguinal fat depot) (Song et al., 2016). Conversely, Gealekman and colleagues reported that SAT, taken from the lower abdominal wall, has a greater angiogenic capacity and higher expression of the pro-angiogenic Angiopoietin-Like Protein 4 (ANGPTL4) than VAT, taken from the greater omentum, in human AT explants. Furthermore, morbid obesity decreased SAT, but not VAT, angiogenic capacity and capillary density negatively

correlated with insulin sensitivity in humans (Gealekman et al., 2011). Cullberg and colleagues have shown that the levels of serum VEGF, Ang-1 and Ang-2 are influenced by weight changes, with weight loss being associated with reduced angiogenic activity in the circulation (Cullberg et al., 2013). Silha and colleagues similarly reported elevated serum levels of pro-angiogenic factors VEGF, VEGFR2, Ang-2, HGF and anti-angiogenic endostatin in overweight and obese study participants, with higher levels in females compared to males (Silha et al., 2005). Specifically, AT-derived VEGF plays an important role in maintaining proper AT function in times of tissue expansion. Overexpression of AT VEGF prevented AT hypoxia in mice fed a HFD (Elias et al., 2012). Sung and colleagues demonstrated that the loss of VEGF in mice fed a HFD lowered AT capillarity, percentage body fat and fat pad mass and restoring its expression reversed negative metabolic outcomes (i.e. insulin sensitivity and glucose tolerance) and reinstated adequate vessel density (Sung et al., 2013). Interestingly, female mice have been reported to gain less weight and show greater levels of pro-angiogenic factors and vascularity than males when placed on a HFD for sixteen weeks (Rudnicki, Abdifarkosh, Rezvan, et al., 2018).

1.3f. Exercise-Driven Angiogenesis

High-intensity, repeated, aerobic exercise (i.e. training) is a well-established stimulus of angiogenesis in the skeletal muscle. Repeated exercise during training serves as a strong physiological stressor, shifting towards a pro-angiogenic profile to induce changes in skeletal muscle capillarization (Andersen & Henriksson, 1997; Bloor, 2005; Carrow et al., 1967; Egginton, 2009; Gustafsson & Kraus, 2001). There are a multitude of studies investigating exercise-driven angiogenesis in rodent AT after weeks of aerobic exercise training. For instance, Hatano and colleagues described an increase in VEGF mRNA from visceral (epididymal) WAT after nine weeks of endurance training in male Wistar rats. Slower weight gain, increased number of EC per

adipocyte, and lower final weight were also reported (Hatano et al., 2011). Interestingly, six weeks of voluntary wheel running has been shown to alter angiogenic gene expression in a fat depot specific manner in male C57Bl/6 mice. For instance, VEGF expression was increased in subcutaneous (inguinal) WAT, whereas it was downregulated in the visceral (epididymal) WAT (H. J. Lee, 2018). Zachwieja and colleagues investigated the effects of AT VEGF deficiency on exercise capacity and reported a significant decline in endurance running capacity in AT VEGF deficient mice. The authors also noted a 40% reduction in AT capillarity and the blunting of circulating FFA after an acute bout (1h) of submaximal maxFOR exercise, elucidating the role of adipose VEGF in AT vascularization and metabolism (Zachwieja et al., 2015).

Interestingly, an acute bout of exercise can promote the release of both pro- and anti-angiogenic factors in the muscle (Gavin & Wagner, 2001; Hoier et al., 2012; Richardson et al., 1999). For instance, Hoier and colleagues describe increased levels of VEGF, Matrix Metalloproteinase 9 (MMP9), Ang-2, Tie2 and TSP-1 mRNA in the vastus lateralis of healthy male subjects after acute exercise on a cycle ergometer at ~60% VO_2 max (Hoier et al., 2012). The effects of acute exercise on the angiogenic response of the AT remain to be elucidated, as most studies focus on the effects of exercise on AT metabolism. Ludzki and colleagues have reported that acute endurance exercise (2h swimming) increased VEGF mRNA expression in the AT of rats during the early stages of weight gain (Ludzki et al., 2018). In young healthy male subjects, a 15-minute bout of endurance exercise performed at 80% VO_2 max induced changes in the regulation of genes related to angiogenesis, positive regulation of cell proliferation and cell migration in subcutaneous WAT (Fabre et al., 2018).

1.3g. Gaps of Knowledge

To date, it is unknown how exercising at maxFOR, in humans, impacts AT EC despite the important role of AT capillaries in supporting increased ATBF and delivery of FFA from the AT to working skeletal muscles, as depicted in **Figure 5**.

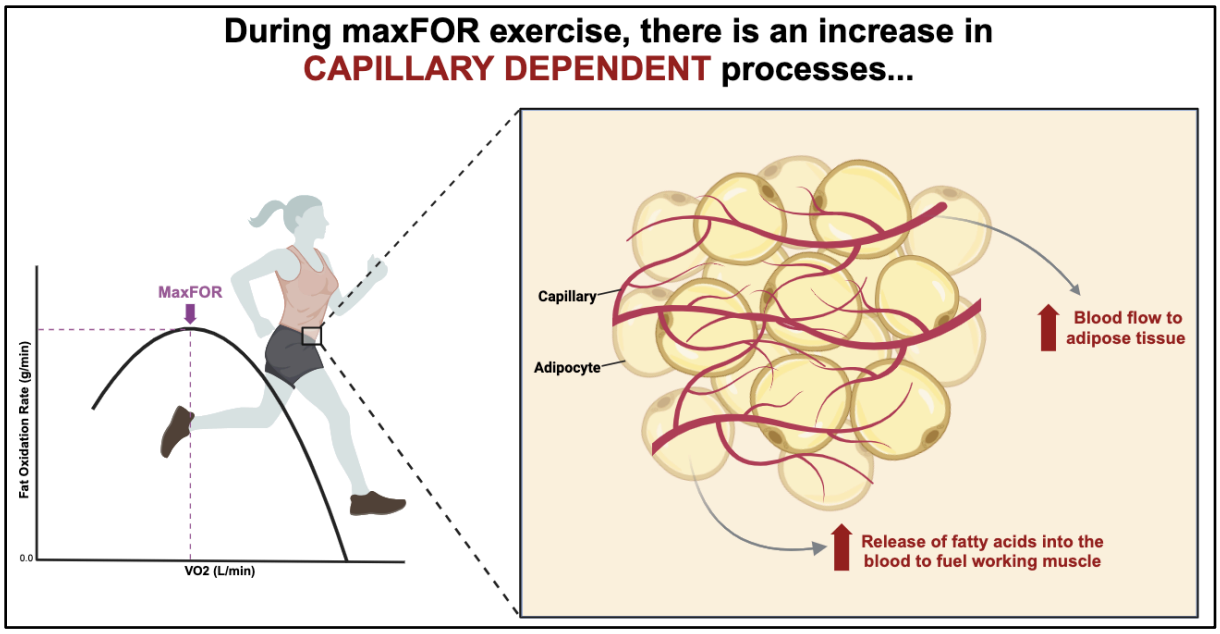


Figure 5. The Effects of MaxFOR Exercise on the Adipose Tissue

2.0. STUDY HYPOTHESIS AND OBJECTIVES

2.1. Hypothesis

It is hypothesized that serum collected after an acute bout of exercise performed at maxFOR has the capacity to alter HAMEC behavior via:

a) The promotion of proliferation and migration, key functions of angiogenesis.

b) Positively impacting gene expression related to FFA transport and vessel permeability.

2.2. Objectives

In vivo objectives:

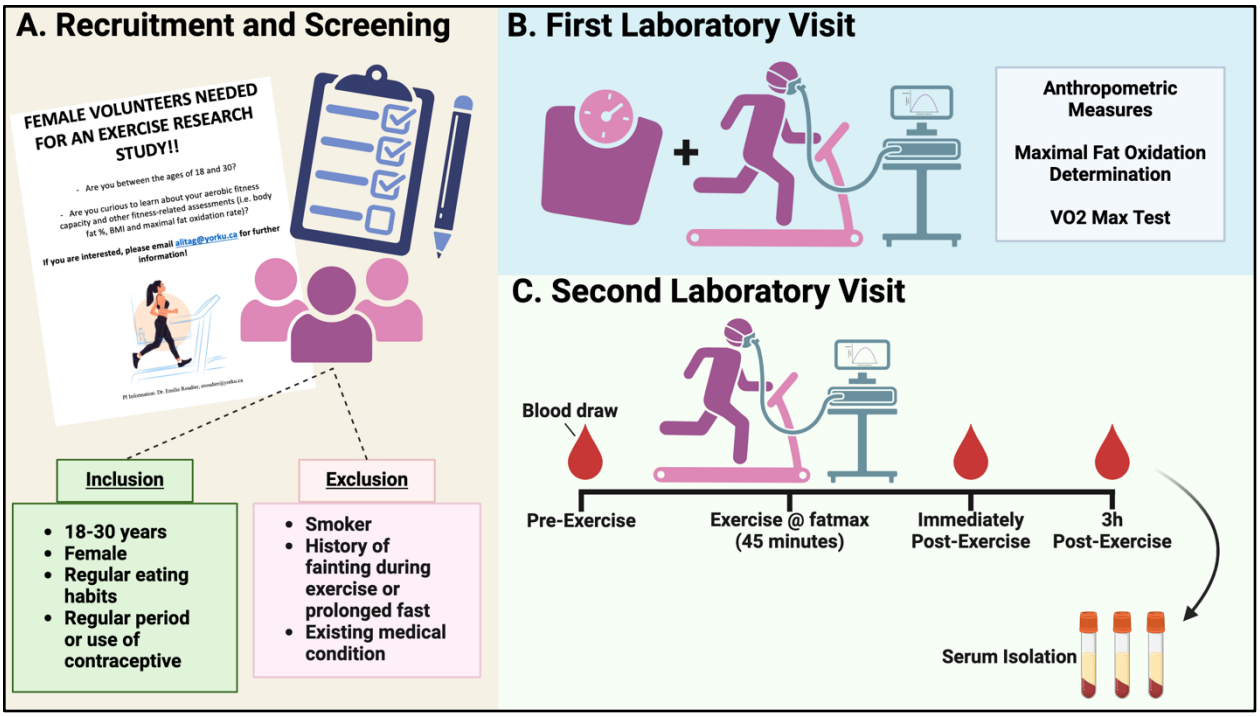
- Determining true maxFOR workload for all participants.
- Confirming that this type of exercise stimulates the release of FFA into the blood.

In vitro objectives

- Assessing proliferation and migration in cultivated HAMEC after treatment with pre- and post- maxFOR exercise serum samples.
- Assessing genes related to FFA transport and endothelium permeability in cultivated HAMEC after treatment with pre- and post- maxFOR exercise serum samples.

3.0. METHODS
3.1- PART 1: PARTICIPANTS (Figure 6A and B)
3.1a- Participant Information

Eleven healthy females, aged 18-30 years, were recruited from York University using approved posters posted on campus (**Appendix A**), through classroom presentations, and by word of mouth. Participants were screened to ensure they met the inclusion criteria which included: no existing cardiovascular diseases or metabolic conditions, no existing hormonal imbalances, having a regular period for the past 6 months, no history of fainting during exercise or prolonged fasting, being a non-smoker and no irregular eating habits- defined as dieting, binging or purging. Screening questions were sent to participants via email (**Appendix B**). Participants who met the eligibility criteria were informed about the study protocol and then provided written informed consent (**Appendix C**). The Physical Activity Readiness Questionnaire for Everyone (www.eparmedx.com) was administered to ensure each participant was safe to engage in unrestricted progressive intensity physical activity (**Appendix D**). This research study was approved by York University's Human Research Ethics Board (certificate # e2023-318). All study participants were asked to track their menstrual cycle and maintain the same dietary and physical activity habits for the duration of the study (**Figure 6A**).



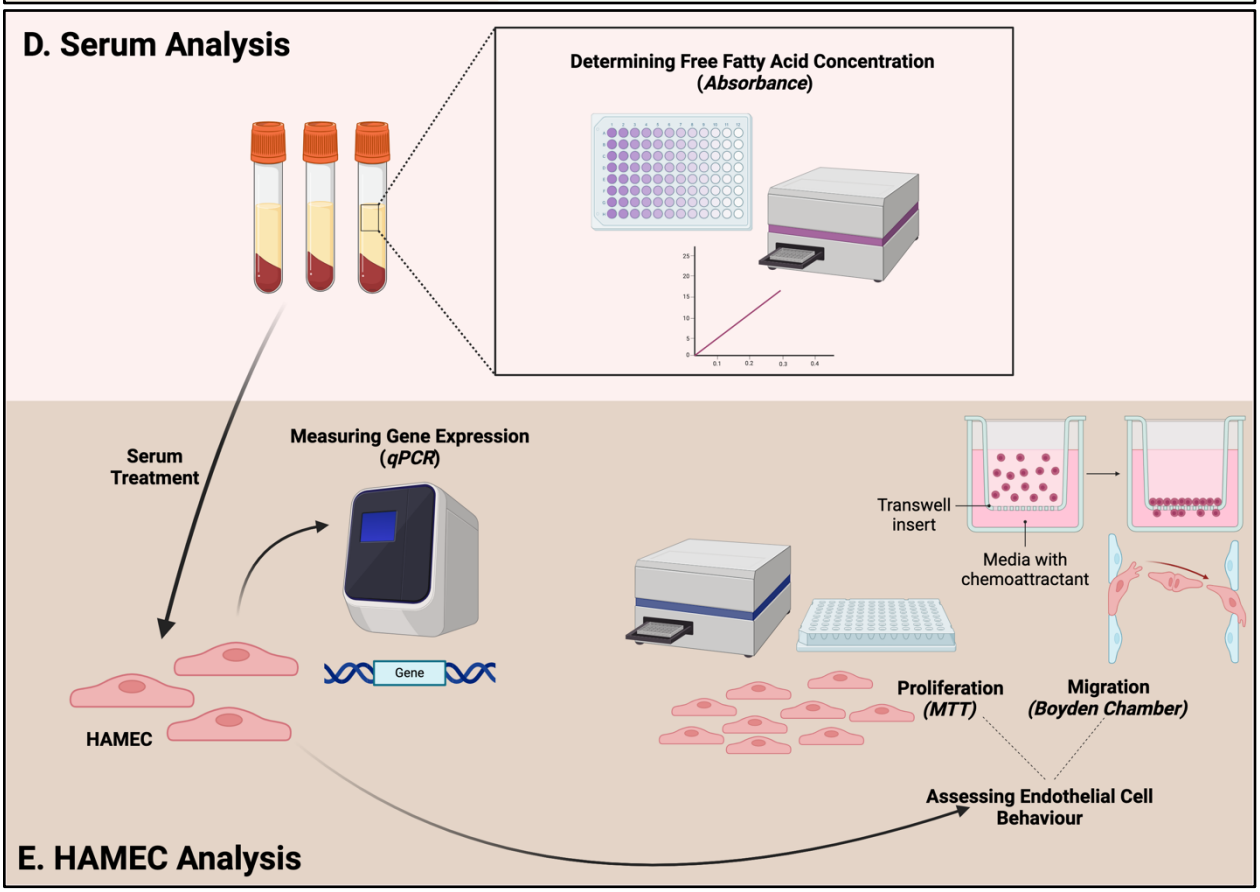


Figure 6 - Overview of Methods

3.1b- Laboratory Visits Overview

Participants were asked to attend two laboratory visits. All visits were conducted in the morning (between 7am-10am) to account for circadian rhythm effects (Froy, 2010). During the first visit, anthropometric measures, maxFOR and VO_2 max were assessed (**Figure 6B**). During the second visit, participants exercised at their maxFOR and blood draws were performed (**Figure 6C**). These visits were conducted with a minimum 48h between each visit to allow for washout. Prior to each experimental day, participants were asked to arrive to the laboratory following a 16h fast. Water consumption was permitted (no caffeine or alcohol). Participants were required to refrain from vigorous physical exercise 24h before each visit. Participants were also asked to keep a food log the day prior to their first visit and prompted to follow the same food log the day prior to their second visit. Visits were scheduled when participants were in their menstruation or late luteal pahse – periods of the cycle where estrogen levels were reported to be lowest. Menstrual cycle day and length were noted for each participant during their visits. Use of a contraceptive method was noted for each study participant if applicable. All applicants were given a standardized explanation of the protocol prior to commencement of any task.

3.1c- First Laboratory Visit

3.1c.1- Anthropometric Assessments

Anthropometric data were measured using standardized laboratory procedures (Jamnik & Glendhill, 2015) and were all performed by the same researcher for all participants. Body mass (kg) was measured using the Seca Alpha Scale (Model 770). Height was measured without footwear using a wall-mounted stadiometer and recorded to the nearest centimeter. Body mass and height measurements were used to calculate participants' Body Mass Index (BMI, $kg \bullet m^{2\ -1}$). Percent Body Fat (%BF) and estimated Skeletal Muscle Mass (SMM, kg), was assessed via

bioelectrical impedance analysis (InBody 270 BIA system; InBody Co. Ltd.). SMM and height measurements were used to calculate participants Skeletal Muscle Index (SMI, $kg \bullet m^{2\,-1}$). Skinfolds (mm; triceps, biceps, subscapularis, iliac crest, and medial calf) were measured on the right side of all participants using Harpenden fat calipers (Baty International) according to the Physical Activity and Lifestyle "R" Medicine (PALM) protocol to determine body adiposity (Jamnik & Gledhill, 2015) and measured to the nearest millimeter. Waist Circumference was measured following the standard National Institutes of Health (NIH WC) protocol, with a tape measure around the waist, on the skin, and at the iliac crest level and recorded in centimeters. The average of two skinfold and NIH WC measurements were recorded and completed by the same research personnel. Pre-exercise Blood Pressure (BP) and Heart Rate (HR) measurements were determined in the seated position in a private room using an automated device (BpTRU Medical Devices Ltd.). Following a 5-minute seated rest period, the BpTRU™ recorded six sequential measurements, 1 minute apart. The BpTRU™ device generated an average value for the pre-exercise systolic plus diastolic BP and pulse rate using the last five of the six measurements.

3.1c.2- MaxFOR and VO_2 Max Assessments

Study participants were then fitted with a chest-mounted HR monitor (Polar Electro) and instructed on the exercise protocol. Participants' maxFOR and VO_2 max were determined via indirect calorimetry, assessing the expired fraction of Oxygen (O_2) and Carbon Dioxide (CO_2). Participants performed a continuous ramp graded exercise test on the treadmill. Each workload lasted 3 minutes and there was a consistent increase in intensity at every stage. At the start of the protocol, two resting VO_2 measurements were collected. The exercise began at 1.6 Miles Per Hour (mph) at a 1% incline. Speed then increased by 0.2mph every 3 minutes until the participant reached a comfortable brisk walking pace (3.2 - 3.6mph). Once the maximum walking speed was

attained, the incline was increased by 2% every 3 minutes thereafter, until a Respiratory Exchange Ratio (RER) of 1.0 or greater was obtained. Frayn's equation (**Equation 7**) was used to calculate and monitor FOR throughout the exercise to determine when maximal FOR has been reached. Nitrogen excretion rate was assumed to be negligible for the purpose of the calculations. The FOR equation employed is as follows: **Equation 7***: Fat (g·min-1) = 1.67 x VO_2 (L·min-1) – 1.67 x VCO_2 (L·min-1).*

Once maxFOR was reached, the incremental-to-maximal effort treadmill test was performed for the determination of VO_2 max. The speed of the treadmill was increased every 2 minutes by 1mph until the participant reached a comfortable running pace (6 – 8mph). Participants who were unable to run completed a modified walking protocol of the test. Once this speed was achieved, the incline of the treadmill was increased by 2% every 2 minutes. The participants were instructed to remain on the treadmill until their work tolerance was compromised (they could no longer keep pace or requested to stop), at which point they received a 2-minute low-intensity active recovery period. Following recovery, the participants completed another incremental workload followed by a 2-minute recovery. This discontinuous sequence was repeated until the VO_2 of the subsequent workload was equal to or lower than the previous, indicating attainment of VO_2 max (Achten et al., 2002; Howley et al., 1995; Jamnik & Gledhill, 2015). This could also be referred to as verification or supramaximal testing (Hancock et al., 2023; Howley et al., 1995). The VO_2 max test was terminated if the study participant could no longer complete the workload due to volitional fatigue or if VO_2 max criteria were met, including

1) No further increase in VO_2 with increased workload;

2) Participant was unable to continue;

3) RER > 1.0;

4) HR > 90% age predicted HR (**Equation 1:** *age predicted HR = 220 – age*);

Following completion of the test, participants went through a 5-minute cool down period on the treadmill (3mph at 1% incline).

The FOR and VO_2 were determined from measurements obtained during the last 30 seconds of each workload via direct analysis of mixed expired gases. The discrete component system consisted of; a 120L Tissot Gasometer (Warren E Collins Ltd.), Rapid Response O_2 and CO_2 Gas Analyzers (Applied Electrochemistry, Model S-3A and CD-3S), a flexible plastic hose, two-way Y-valve (Ewald Koegal Co.), mouthpiece, and nose plugs. The mouthpiece was positioned between the participants' gums and teeth, and they were required to breathe in and out of the mouthpiece with their noses plugged throughout the collection period. The two-way Y-valve allowed the participants to inhale atmospheric air freely, then directly exhale air into the hose and tank, where the gases were collected and mixed. Once the expired gases were collected in the Tissot Gasometer, they were analyzed using the Rapid Response O_2 and CO_2 Gas Analyzers. Between all measurements, gas calibrations were performed by the same researcher. Collected variables- Minute Ventilation (V_E; $mL \bullet min^{-1}$) and fractions of expired O_2 and CO_2- were then used to calculate the participants' VO_2, RQ (**Equation 3:** *RQ = Volume CO_2 (VCO_2) / Volume O_2 (VO_2)*) and EE (**Equation 2**: *EE (kcal) = (3.815 + (1.232*RQ)*VO_2)*duration of exercise (min)*) (Hancock et al., 2023). The criterion HR was measured throughout using a Polar HR Chest Monitor (Polar Electro).

3.1d- Second Laboratory Visit

3.1d.1- Exercising at Identified MaxFOR

Participants were asked to return to the laboratory following a 16h overnight fast to complete a 45-minute bout of exercise at their identified maxFOR. The maxFOR exercise

intensities for all participants are outlined in **Supplementary Table 1.** The Discrete Open-Circuit Spirometry System was utilized to monitor maxFOR. Resting and exercise HR were measured via a Polar HR Chest Monitor (Polar Electro). Resting FOR and VO_2 measurements were obtained with the participants seated. Expired gases were collected and analyzed in the same manner as during the first laboratory visit and then used in Frayn's equation (**Equation 7**) to calculate FOR throughout the exercise. The protocol began with a 10-minute warm-up at 3.0mph and a 1% incline. Speed and incline were then set to the corresponding FOR determined for each participant during their first visit. The expired gases were collected via indirect calorimetry in the last thirty seconds of the 15-, 20-, 30-, 40- and 45-minute marks to calculate FOR using Frayn's equation (**Equation 7**). The urinary nitrogen excretion rate was assumed to be negligible for the purpose of the calculations. At the end of each workload, participants' HR was recorded. Body mass (kg) was measured using the Seca Alpha Scale (Model 770) pre-, immediately post-, and 3h-post exercise for all participants. Water consumption (mL) was monitored post-exercise until the last blood draw was performed (**Supplementary Table 2)**.

3.1d.2- Blood Draws and Serum Isolation

Venous blood samples were collected from a vein located in the antecubital fossa of each participants' arm by trained study personnel using a standardized venipuncture technique pre-, immediately post-, and 3h-post exercise. For all blood draws, approximately 20mL of blood was collected in Red Top 6mL VACUETTE® Serum Clot Activator Vacutainer Collection Tubes (Cat. 456089, Greiner Bio-One). Blood samples were kept at room temperature for 30 minutes to clot before being centrifuged (Rotina 38R) at 1300 Relative Centrifugal Force (RCF) at 4°C for 15 minutes. Serum was then aliquoted into multiple small (1.5 mL) Eppendorf cryotubes and stored at -80°C until all trials were completed. All blood draws were performed in a fasted state to avoid

confounding effects of food metabolism on study results. Exercise start/finish and blood draw timepoints are outlined in **Supplementary Table 3.**

3.1d1.3- Hemoglobin and Hematocrit Measurements

Participants' hemoglobin ($g \bullet dL^{-1}$) was assessed using capillary blood. Briefly, participants' hands were warmed, relaxed, and palpated to allow optimal blood flow to the fingers. The middle finger was punctured using a Microtainer® Contact-Activated Lancet (Cat. 366594, Becton, Dickinson & Co.) and the HemoCue Hb201 Microcuvette (Cat. 361057596, Stevens Company Ltd.) was filled in one continuous process. The microcuvette was then placed in the HemoCue Hb 201+ Analyzer (HemoCue) to allow for hemoglobin reading. Hematocrit was then measured by multiplying the hemoglobin value by three.

3.2- PART 2: SERUM AND HAMEC ANALYSES (Figure 6D and E)

3.2a- Serum Free Fatty Acid Determination

Serum FFA (uM) were measured using the EnzyChrom™ Free Fatty Acid Assay Kit (cat. EFFA-100. BioAssay Systems) according to manufacturer's instructions. Briefly, serum samples were diluted 2-fold in water and assayed directly, measuring the optical density at 570nm. Palmitic acid was used to create a standard curve to calculate the FFA concentration of each serum sample according to **Equation 8**. All serum samples were loaded in duplicates and the average of both readings was used to report serum FFA.

Equation 8: $[Free\ Fatty\ Acid\ of\ Sample] = ((R_{SAMPLE} - R_{BLANK}) / Slope\ (uM^{-1})) \times 2$,
where R is the reading of the sample.

3.2b- Cell Culture

Primary Human Adipose Microvascular Endothelial Cells (HAMEC) (Cat. 7200, Lot. 19427, ScienCell Research Laboratories) were cultured in complete Endothelial Cell Media (ECM; Kit Cat. 1001, ScienCell Research Laboratories) containing 500mL of basal medium

supplemented with 5% Fetal Bovine Serum (FBS; Cat. 0025, ScienCell Research Laboratories), 1X Endothelial Growth Supplement (ECGS; Cat. 1052, ScienCell Research Laboratories) and 1X Penicillin/Streptomycin Solution (P/S; Cat. 0503, ScienCell Research Laboratories). Cells were plated on 50ug/mL collagen-coated dishes (Gibco Collagen I Rat Protein Tail; Cat. A1048301, ThermoFisher Scientific) for all experiments. Cells were cultured at 37℃ and 5% CO_2 and used between passages P4 and P7 and settled at a density of ~23,000 cells/cm^2. All experiments were conducted using cells from the same lot number (Lot. 19427), originally collected from a female donor (**Appendix E**).

3.2c- HAMEC Treatment with Human Serum

For all experimental conditions, sub confluent (~60-80%) HAMECs were cultured in reduced serum medium (i.e. starved) for 18h in Opti-Minimal Essential Media (opti-MEM; Gibco, Cat. 31985062, ThermoFisher Scientific) supplemented with 1X Penicillin/Streptomycin Solution (P/S; Cat. 0503, ScienCell Research Laboratories) before serum treatment. Hereafter, this condition will be referred to as "starvation". Cells were treated with serum by replacing 10% of the media volume with serum collected from participants. All volumes were calculated according to the surface area (cm^2) of the dish used for the experimental procedure, using 10mL in a T75 cell culture flask (75cm^2) as the standard measure (0.13mL / cm^2).

3.2d- Boyden Chamber Chemotaxis Migration Assay

EC migratory response was assessed by performing Boyden Chamber Chemotaxis Assays as previously described (Aiken et al., 2016). Following trypsinization, HAMECs were loaded in the upper chamber at a density of 500cells/µL Cells migrated through an 8-µm polycarbonate membrane filter (NeuroProbe, Inc., Cat. PFB8, Cedarlanes) coated with 50µg/ml collagen (Gibco, Cat. A10438-01, ThermoFisher Scientific) in 0.02M acetic acid (Cat. 320099, Sigma-Aldrich). In

each Boyden Chamber experiment, the basal level of HAMECs migration was assessed by loading the bottom chamber with 28µl of opti-MEM supplemented with 1% FBS (Cat. 0025, ScienCell Research Laboratories). A positive control for migration was incorporated on each assay by loading the bottom chamber with 28µl of opti-MEM supplemented with 10% FBS and 5% ECGS (Cat. 1052, ScienCell Research Laboratories). HAMECs migration was then assessed in response to stimulation by 10% serum collected pre-, immediately post- or 3h post-exercise. After 6h of migration time at 37℃, the nitrocellulose membrane filter was washed with cold Phosphate Buffered Saline (PBS; Gibco, Cat. 10010023, ThermoFisher Scientific), fixed in cold methanol, stained with 10% Giemsa stain diluted in water (Cat. R03055-74, Sigma-Aldrich), and mounted on a glass microscope slide.

For every Boyden experiment run (1 experiment = 48 total wells, as shown in **Figure 2**), one HAMEC flask was grown to appropriate passage and cell number and used. A total of 3 Boyden experiments were conducted to analyze serum treatment from all participants. 'Stim' and 'No-stim' conditions were included in every Boyden experiment (n=3 well each). For every participant and for every serum condition, n=3 wells were run. For each well, four individual fields of view were counted under 400X magnification, and results were presented as the average of all fields of view (**Figure 7**).

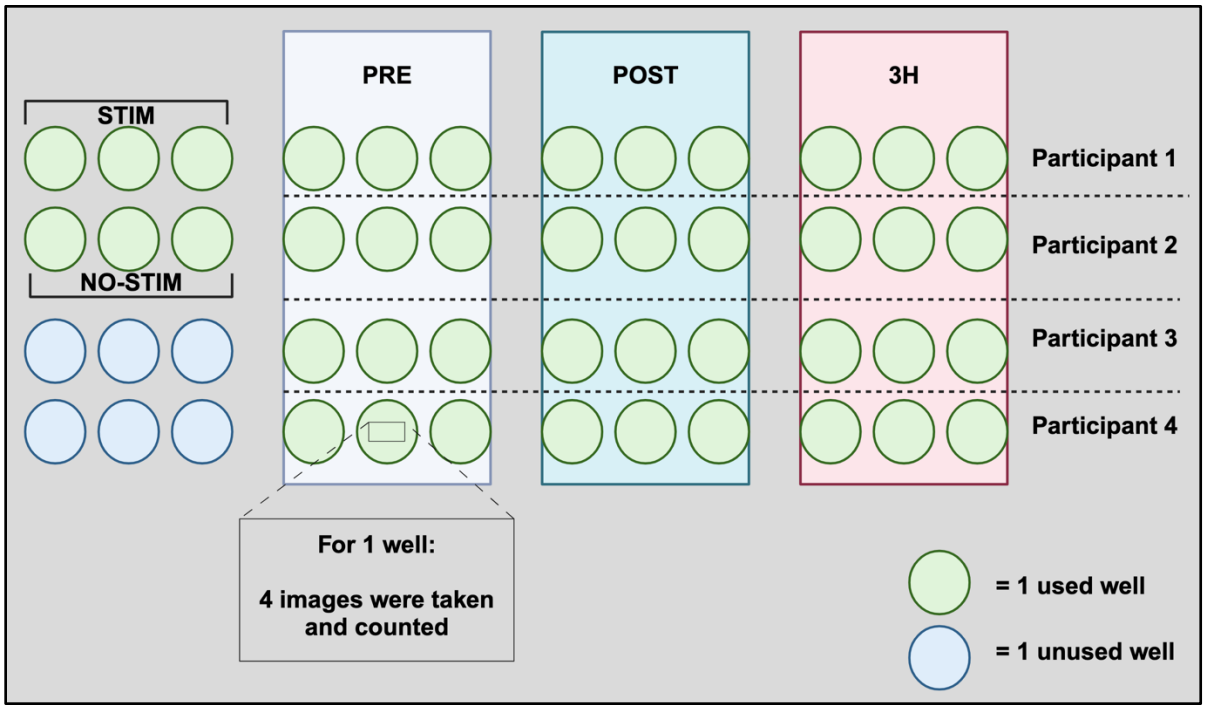


Figure 7. Example of One Boyden Chamber Experiment.

3.2e- HAMEC Proliferation

HAMECs were seeded on a 96-well plate at a density of 7,300 cells/well and cultured at 37℃ for 48h (until wells reached ~60% confluency). Cells were then treated with 10% participant serum for either 24h or 48h following an 18h starvation. Cells proliferation was quantified by using the cyQUANT® MTT Cell Proliferation Assay Kit (Invitrogen, Cat. V13154, ThermoFisher Scientific) according to the manufacturer's instructions using the rapid protocol. Briefly, metabolically active cells cause the conversion of water-soluble MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) to insoluble formazan. Formazan is then solubilized using Dimethyl sulfoxide (DMSO; Cat. D12345, ThermoFisher Scientific) and the assessment of cell proliferation is based on quantifying the absorbance of the colorimetric probe by measuring optical density at 540nm. Two independent cell conditionings were performed with six cell culture wells

per condition (n=6 pre-, post-, and 3h post-exercise for each participant) and per time-point (24h and 48h).

3.2f- Total RNA Isolation and Real-Time Polymerase Chain Reaction (qPCR)

Total RNAs were isolated from HAMEC cell lysate following an 18h starvation and then a 1h and 24h treatment with 10% serum using the QIAzol Lysis Reagent (Qiagen, Cat. 79306) as previously described (Lam et al., 2021). For control and $VEGF_{165}$ conditions, 3 independent wells were platted for RNA isolation. For participants, 3 wells were platted per participant, with 1 well for every serum condition (pre, post and 3h).

To convert isolated RNA to 400ng of cDNA per sample, RNA concentration was determined by measuring absorbance at 260 and 280 nm. RNA was then reverse-transcribed using the High-Capacity RNA-to-cDNA kit using appropriate volumes for each sample based on quantification (Applied Biosystems, Cat. 4387406, ThermoFisher Scientific). Semi-quantitative real-time PCR was performed for 10ng of sample in triplicate using TaqMan® Gene Expression Assay Probes and TaqMan® Universal Master Mix II (Applied Biosystems, Cat. 4440042, ThermoFisher Scientific) for 40 cycles of denaturation at 95℃ for 3 seconds, followed by annealing and extension at 60℃ for 30 seconds. Early Growth Response 3 (*EGR3;* Hs04935588_m1), Fatty Acid Binding Protein 4 (*FABP4;* Hs01086177_m1), Carnitine Palmitoyl Transferase I (*CPT1A;* Hs00912671_m1), Cluster of Differentiation 36 (*CD36;* Hs00354519_m1), VE-cadherin (*CHD5;* Hs00901465_m1) and Claudin-5 (*CLDN5;* Hs00533949_s1) were measured and the ΔΔCt method was used to express the relative changes. mRNA changes were normalized to the expression of the Hypoxanthine-Guanine Phosphoribosyl Transferase *(HPRT)* housekeeping gene. Data is expressed as $2^{-\Delta\Delta Ct}$ and all pre-serum samples were used to calculate the calibrator.

After1h treatment, *EGR3* gene expression was analyzed. All other gene expression (*FABP4, CPT1A, CD36, CDH5* and *CLDN5*) was measured after 24h serum treatment. For the 1h treatment, where we aimed to measure *EGR3,* two sets of experiments were conducted, as outlined in **Figure 8**. Hereafter, experiments will be referred to as 'set 1' and 'set 2' for this gene. While for the 24 hours treatment, all experiments were conducted on cell culture tissue from a single vial.

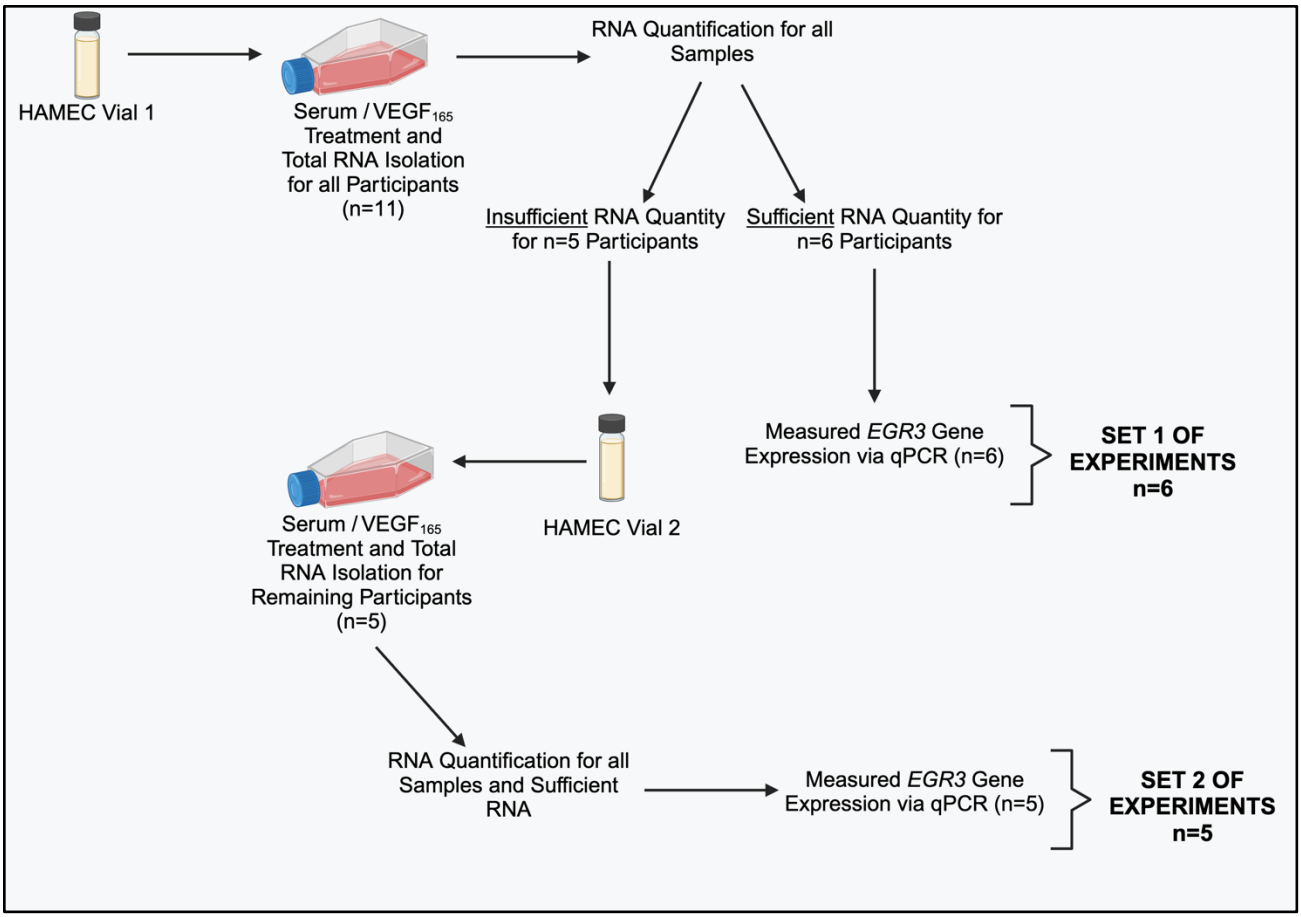


Figure 8. Schematic of Experiments Conducted for *EGR3* Gene Expression (1h serum treatment).

For every sample, genes of interest and housekeeping genes were measured by qPCR in triplicate. Triplicates were averaged to determine the Ct of genes of interest and housekeeping gene. Triplicates were all included when the SD of triplicate was below or equal to 0.3.

Alternatively, a triplicate was excluded if obvious outliner (when value was mean ± 2SD). When needed, qPCR was re-run in triplicate again if Ct could not be determined based on these principles.

3.3- Statistical Analyses

Statistical analyses were performed using Prism version 10.3.1 (GraphPad software). For anthropometric measures, descriptive statistics were conducted (mean ± SD, minimum and maximum values, range and 95% CI of the mean). Outliers were tested using ROUT (Q=1%). Relationship between two variables were tested via linear regression analysis. Effect between two groups were analyzed using an unpaired two-tailed t-test. For all serum treatments, one-way ANOVA mixed analysis effects (parametric) or Kruskal-Wallis (non-parametric) tests were conducted. Dunnett's post-hoc test was used to compare differences between control and treatment conditions. Tukey's post-hoc test was use for multiple comparisons between all groups. Fisher's LSD or Dunnett's uncorrected post-hoc tests were used to determine the least significant difference between groups. $p < 0.05$ was considered statistically significant.

4.0. RATIONALE FOR STUDY DESIGN

4.1. Female Participants

This study was conducted in female participants for multiple reasons. Firstly, the *in-vitro* primary cell model used to assess endothelial cell behaviour was derived from a female donor (**Appendix D**). Treating endothelial cells from a female donor with serum from female participants provided the most physiologically relevant context for the study. Secondly, there are known sex differences in endothelial transcription and cell behaviour (Lorenz et al., 2015; Rudnicki et al., 2023). Keeping sex consistent limited the study aim to characterizing a response to an acute bout of exercise rather than investigating sex differences. Finally, a choice was made to use young, healthy female participants to complete the analysis as young males have been predominantly used in the field (James et al., 2023) and due to the better response of exercise in women, causing greater gains in the reduction of all-cause and cardiovascular mortality risk from similar PA participation compared to men (Ji et al., 2024).

4.2. Inclusion and Exclusion Criteria

Young, healthy and being a non-smoker - This is the first study that is examining the response of EC to an acute bout of exercise performed at maxFOR. Notably, the presence of saturated FFA was associated with endothelial dysfunction in patients with type 2 diabetes mellitus (Perassolo et al., 2008) emphasizing the importance of recruiting a healthy study population. Future studies can investigate the impact of age, sex, smoking and presence/absence of disease.

Absence of existing cardiovascular diseases, metabolic conditions, and history of fainting during exercise or prolonged fasting - As outlined in the Par-Q+ (**Appendix C**), if participants indicated the presence of general health concerns or issues, the participants were advised to seek medical advice before engaging in any physical activity.

Presence of a regular period and absence of hormone imbalances - Hormone imbalances may present a confounding effect on study results. Although menstrual cycle phase does not affect FOR (Frandsen et al., 2020) or FFA composition in the serum (Mattsson et al., 1985), estrogen has been shown to be a key player in lipid and glucose metabolism (Katzer et al., 2021) and direct modulator of angiogenesis (Losordo & Isner, 2001). Participants were asked to perform all tasks when estrogen levels were reported to be at their lowest, either during menstruation or in the late luteal phase, as estrogen is a known pro-angiogenic factor (Losordo & Isner, 2001). By recruiting females with regular menstrual cycles or using contraceptives, hormone levels were better predicted and accounted for during the study period.

Absence of irregular eating habits, fasting requirement, and following a similar food log before each testing day – Substrate use during exercise is greatly determined by an individual's diet. For instance, dietary carbohydrate appears to be a predictor of fat oxidation during exercise (Brun et al., 2022). Støa and colleagues aimed to assess the variability in fat oxidation after only one day of change in diet composition. The authors reported no difference in fat oxidation when participants were given the same habitual diet on two separate days before fat oxidation testing. Yet, fat oxidation decreased after a carbohydrate-rich diet (50% carbohydrate) compared to both the habitual diet, and fat-rich diet (50% fat) (Støa et al., 2016). By recruiting participants with regular eating habits and prompting participants to follow the same food log before each laboratory visit, variability in fat oxidation and substrate use during exercise may be considered. Finally, having participants perform the exercise in a fasted state increased fat oxidation during exercise and blood flow to the adipose tissue (Ratliff et al., 2022).

4.3. Performing all Exercise at Same Time of Day

Numerous physiological processes are influenced by a circadian rhythm. For instance, some studies show that substrate oxidation can differ depending on the time of the day (Froy, 2010). Other studies reveal that higher maxFOR values may be obtained in the evening compared to the morning, although this was only shown in men (Mohebbi & Azizi, 2011). To avoid potential differences between our study participants, all laboratory visits were conducted in the morning (**Supplementary Table 4**). The morning was chosen to facilitate the fasting period for participants.

4.4. Blood Draw 3h Post-Exercise

A study by He and colleagues aimed to measure changes in several biomarkers in response to either an acute bout of exercise performed at the maxFOR or exercise at higher intensity. A significant increase in the autocrine glycoprotein follistatin and the adipokine omentin, and significant reduction in the adipokine resistin, were observed 3h post-exercise at maxFOR, serving as the basis of the recovery time chosen in this study (He et al., 2019).

Interestingly, a fasted 4-hour recovery period after a bout of moderate-intensity exercise has been reported to impact FFA metabolism and ATBF (Al Mulla et al., 2000). Furthermore, a study by Fabre and colleagues investigated the effects of an acute bout of exercise training on the genomic response in adipose tissue. The authors noted that 4h after an acute bout of exercise, genes involved in AT angiogenesis, cell-cell signaling, and chemotaxis were altered (Fabre et al., 2018). Putting these findings together, a 3-hour recovery period was the most reasonable for our study.

4.5. Performing a Single Bout of Exercise versus Training

Studying an acute bout of exercise is of interest as this is where the body experiences the highest stress response. As an individual repeatedly trains, their body adapts and there is a decrease

in response to the same stimulus (Rivera-Brown & Frontera, 2012). Investigating the systemic effects of exercising at the maxFOR may allow the characterization of the initial stress response.

4.6. Measuring Hemoglobin and Hematocrit

An individual's VO_2 max can be limited by the ability of the cardiorespiratory system to take up and transport O_2 to the muscles (Wittenberg, 1970) and the ability of the muscles to take up and use the O_2 (Poole & Musch, 2023). The iron-rich protein hemoglobin found in red blood cells is required to bind O_2 and transport it to our tissues. For instance, VO_2 max was found to be greater two days post induced erythrocythemia (i.e. blood doping) in women. Hemoglobin levels also increased (Robertson et al., 1984). By measuring hemoglobin and hematocrit in study participants, potential individual differences in O_2 transport were considered.

Another aspect of O_2 transport that varies between individuals is the extraction and utilization of O_2 at the level of the skeletal muscle. Once O_2 has been delivered to myocytes via the blood stream, the iron-rich protein myoglobin acts as an intracellular O_2 storage site for the muscle, binding to O_2 within the myocyte (Ordway & Garry, 2004). Once muscle activity begins, myoglobin desaturates, helping to increase the O_2 diffusion gradient from the capillaries to the myocyte (Kamga et al., 2012; Ordway & Garry, 2004). Once O_2 is released, it enters the mitochondria for oxidative phosphorylation (Memme et al., 2021). Greater mitochondrial density in the muscle allows for greater and more efficient energy production and O_2 utilization (MacInnis & Gibala, 2017). These are factors that may attribute to variability in participants' VO_2 max that have not been measured in our study.

4.7. Monitoring Water Consumption Post-Exercise

An individual's hydration status can impact their blood volume. As part of this study measured the concentration of factors found in the serum, water consumption post-exercise was

considered. Potential dilution effects could show no change in factor concentration observed in the 3h recovery period. For instance, Endo and colleagues reported biphasic changes in blood composition in humans after drinking 1L of water post-overnight dehydration. Conversely, Benozzi and colleagues reported that water intake of 300mL 1h prior to phlebotomy did not interfere with glucose, total protein, urea, total cholesterol, total bilirubin, uric acid or triglycerides at a clinically significant level (Benozzi et al., 2018). On average, participants in this study drank 400mL of water post-exercise (**Supplementary Table 3**). Based on volume of water consumed and the fact that participants were allowed to drink water *ad libitum* for the duration of the study, adjusting for dehydration and changes in factor concentration post-exercise were not needed in this study.

4.8. Cellular Model

Primary HAMEC were selected as the *in-vitro* model because both the cells and the serum collected were derived from humans. Secondly, as this study aimed to investigate the effects of an acute bout exercise performed at the maxFOR, on the angio-adaptation of blood vessels in the AT, EC derived from this tissue would be most appropriate.

4.9. Serum Treatment Conditions

Previous work in the laboratory used 10% muscle homogenate to assess effects of exercise stimulation (Aiken et al., 2016). For this study, we treated cells with 10% serum, allowing us to observe a change at the level of gene expression in the cells. Prior to gene expression and proliferation assay, HAMECs were starved for 18h to ensure all cells were at the same point of the cell cycle and promote cell synchronization when treated with serum.

5.0. RESULTS

NOTE: Each participant is represented either by a number (participant ID) or colour (as indicated in **Table 2**). Where possible, participants are organized from lowest to highest VO_2 max (1- lowest, 11- highest). **Table 2** matches both participant ID and colour to help easily identify participants.

5.1. Participant Characteristics

Table 1 presents the baseline anthropometric and select fitness assessments of the 11 women (aged 23.6 ± 3.0 years) recruited. **Table 2** presents characteristics that may influence VO_2 max (hemoglobin; g/dL, skeletal muscle index; SMI $kg \cdot m^{2\,-1}$ and body mass index; BMI $kg \cdot m^{2\,-1}$) for every participant.

Table 1: Participants Anthropometric and Fitness Assessments (n=11)

Characteristics	Value
Age, years, mean ± SD	23.6 ± 3.05
Hemoglobin, $g \cdot dL^{-1}$, mean ± SD	12.9 ± 1.22
Body Mass Index (BMI), $kg \cdot m^{2\,-1}$, mean ± SD	22.8 ± 2.71
Waist Circumference, cm, mean ± SD	79.3 ± 5.53
Sum of 5 Skinfolds (SO5S), mm, mean ± SD	66.3 ± 27.8
InBody Body Fat, %, mean ± SD	27.1 ± 6.37
Skeletal Muscle Index (SMI), $kg \cdot m^{2\,-1}$, mean ± SD	9.21 ± 1.21
Maximal Fat Oxidation, $g \cdot min^{-1}$, mean ± SD	0.41 ± 0.11
MaxFOR, % VO_2 max, mean ± SD	57.4 ± 8.61
MaxFOR, $mL \cdot kg^{-1} \cdot min^{-1}$, mean ± SD	22.2 ± 4.08
VO_2 Max, $mL \cdot kg^{-1} \cdot min^{-1}$, mean ± SD	38.9 ± 6.71
Menstrual Cycle Phase, *n*, %*****	
o Menstruation (Days 1-7)	8, 72
o Follicular Phase (Days 7 -14)	0, 0
o Ovulation (Day 14)	0, 0
o Luteal Phase (Days 14-35)	
Days 14-21	0, 0
Days 21-35	3, 27
Use of Contraceptive, *n*, %	
o **Yes (pill)**	2, 18
o **No**	9, 81

***Menstrual cycle phase was noted on the day participants exercised at MaxFOR**

Table 2. Characteristics Related to VO_2 Max Workload Depicted for Every Participant

Participant ID	Hemoglobin ($g \cdot dL^{-1}$)	Skeletal Muscle Index ($kg \cdot m^{2\,-1}$)	Body Mass Index ($kg \cdot m^{2\,-1}$)	Max Fat Oxidation ($g \cdot min^{-1}$)	MaxFOR (%VO_2 Max)	VO_2 Max ($mL \cdot kg^{-1} \cdot min^{-1}$)
1	12.4	9.32	22.1	0.43	61.7	31.4
2	13.0	9.38	26.0	0.33	40.9	33.1
3	11.2	8.09	22.4	0.36	54.0	33.3
4	11.7	9.01	24.6	0.34	66.0	33.9
5	12.5	6.96	16.6	0.28	69.5	34.5
6	12.8	8.84	21.0	0.25	57.1	37.0
7	14.9	9.53	22.7	0.44	64.5	41.2
8	12.0	8.54	22.1	0.57	61.4	43.2
9	14.7	9.89	26.4	0.53	58.4	43.4
10	12.7	11.79	24.9	0.39	48.7	44.9
11	14.3	9.91	22.5	0.59	48.8	53.1

The average hemoglobin concentration was 12.9 ± 1.22 $g \cdot dL^{-1}$ and is within the normal range for this population. When assessing individual hemoglobin values, nine of the women were non-anemic (≥12.0 $g \cdot dL^{-1}$) and two of the women were mildly anemic (11.0-11.9 $g \cdot dL^{-1}$) (WHO, 2000). The average BMI for the women was 22.8 ± 2.71 $kg \cdot m^{2\,-1}$. One woman was underweight (<18.5 $kg \cdot m^{2\,-1}$), seven had a healthy weight (18.5 – 24.9 $kg \cdot m^{2\,-1}$), two were overweight (24.9 – 30 $kg \cdot m^{2\,-1}$), and none were obese (>30 $kg \cdot m^{2\,-1}$) (Enderle et al., 2023; Ofenheimer et al., 2020). Values above the 80cm threshold for waist circumference are associated with increased cardiovascular risk. Three participants from our cohort were above this threshold, although the average value (79.3 ± 5.53 cm) for all the women fell below this threshold (Ross et al., 2020). There are no official reference values of Sum of 5 Skinfolds (SO5S) measurements. Overall, in our population, the average SO5S was 66.3 ± 27.8 mm. The average measurements for all skinfolds are as follows: Triceps, 15.5 ± 7.83mm; Biceps, 7.25 ± 3.22mm; Subscapular, 14.1 ± 5.9mm; Suprailiac, 15.5 ± 7.22mm and Medial Calf, 14.02 ± 6.74mm. The average percent body fat for

our group was 27.1 ± 6.4%, corresponding to a percentage that is associated with the "average" category compared to the general population (ACSM, 2019).

The European and Asian Working Group for Sarcopenia (AWGS) Skeletal Muscle Index (SMI) cut-off value to evaluate the loss of muscle mass in the diagnosis of sarcopenia is 5.7 $kg•m^{2\ -1}$ in females (Cruz-Jentoft et al., 2010; Oshita & Myotsuzono, 2021). The average SMI in our population was 9.21 ± 1.21 $kg•m^{2\ -1}$. None of the women in our population fell below the sarcopenia cut-off value. Finally, the VO_2 max of our population of study falls within the normal average for women of this age group (38.9 ± 6.71 $mL•kg^{-1}•min^{-1}$), corresponding to the 10^{th} - 25^{th} percentile for CRF according to the FRIEND database (Kaminsky et al., 2015).

Next, we tested whether any participant would deviate for a normal distribution for every morphometric assessment performed. No outliers were identified therefore, we included all participants for follow up experiments.

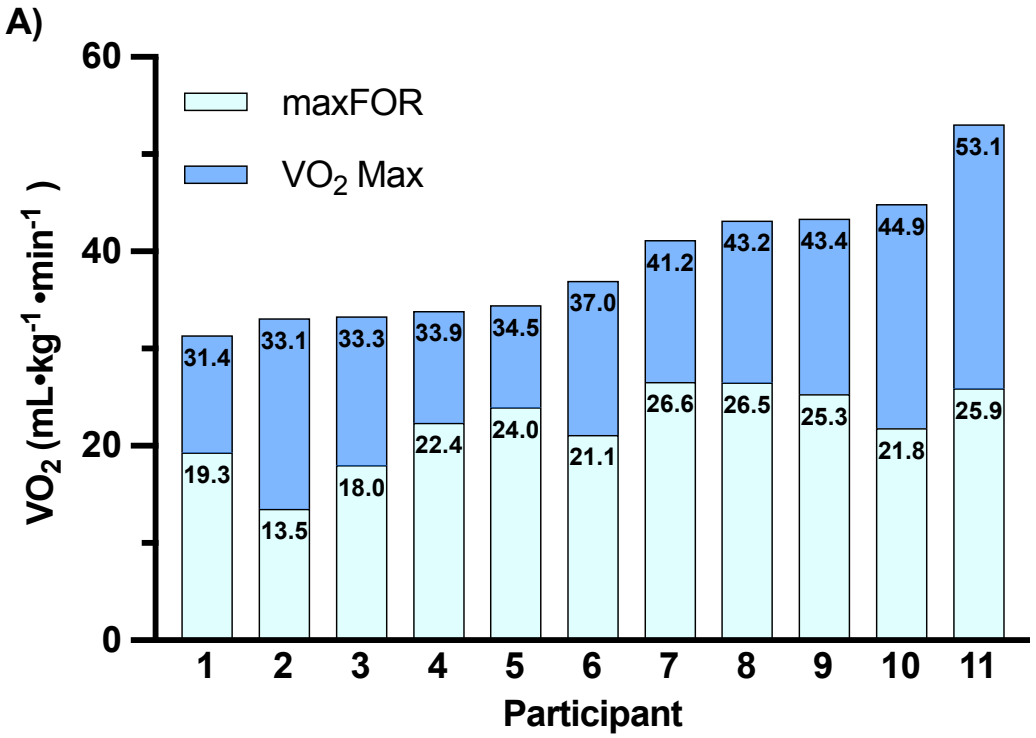
5.2. MaxFOR and VO_2 Max Workloads for Each Participant

Our cohort had a mean ± SD VO_2 max of 38.9 ± 6.71 $mL•kg^{-1}•min^{-1}$ (**Table 1,** n=11). The 95% CI was: [34.47 – 43.49] $mL•kg^{-1}•min^{-1}$. The lowest VO_2 max was 31.4 $mL•\ kg^{-1}•\ min^{-1}$ (**Figure 9A and Table 2**, participant 1) and the highest VO_2 max was 53.1 $mL•\ kg^{-1}•\ min^{-1}$ (**Figure 9A and Table 2**, participant 11) (Range: 21.74 $mL•kg^{-1}•min^{-1}$). All other VO_2 max, expressed in $mL•\ kg^{-1}•\ min^{-1}$, are depicted in **Figure 9A** (dark blue bars) **and Table 2**.

The mean ± SD VO_2 at the maxFOR workload (n=11) was 22.2 ± 4.08 $mL•\ kg^{-1}•\ min^{-1}$ (**Table 1**). The 95% CI of the mean was: [19.48 – 24.96] $mL•kg^{-1}•min^{-1}$. The lowest VO_2 at maxFOR was 13.5 $mL•kg^{-1}•min^{-1}$ (**Figure 9A and Table 2**, participant 2) and the highest VO_2 at

maxFOR was 26.6 •kg^{-1} •min^{-1} (**Figure 9A and Table 2**, participant 7) (Range: 13.01 mL•kg^{-1} •min^{-1}). All other VO_2 workloads, expressed in mL• kg^{-1} • min^{-1} are depicted in **Figure 9A** (light blue bars).

When expressing the maxFOR workload as a percentage of an individual's VO_2 max, the mean ± SD (n=11) is 57.4 ± 8.61 %VO_2 max (**Table 1**). The 95% CI of the mean was: [51.57 – 63.14] %. The lowest maxFOR workload was 40.9 %VO_2 max (**Figure 9B** and **Table 2**, participant 2) and the highest maxFOR workload was 69.5 %VO_2 max (**Figure 9B** and **Table 2**, participant 5) (Range: 28.65%). All other FOR workloads, expressed as %VO_2 max, are depicted in **Figure 9B** (green bars) and **Table 2**.


A)
maxFOR
VO2 Max
VO2 (mL•kg-1•min-1)
Participant
0
20
40
60
1
2
3
4
5
6
7
8
9
10
11
31.4
19.3
33.1
13.5
33.3
18.0
33.9
22.4
34.5
24.0
37.0
21.1
41.2
26.6
43.2
26.5
43.4
25.3
44.9
21.8
53.1
25.9

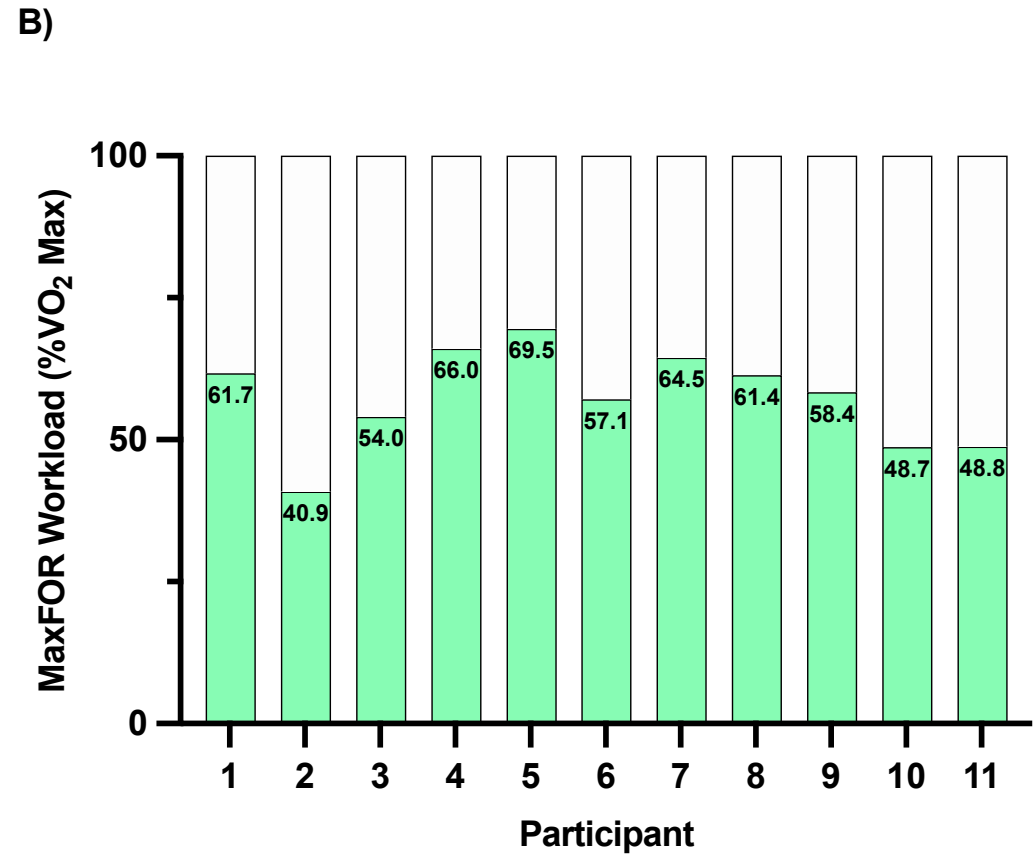

B)
MaxFOR Workload (%VO2 Max)
Participant
0
50
100
1
2
3
4
5
6
7
8
9
10
11
61.7
40.9
54.0
66.0
69.5
57.1
64.5
61.4
58.4
48.7
48.8

Figure 9. Visualisation of MaxFOR and VO_2 Max Workloads for Each Participant
A) MaxFOR (light blue) and VO_2 max (dark blue) ($mL \bullet kg^{-1} \bullet min^{-1}$; y-axis) depicted for each participant. Every participant is represented by a different number on the x-axis. Participants are organized from lowest to highest VO_2 max; [31.4 - 53.1] $mL \bullet kg^{-1} \bullet min^{-1}$. The bottom portion of each bar represents the VO_2 at maxFOR whereas the upper portion represents the VO_2 max for a given participant. **(B)** maxFOR VO_2 (green; y-axis) represented as a percentage of every participant's VO_2 max. White bars for every participant represent 100% of their VO_2 max. The numbers in each green bar represent the VO_2 at maxFOR (%VO_2 max) for a given participant. Every participant is represented by a different number on the x-axis. Participants are organized from lowest to highest VO_2 max; [31.4 - 53.1] $mL \bullet kg^{-1} \bullet min^{-1}$.

5.3. Every Participant Reached their Maximal Fat Oxidation Rate

As exercise intensity increased (~0-55% VO_2 max), the FOR ($g \bullet min^{-1}$) increased until it reached a peak, markedly decreasing as exercise intensity continued to increase (>55-100% VO_2 max) (**Figure 10A**). The workload at which participants reached their maxFOR varied but fell between 40.9 - 69.5 %VO_2 max (**Figures 9** and **10**). The mean maxFOR was 0.41 ± 0.11 $g \bullet min^{-1}$ (n=11, **Table 1**). The 95% CI of the mean was: [0.33 – 0.49] $g \bullet min^{-1}$. Every participant reached their maxFOR as depicted by the peak of the curves in **Figure 10A,** or the values listed in **Table 2**. The lowest maxFOR was 0.25 $g \bullet min^{-1}$ (**Figure 10A** and **Table 2**, participant 6) and the highest maxFOR was 0.59 $g \bullet min^{-1}$ (**Figure 10A** and **Table 2**, participant 11) (Range: 0.34 $g \bullet min^{-1}$).

We measured the average Respiratory Quotient (RQ) of each participant to help determine substrate use during exercise (**Equation 3**). In our cohort, the average RQ measured at the maxFOR (n=11) was 0.83 ± 0.04, validating the use of fat as the main substrate source of energy (**Table 3**). The 95% CI of the mean was: [0.81 – 0.86]. The lowest RQ was 0.78 (**Table 3**, participant 3) and the highest RQ was 0.89 (**Table 3**, participant 6) (Range: 0.19). All other RQ values are depicted in **Table 3**.

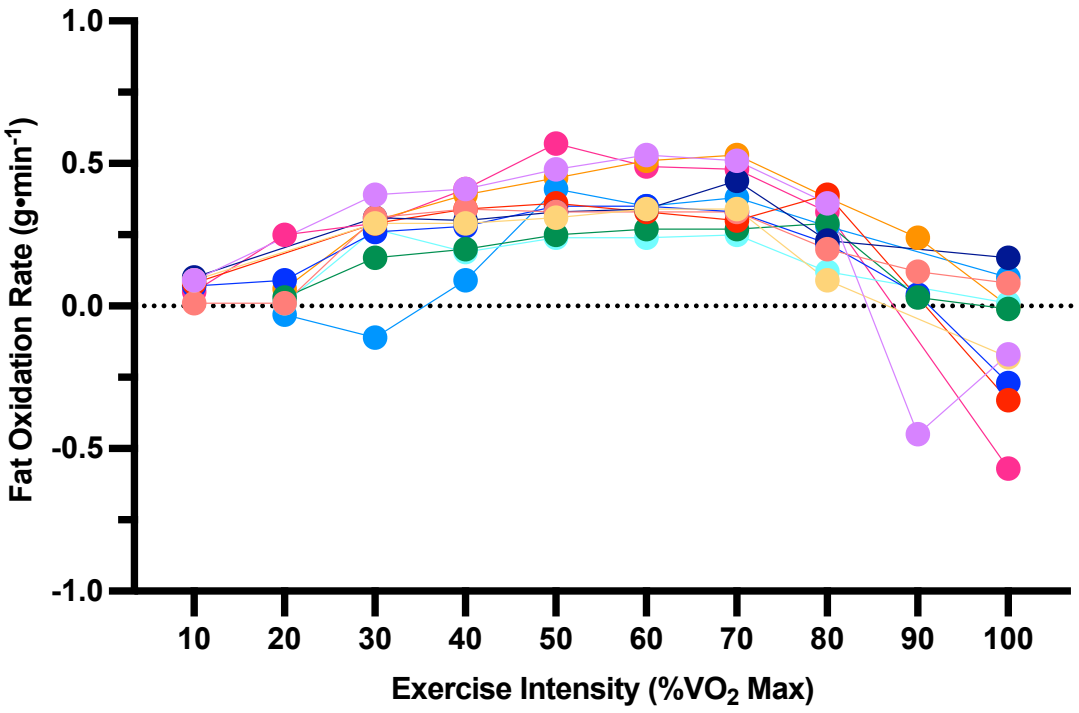


Figure 10. Maximal Fat Oxidation Rate Curves for Each Participant

Curves of estimated fat oxidation rates (g•min^{-1}, y-axis) plotted as a function of exercise intensity (% VO_2 max; x-axis) for every participant enrolled in the study (n=11) and color-coded as indicated in **Table 2**. Every point represents the average fat oxidation for a participant within a given workload range. Curves are used to illustrate the intensity of exercise where participants reached their maximal fat oxidation rate and to demonstrate variability in maximal fat oxidation rates between individuals. Values on the y-axis represent the upper limit of the exercise intensity, in intervals of ten.

Table 3. Respiratory Quotient for Every Participant at MaxFOR

Participant ID	Respiratory Quotient (RQ)
1	0.79
2	0.86
3	0.78
4	0.87
5	0.85
6	0.89
7	0.85
8	0.79
9	0.82
10	0.86
11	0.78

5.4. VO_2 Max Positively Correlates with MaxFOR, Hemoglobin Content and Energy Expenditure During Exercise at the MaxFOR

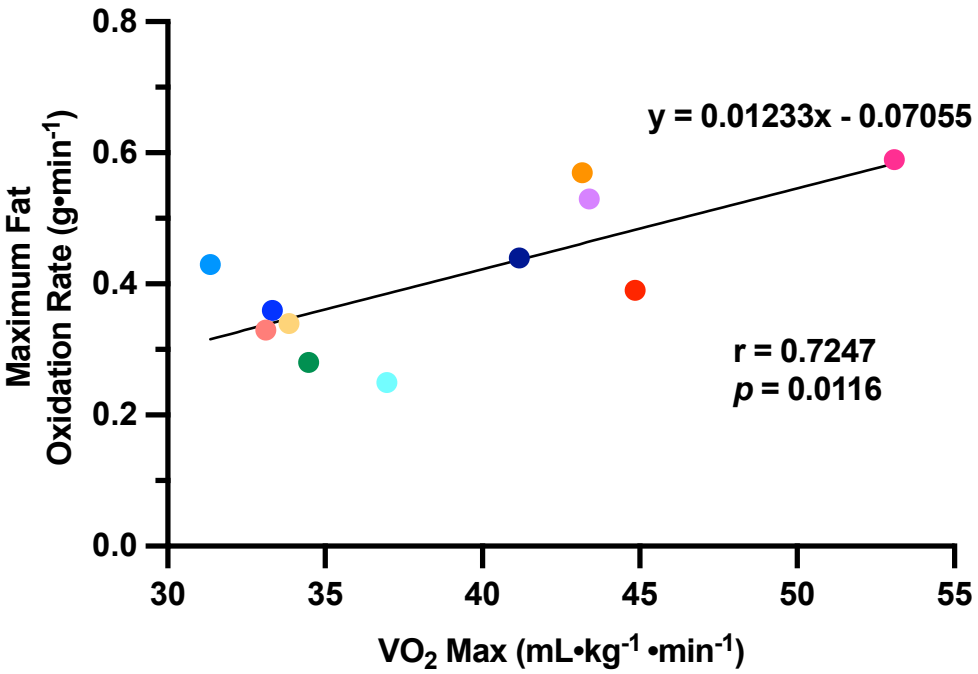
There is a known relationship between maxFOR and VO_2 max. To further validate the maxFOR of participants in this study, the relationship between maxFOR and VO_2 max was tested. A strong positive correlation was found between VO_2 max and maxFOR. As VO_2 max increased, maxFOR also increased (**Figure 11A**, r = 0.7241, p = 0.0116).

Outside of training status, there are a multitude of factors known to affect an individual's VO_2 max. These can include skeletal muscle mass, hemoglobin content, myoglobin, mitochondrial density, whole-body mass and lean body mass. Some of these factors are outlined in **Table 2**. In this cohort (n=11), there was a moderate positive correlation between VO_2 max and hemoglobin content ($g \cdot dL^{-1}$). As VO_2 max increased, hemoglobin content also increased (**Figure 11B**, r = 0.5897, p = 0.00562). Other measured factors were not significantly correlated with VO_2 max.

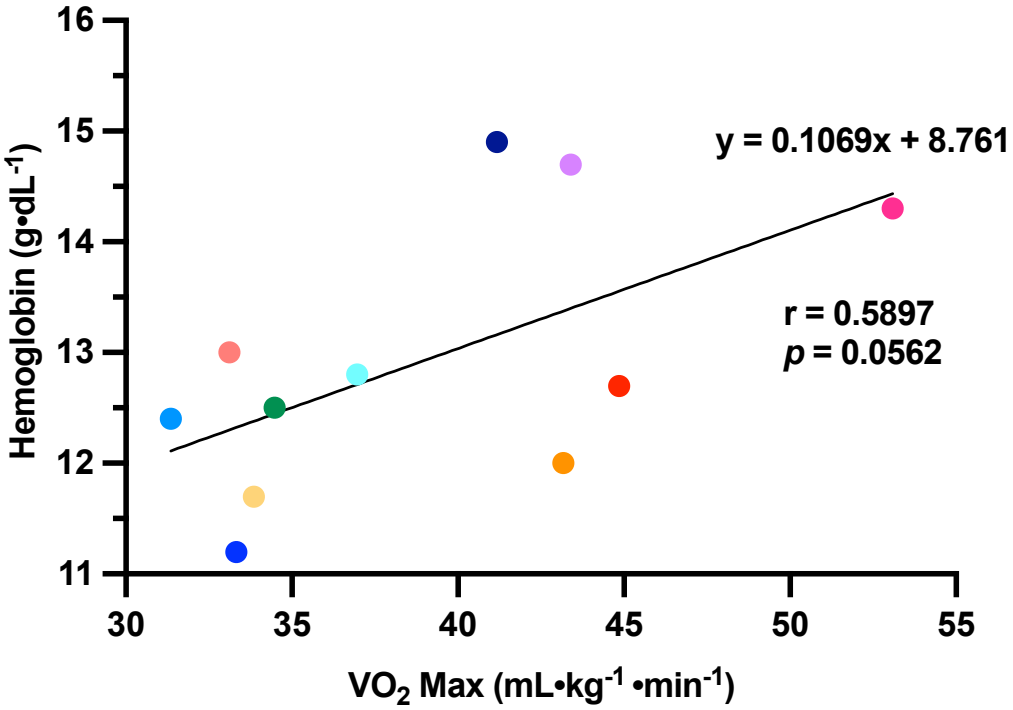
Myoglobin and mitochondrial densities were not measured and therefore, no analyses were conducted on these factors.

The EE during the 45-minute bout of exercise performed at the maxFOR was calculated for all participants (**Equation 2**). The relationship between VO_2 max and EE was then tested. A strong positive correlation was determined between VO_2 max and EE. As VO_2 max increased, the number of calories expended at the maxFOR workload also increased. (**Figure 11A**, r = 0.705; p = 0.0154).

A)


0.8
0.6
0.4
0.2
0.0
Maximum Fat Oxidation Rate (g•min-1)
y = 0.01233x - 0.07055
r = 0.7247
p = 0.0116
30
35
40
45
50
55
VO2 Max (mL•kg-1 •min-1)

B)


16
15
14
13
12
11
Hemoglobin (g•dL-1)
y = 0.1069x + 8.761
r = 0.5897
p = 0.0562
30
35
40
45
50
55
VO2 Max (mL•kg-1 •min-1)

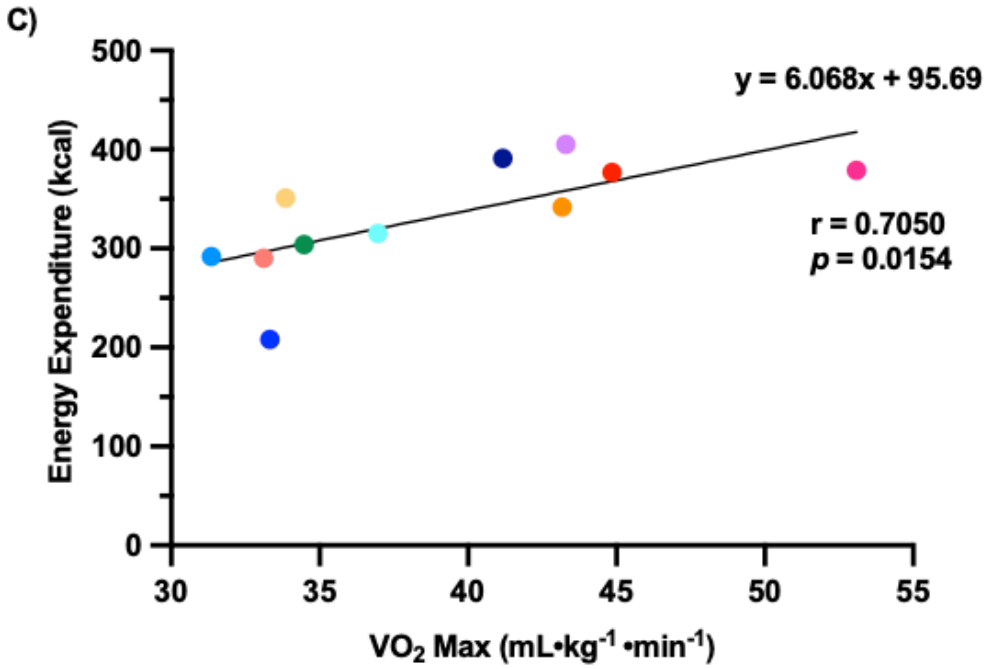


Figure 11. Relationship Between VO_2 Max, Maximal Fat Oxidation Rate, Hemoglobin and Energy Expenditure

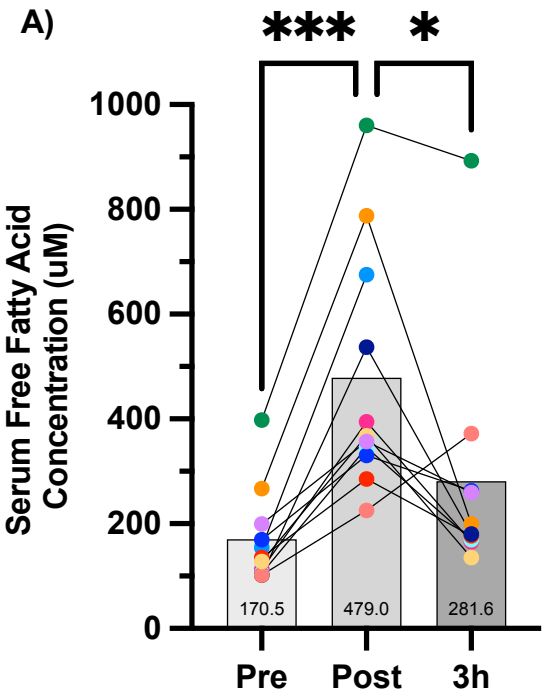
Each participant was colour-coded as indicated in **Table 2. A)** A linear regression tested the relationship between VO_2 max ($mL \bullet kg^{-1} \bullet min^{-1}$; x-axis) and maximal fat oxidation rate ($g \bullet min^{-1}$; y-axis) ($r = 0.7241$, $p = 0.0116$; $y = 0.01233x - 0.07055$). **B)** A linear regression tested the relationship between VO_2 max ($mL \bullet kg^{-1} \bullet min^{-1}$; x-axis) and hemoglobin content ($g \bullet dL^{-1}$; y-axis) ($r = 0.5897$, $p = 0.00562$; $y = 0.1069x + 8.761$). **C)** A linear regression tested the relationship between VO_2 max ($mL \bullet kg^{-1} \bullet min^{-1}$; x-axis) and energy expenditure during a 45-minute bout of maxFOR exercise (kcal; y-axis) ($r = 0.7050$, $p = 0.0154$; $y = 6.068x + 95.69$).

5.5. Serum Free Fatty Acid Increased in All Participants Post-MaxFOR Exercise and Higher Skeletal Muscle Mass is Associated with Lower Pre-Exercise Serum Free Fatty Acid

Next, we tested whether exercising at maxFOR had an impact of circulating FFA. There is an overall impact of time of serum collection on serum FFA concentration, with a significant increase in FFA concentration when comparing pre-exercise and post-exercise conditions ($p = 0.0003$, one-way ANOVA mixed analysis effects). FFA concentration increased by 1.81 times

between pre- and post-exercise serum. Serum concentration returned close to pre-exercise concentrations 3h post- exercise performed at the maxFOR, decreasing by 41% when comparing immediately post-exercise to 3h post-exercise (p = 0.0447, Tukey's post-hoc test). There was no significant difference between pre-exercise and 3h post-exercise serum concentration (p = 0.1309, Tukey's post-hoc test). Serum FFA concentrations at each time point varied greatly between participants (Range in μM: pre-exercise, 296.3; post-exercise, 735.0; 3h post-exercise, 757.7; and 95% CI of mean in μM; pre-exercise [109.6 – 231.4], post-exercise [322.2 – 635.9]; 3h post-exercise [120.2 – 443.0] (**Figure 12A**).

Next, we tested whether baseline FFA was correlated with baseline anthropometric measures. Linear regression analysis between SMI ($kg \cdot m^{2\,-1}$) and pre-exercise serum FFA revealed a strong negative correlation. As skeletal muscle mass increased, serum FFA concentration pre-exercise decreased (**Figure 12B**, r = -0.6270, p = 0.0390; y = -46.78x + 601.3). No other significant correlations between serum FFA concentrations and other anthropometric measures (% body fat, waist circumference) or serum from other conditions (post-exercise and 3h post-exercise) were found.


A)
***
*
Serum Free Fatty Acid Concentration (uM)
1000
800
600
400
200
0
170.5
479.0
281.6
Pre
Post
3h

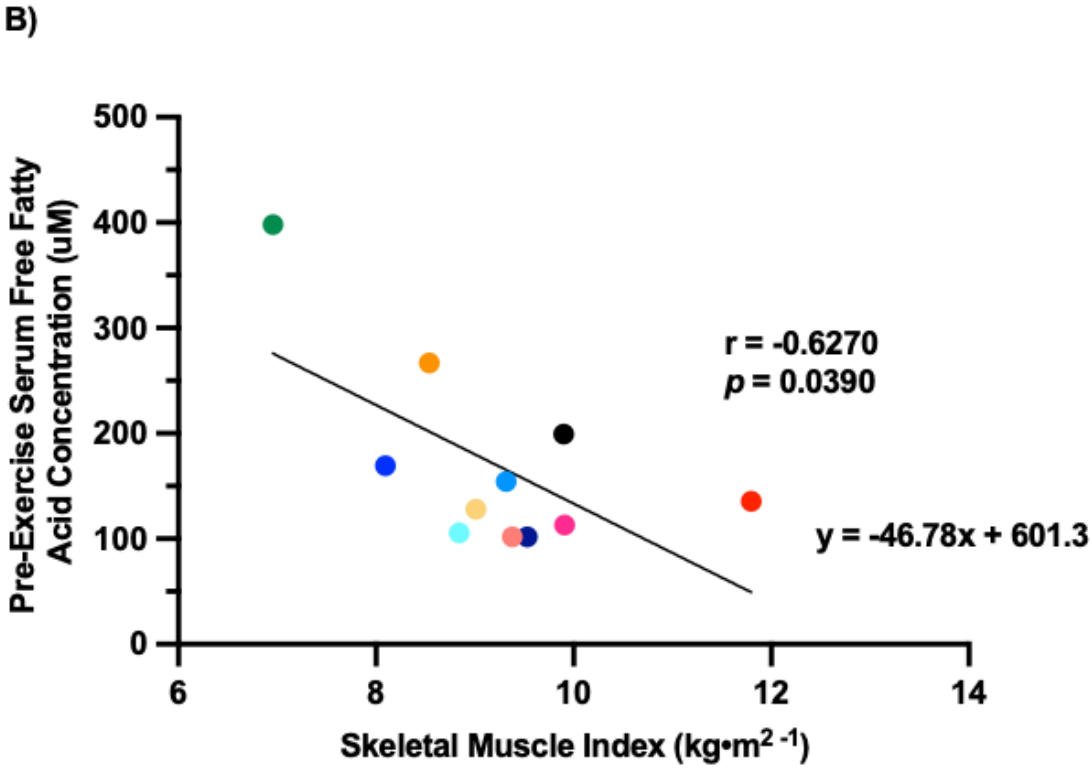

B)
Pre-Exercise Serum Free Fatty Acid Concentration (uM)
500
400
300
200
100
0
r = -0.6270
p = 0.0390
y = -46.78x + 601.3
6
8
10
12
14
Skeletal Muscle Index (kg•m2 -1)

Figure 12. Effect of Exercise Performed at the Maximal Fat Oxidation Rate on Circulating Free Fatty Acids

Enzymatic assay quantified FFA in the serum of women collected before (pre), immediately after (post) and 3 hours (3h) after a bout of exercise performed at maximal fat oxidation rate. **A)** Data shows the FFA concentration (uM) as bars that represent mean serum concentrations observed at the 3 timepoints (n=11 except for 3h post-exercise where n=10). Mean values are also depicted at the bottom of each bar. In the scatter plot, every participant is represented by a different point, colour coded as indicated in **Table 2**. All dots for a single participant are connected by a black line. Significant differences were tested using a one-way ANOVA mixed-effect analysis. Differences between group were tested with a Tukey's post-hoc analysis. Significant difference between groups: $*p \leq 0.05$; $***p \leq 0.005$. **B)** A linear regression analysis tested the relationship between skeletal muscle index (SMI; $kg \bullet m^{2\ -1}$; x-axis) and pre-exercise serum FFA (uM; y-axis) ($r = -0.6270$, $p = 0.0390$; $y = -46.78x + 601.3$). Every participant is depicted by a different coloured circle, as indicated in **Table 2**.

5.6. Serum Collected 3h Post-Exercise Had a Lower Capacity to Stimulate HAMEC Migration Compared to Serum Collected Pre-Exercise.

HAMEC migratory response was assessed by performing a Boyden Chamber Chemotaxis Assay. First, we tested the impact of cells treated with 'non-stim' (opti-MEM supplemented with 1% FBS) versus the 'stim' condition (opti-MEM supplemented with 10% FBS and 5% ECGS). We used the 'stim' condition as a positive control for migration. HAMEC treated with the 'stim' condition increased migration by 72% compared to cells treated with the 'non-stim' condition. An unpaired two-tailed t-test revealed a significant increase in HAMEC cell count when cells were treated with the 'stim' condition compared to 'non-stim' treatment (**Figure 13A**, $p = 0.0173$), validating the assay and migratory capacity of our cell model. The cell images on the right (**Figure 13A**) reveal an increased number of cells (purple dots indicated by red arrow) in the 'stim' vs 'non-stim' condition.

Next, we treated HAMEC with 10% serum collected pre-, immediately post- or 3h post-exercise to assess whether changes induced by exercising at the maxFOR could modify the

migratory capacity of these cells. When analysing the impact of participants' serum, the time of collection had a significant negative impact on migration (**Figure 13B**, p = 0.0429 one-way ANOVA mixed analysis effects). HAMEC treated with serum collected 3h post-exercise had a significantly reduced migratory capacity compared to cells treated with pre-exercise serum (**Figure 13B**; 11.56 mean difference of cell counts, p = 0.0407, Fisher's LSD post-hoc test). A non-significant decrease was also observed for HAMEC treated with post-exercise serum (**Figure 13B**; 8.19 mean difference of cell counts, p = 0.0517 Fisher's LSD post-hoc test). There were no significant differences found in HAMEC migratory capacity when comparing both the 'non-stim' and 'stim' conditions to all serum treated HAMEC conditions (data not shown, Kruskal-Wallis test, p = 0.2312).

A heat map was used to depict the impact of serum on cell migration for every participant. This revealed great variability in HAMEC migration between participants. Red indicates more migration and white indicates less migration. The X in the top right corner indicates missing data as no blood was collected at this time point from this participant (**Figure 13C**). Representative cell images from one participant (**Figure 13D**, participant ID 11) reveal a lower cell count in the post-exercise and 3h post-exercise images compared to pre-exercise. Red arrow in cell images point to cells (purple) that were counted in each chamber.

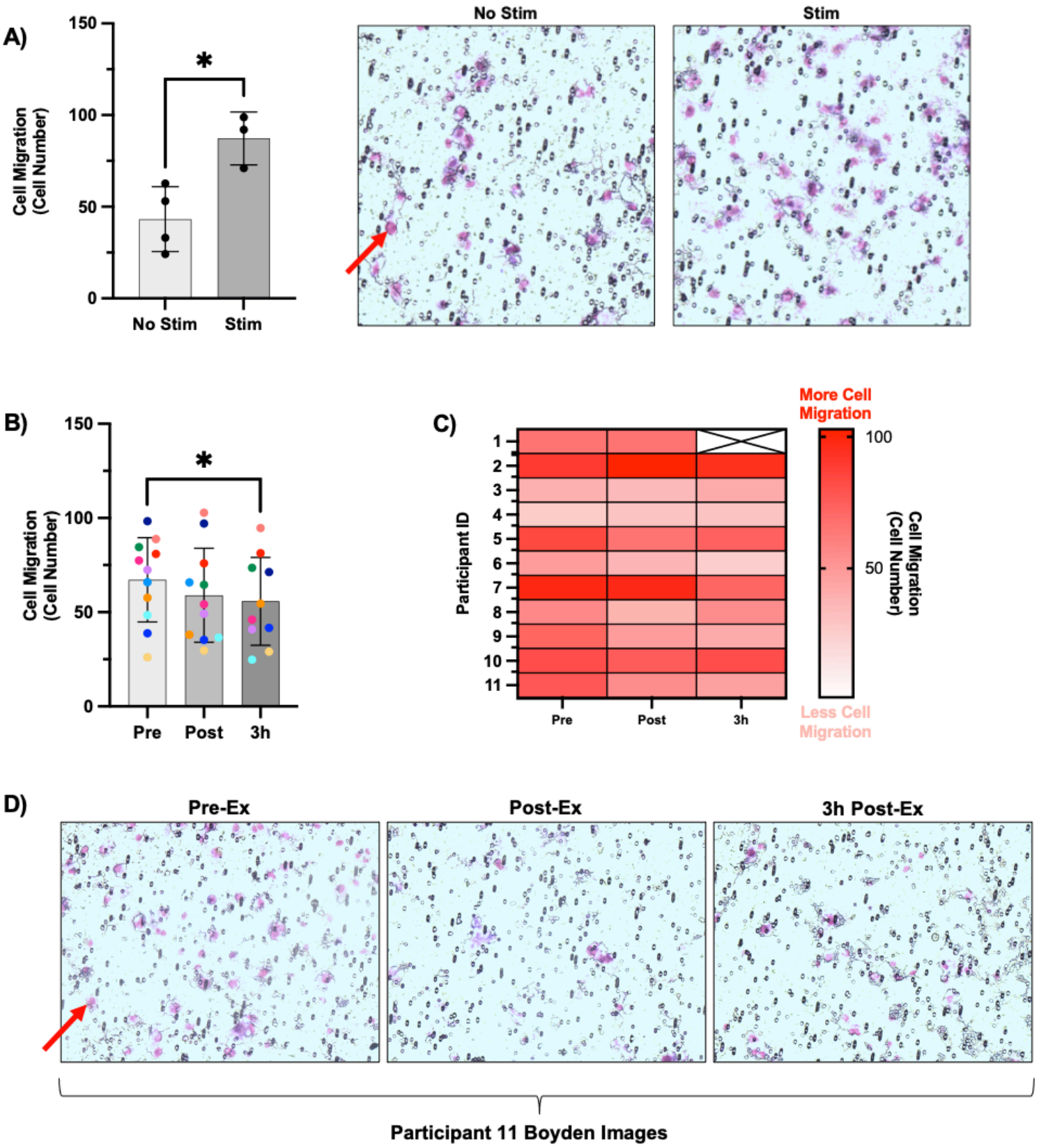

A)
Cell Migration (Cell Number)
150
100
50
0
*
No Stim
Stim
No Stim
Stim
B)
Cell Migration (Cell Number)
150
100
50
0
*
Pre
Post
3h
C)
Participant ID
1
2
3
4
5
6
7
8
9
10
11
Pre
Post
3h
More Cell Migration
Less Cell Migration
100
50
Cell Migration (Cell Number)
D)
Pre-Ex
Post-Ex
3h Post-Ex
Participant 11 Boyden Images

Figure 13. Effect of Serum Treatment on HAMEC Cell Migration

Boyden Chamber Chemotaxis tested the impact 10% serum treatment on the migratory capacity of HAMEC. 'Non-stim' (1% FBS) was used to determine baseline migration. HAMEC were treated with either 'stim' (positive control 10% FBS and 5% ECGS) or 10% serum from participant collected before (pre), immediately after (post) or after a 3h recovery period (3h) after a bout of maxFOR exercise. **A)** Impact of 'stim' condition on HAMEC migration. Bars represent mean ± SD of HAMEC cell migration, assessed by counting the number of cells per field of view, for both 'non-stim' and 'stim' conditions. Significant difference between groups: $*p \leq 0.05$ (unpaired two-tailed t-test). On the right, representative images for each condition. Red arrow in cell images point to cells (purple) that were counted in each chamber. **B)** Impact of serum collected pre-, immediately post- or 3h post-maxFOR exercise on HAMEC migration. Bars represent the mean ± SD cell migration assessed by counting the number of cells per field of view. Every colour circle represents a different participant (colour-coding in **Table 2**). One-way ANOVA mixed effects analysis revealed a significant effect of serum treatment ($p = 0.035$). Significant difference between groups: $**p \leq 0.05$ (Tukey's post-hoc test). **C)** A heat map depicting the difference in cell migration in every participant for every condition. Red indicates more migration and white indicates less migration (y-axis, right). Every participant is represented by a different number on the y-axis (left). Participants are organized from lowest to highest VO_2 max; [31.4 - 53.1] $mL \bullet kg^{-1} \bullet min^{-1}$ **D)** Representative cell images from one participant (participant ID 11). Red arrow in cell images point to cells (purple) that were counted in each chamber. The X in the top right corner indicates missing data as no blood was collected at this time point from this participant.

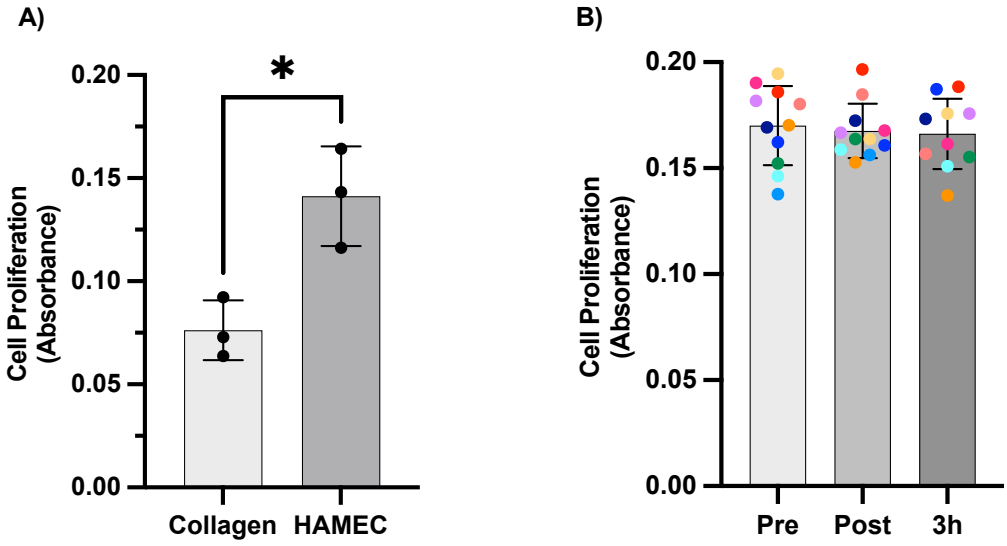
5.7. HAMEC Cell Proliferation Varies Greatly Between Participants – Inconclusive Effect of 24h and 48h Serum Treatment on HAMEC Proliferation

Since an effect on HAMEC migration was observed with serum treatment, we were interested to assess the effect of serum treatment on HAMEC proliferation. The cyQUANT® MTT Assay used to assess proliferation based on HAMEC metabolic activity. We first verified that the assay was efficient in detecting cell metabolic activity without reaching saturation of signal (maximal absorbance). We compared background absorbance of wells platted with collagen only to wells where HAMEC were platted and grown for 24 hours. A greater absorbance was observed in the wells containing HAMEC compared to wells containing only collagen, representing an increase in cell proliferation in wells containing HAMEC (**Figure 14A**, $p = 0.0162$, unpaired two-tailed t-test). These results validated the use of the cyQUANT® MTT Assay to test proliferation of HAMEC.

Next, we aimed to assess the effect of 24h serum treatment on HAMEC proliferation. Proliferation was not significantly different between HAMEC treated with 10% serum collected pre-, post- and 3h post-exercise performed at maxFOR (**Figure 14B**, $p = 0.5941$, one-way ANOVA mixed effects analysis).

We aimed to determine whether serum collected were enhancing proliferation compared to standard culture conditions (i.e. HAMEC) and basal HAMEC proliferation. A Kruskal-Wallis test was used to analyze differences between groups followed by a Dunn's test. None of the serum conditions significantly increased HAMEC proliferation compared to HAMEC basal proliferation levels (**Figure 14C**, HAMEC vs pre, $p = 0.1660$; HAMEC vs post, $p = 0.3269$; HAMEC vs 3h. $p = 0.3766$). Thus, time of serum collection had no impact on the proliferation measured at 24 hours.

Finally, a heat map was used to depict the variability in cell proliferation in every participant for every condition. Red indicates more proliferation and white indicates less proliferation. The X in the top right corner indicates missing data as no blood was collected at this time point from this participant. The heat map revealed the great variability (95% CI of mean for absorbance in nm; pre-exercise [0.1575 – 0.1826], post-exercise [0.1590 – 0.1763]; 3h post-exercise [0.1543 – 0.1781]) found in the response of HAMEC proliferation between participant serum, making it difficult to draw an overall conclusion on the effects of serum treatment on HAMEC proliferation with each serum condition (**Figure 14D**).



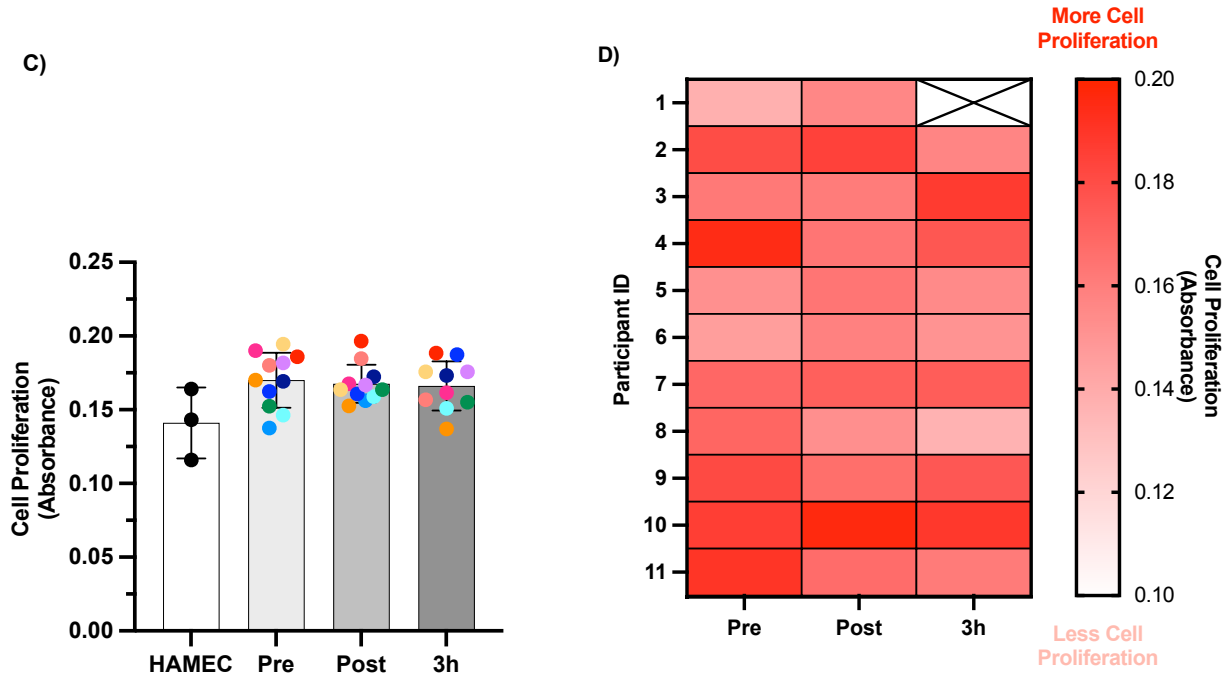


Figure 14. Variable Effect of 24h Serum Treatment on HAMEC Proliferation

Following an 18h starvation, HAMEC were treated with 10% serum for 24h. Wells platted with collagen were used to determine background absorbance and were subtracted from all absorbance readings. Wells with HAMEC only were used to determine baseline proliferation of cells. **A)** Cell proliferation (absorbance at 540nm; y-axis) for wells containing only collagen or HAMEC cells (x-axis). Bars represent the mean ± SD absorbance for each condition. An unpaired t-test was conducted to determine the difference between both groups. Significant difference between groups: $*p \leq 0.05$. **B)** Cell proliferation (absorbance at 540nm; y-axis) for wells where HAMEC were treated with 10% serum (x-axis). Bars represent the mean ± SD absorbance for each condition with every colour circle representing a different participant (colour-coded in **Table 2**). Cell proliferation was analyzed using a one-way ANOVA mixed-effects analysis. No significant differences were found between groups. **C)** Cell proliferation (y-axis) was assessed via a Kruskal-Wallis test followed by a Dunn's test. **D)** A heat map depicting the difference in cell proliferation in every participant for ever condition. Red indicates more proliferation and white indicates less proliferation (y-axis, right). Every participant is represented by a different number on the y-axis (left Participants are organized from lowest to highest VO_2 max; [31.4 - 53.1] $mL \bullet kg^{-1} \bullet min^{-1}$. The X in the top right corner indicates missing data as no blood was collected at this time point from this participant.

Next, we treated HAMEC with serum for a duration of 48h to test whether longer serum treatment would impact proliferation. First, we validated the MTT assay for this 48 hours time point. As with the 24h time point, wells with HAMEC were used to determine baseline proliferation of cells. The higher absorbance observed in wells containing HAMEC cells compared to wells containing only collagen confirmed that the MTT assay can assess proliferation at the 48-hour time point without reaching signal saturation (**Figure 15A**, $p = 0.0108$, unpaired two-tailed t-test).

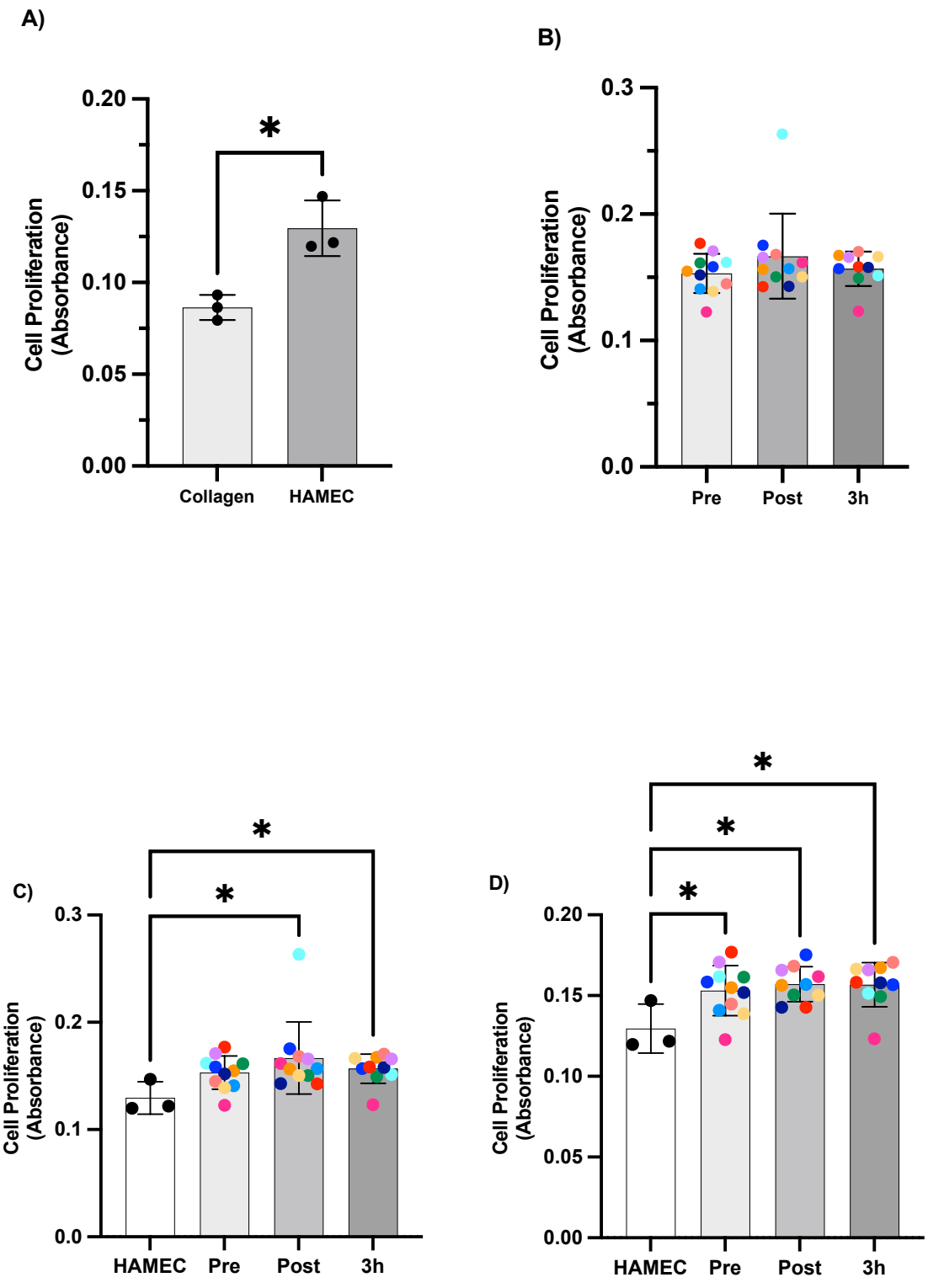
Time of serum collection (pre-, post- and 3h post- exercise at the maxFOR) had no significant effect on proliferation of HAMEC treated with 10% serum from different conditions (**Figure 15B**, $p = 0.3313$, one-way ANOVA mixed effects analysis). An outlier data point was identified in the post-exercise condition running a ROUT test (Q=1%). There was still no significance between groups when the outlier was removed ($p = 0.7096$, one-way ANOVA mixed effects analysis).

To compare the effect of serum treatment on HAMEC basal proliferation, cell proliferation was compared in the basal and serum treated conditions. A Kruskal-Wallis test was used to analyze differences between groups followed by an uncorrected Dunn's test. The post-exercise and 3h post-exercise serum conditions significantly increased HAMEC proliferation compared to HAMEC basal proliferation levels (**Figure 15C**, HAMEC vs post, $p = 0.0163$; HAMEC vs 3h, $p = 0.0179$). An outlier data point was identified in the post-exercise condition running a ROUT test (Q=1%). When this data point was excluded, serum collected before (pre), immediately after (post) and 3 hours after (3h) exercise at maxFOR showed a significant effect on proliferation compared to baseline when analyzed using a Kruskal-Wallis test (**Figure 15D;** Uncorrected Dunn's test,

HAMEC vs pre, $p = 0.0461$; HAMEC vs post, $p = 0.0263$; HAMEC vs 3h, $p = 0.0149$). This might suggest that 48h serum treatment may be optimal to induce HAMEC proliferation.

A heat map was used to depict the impact of serum on cell proliferation for every participant. This revealed great variability in HAMEC proliferation between participants. Red indicates more proliferation and white indicates less proliferation. The X in the top right corner indicates missing data as no blood was collected at this time point from this participant. The heat map reveals the great variability found in the response of HAMEC proliferation between participant serum variability (95% CI of mean for absorbance in nm; pre-exercise [0.1427 – 0.1635], post-exercise [0.1441 – 0.1893]; 3h post-exercise [0.1470 – 0.1666]), making it difficult to draw an overall conclusion on the effects of 48h serum treatment on HAMEC proliferation with each serum condition (**Figure 15E**).

To assess differences in proliferation between the 24h and 48h MTT conditions, HAMEC basal cell proliferation was compared in both conditions. An unpaired two-tailed t-test revealed no significant difference in basal cell proliferation between both time point (**Supplementary Figure 1**, $p = 0.1710$), suggesting that HAMEC basal proliferation levels did not significantly change between both time points unless treated with serum. This may also suggest that HAMEC reached confluency at the 24h timepoint, with potential cell to cell contact inhibition taking place beyond 24h. Due to this, gene expression was assessed after 24h serum treatment.


A)
Cell Proliferation (Absorbance)
0.20
0.15
0.10
0.05
0.00
*
Collagen
HAMEC
B)
Cell Proliferation (Absorbance)
0.3
0.2
0.1
0.0
Pre
Post
3h
C)
Cell Proliferation (Absorbance)
0.3
0.2
0.1
0.0
*
*
HAMEC
Pre
Post
3h
D)
Cell Proliferation (Absorbance)
0.20
0.15
0.10
0.05
0.00
*
*
*
HAMEC
Pre
Post
3h

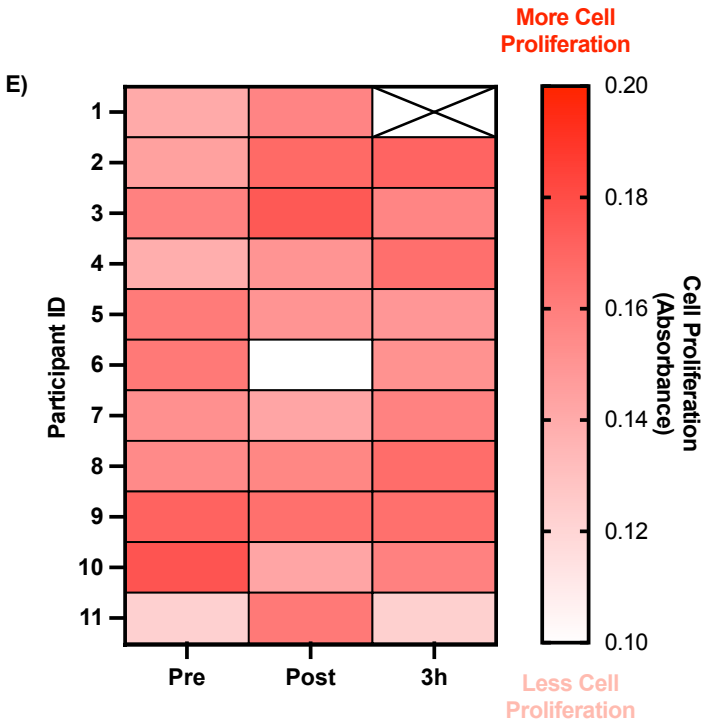


Figure 15. Variable Effect of 48h Serum Treatment on HAMEC Proliferation

Following an 18h starvation, HAMEC were treated with 10% serum for 48h. Wells platted with collagen were used to determine background absorbance and were subtracted from all absorbance readings. Wells with HAMEC only were used to determine baseline proliferation of cells. **A)** Cell proliferation (absorbance at 540nm; y-axis) for wells containing only collagen or HAMEC cells (x-axis). Bars represent the mean ± SD absorbance for each condition. An unpaired t-test was conducted to determine the difference between both groups. Significant difference between groups: $*p \leq 0.05$. **B)** Cell proliferation (absorbance at 540nm; y-axis) for wells where HAMEC were treated with 10% serum (x-axis). Bars represent the mean ± SD absorbance for each condition with every colour circle representing a different participant (colour-coded in **Table 2**). Cell proliferation was analyzed using a one-way ANOVA mixed-effects analysis. No significant differences were found between groups. **C)** Cell proliferation (y-axis) was assessed via a Kruskal-Wallis test followed by an uncorrected Dunn's test. Significant difference between groups: $*p \leq 0.05$. **D)** An outlier point was identified using ROUT (Q=1%). Bars represent the mean ± SD absorbance for each condition with every colour circle representing a different participant (colour-coded in **Table 2**). Statistical analyses were run a second time excluding the outlier from the 'post-exercise' condition. **E)** A heat map depicting the difference in cell proliferation in every participant for ever condition. Red indicates more proliferation and white indicates less proliferation (y-axis, right). Every participant is represented by a different number on the y-axis (left). Participants are organized from lowest to highest VO_2 max; [31.4 - 53.1] $mL \bullet kg^{-1} \bullet min^{-1}$. The X in the top right corner indicates missing data as no blood was collected at this time point from this participant and the white square indicates the outlier that was identified via ROUT.

5.8. HAMEC *EGR3* Gene Expression Response to 1h $VEGF_{165}$ and Serum Treatment Varies Greatly Between Cell Culture Experiments and Participants

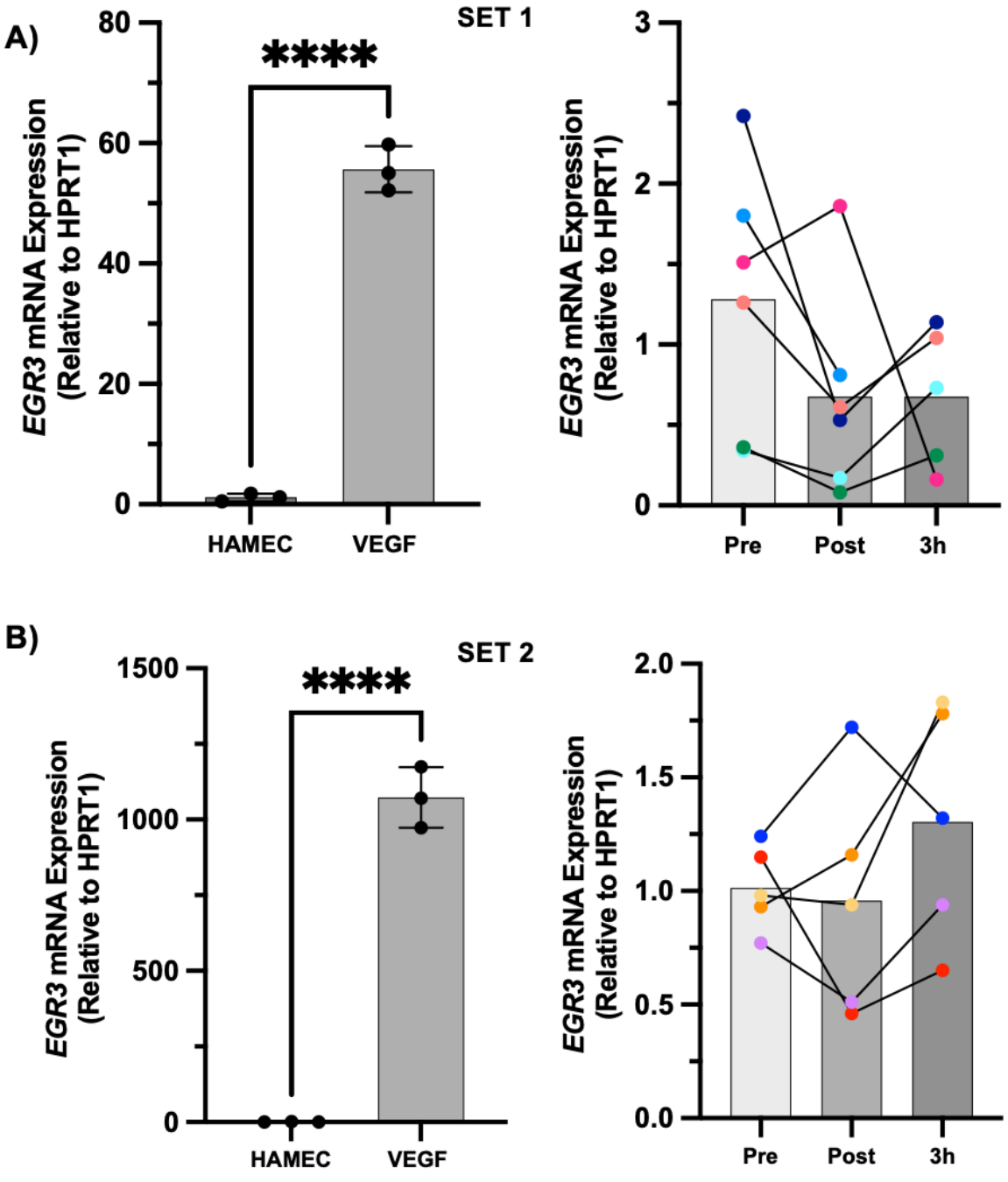
Next, we were curious to assess potential changes in gene expression. Following an 18h starvation, *EGR3* gene expression was measured in HAMEC treated for 1h with either 100ng/mL recombinant $VEGF_{165}$ (positive control) or 10% serum (pre-, post-, 3h post-exercise for all participants). *HPRT* was used as a housekeeping gene.

In the first set of experiments (refer to *methods section 3.2f,* **Figure 8**), a ~50-fold increase in the expression in *EGR3* was observed when HAMEC were treated with $VEGF_{165}$ for 1h (**Figure 16A**, $p < 0.0001$, unpaired two-tailed t-test). The first set of experiments for 6 participants showed no significant difference in *EGR3* expression between serum conditions, although there is a non-significant decrease 3h post-exercise when compared to pre-exercise (**Figure 11A**, $p = 0.2255$, one-way ANOVA mixed effects analysis).

Conversely, the second set of experiments (refer to *methods section 3.2f,* **Figure 8**) revealed a ~1000-fold increase in *EGR3* when treated with $VEGF_{165}$ for 1h (**Figure 16B**, $p < 0.0001$, unpaired two-tailed t-test). In both cases, $VEGF_{165}$ served as a positive control to validate HAMEC treatment conditions and responsiveness. The second set of experiments for the remaining 5 participants also showed no significant change in *EGR3* expression across serum conditions (**Figure 16B**). Interestingly, there is a non-significant increase 3h post-exercise when compared to pre-exercise (**Figure 16B**, $p = 0.3246$, Fisher's LSD post-hoc test).

Figure 16C reveals the combined *EGR3* gene expression in HAMEC treated with serum from all participants. Gene expression was calibrated by combining both sets of experiments and using the 'pre-exercise' gene expression value of *EGR3* from all participants. No significant

differences were found in EGR3 gene expression across all conditions when all the data was combined (**Figure 16C**, $p = 0.3285$, one-way ANOVA mixed effects analysis).



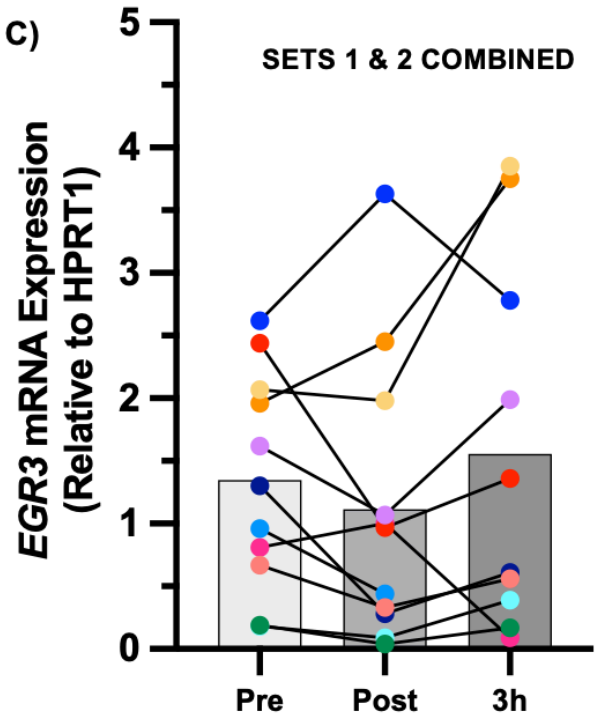


Figure 16. Effect of 1h Serum Treatment on HAMEC *EGR3* Gene Expression
EGR3 gene expression in HAMEC treated with either 100ng/mL recombinant $VEGF_{165}$ or 10% serum (pre-, post-, 3h post-exercise for all participants) for 1h. Bars represent mean ± SD. Treatment experiments for all participants were conducted as two separate sets (refer to *methods section* #). **A-set 1 and B-set 2)** On the left, *EGR3* gene expression normalized to *HPRT* (y=axis) in HAMEC alone or HAMEC treated with 100ng/mL recombinant $VEGF_{165}$ (x-axis). An unpaired two-tailed t-test was conducted to determine the difference between both groups. Significant difference between groups: ****$p \leq 0.0001$. On the right, *EGR3* gene expression normalized to *HPRT* (y=axis) in HAMEC treated with 10% serum (x-axis). Participants are depicted by different colour circles (**Table 2**). A one-way ANOVA mixed-effects analysis was conducted. No significant differences were found between groups. **C)** Combined *EGR3* gene expression (y=axis) in HAMEC treated with 10% serum (x-axis) from all participants (depicted by different colours, **Table 2**). Gene expression was calibrated by combining both sets of experiments and using the 'pre-exercise' gene expression value of *EGR3* from all participants. Bars represent the mean gene expression. An ordinary one-way ANOVA revealed no significant difference between groups.

5.9. HAMEC *FABP4* and *CPT1A* Gene Expression Are Significantly Changed in Response to 24h Serum Treatment

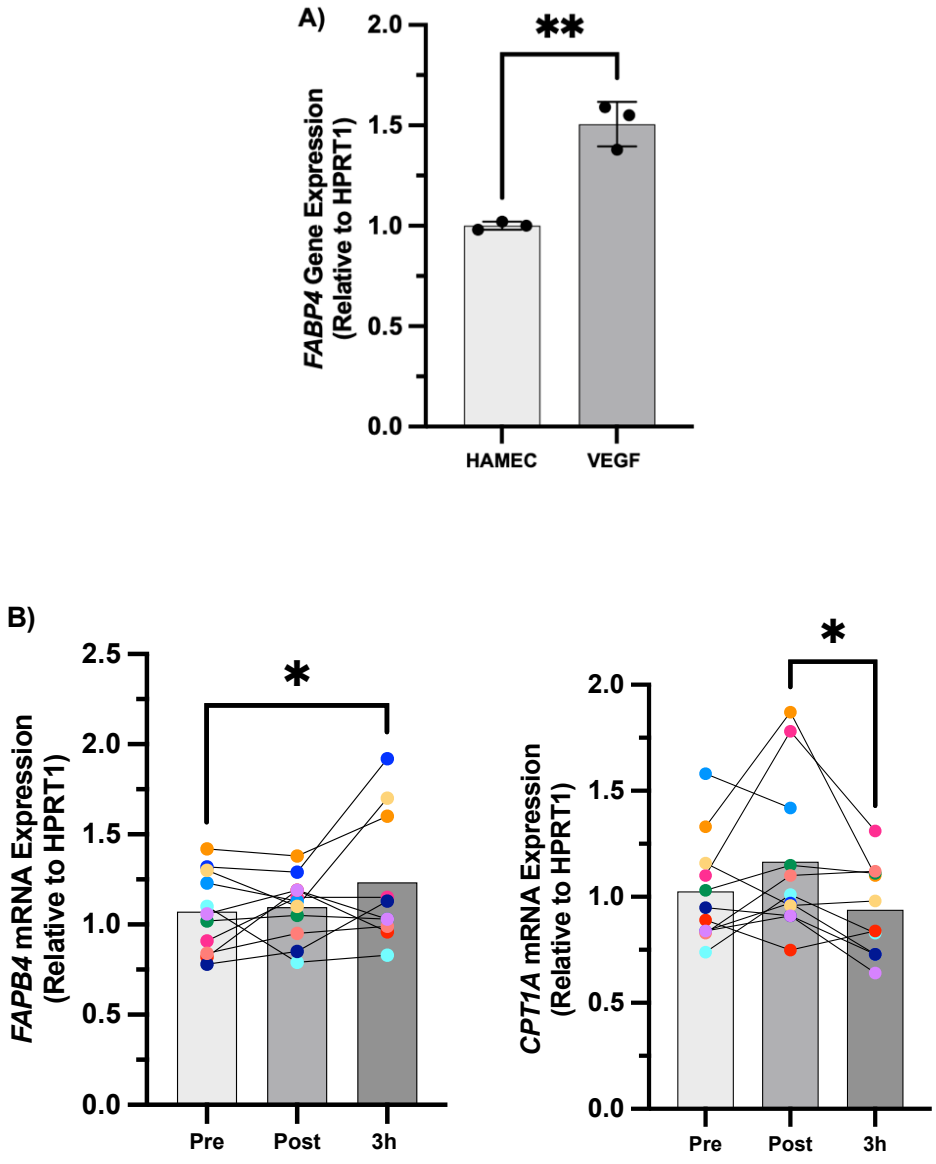
We aimed to assess the impact of this treatment on gene expression. Following an 18h starvation, gene expression in HAMEC treated with either 100ng/mL recombinant $VEGF_{165}$ or 10% serum (pre-, post-, 3h post-exercise for all participants) for 24h was assessed. *HPRT* was used as a housekeeping gene. A significant increase in *FABP4* gene expression after 24h $VEGF_{165}$ treatment indicates that the treatment conditions were ideal and HAMEC were responsive (**Figure 17A**, $p = 0.0015$, unpaired two-tailed t-test). There was no change in expression for all other genes measured with $VEGF_{165}$ treatment.

HAMEC treated with 3h post-exercise serum showed a significant increase by 15% in *FABP4* gene expression compared to pre-exercise treatment (**Figure 17B**, $p = 0.0474$, Fisher's LSD post-hoc test). Conversely, HAMEC treated with 3h post-exercise serum showed a significant decrease by 20% in *CPT1A* gene expression when compared to post-exercise serum treatment (**Figure 17B**, $p = 0.0367$, Fisher's LSD post-hoc test).

HAMEC expression of *CD36*, *CDH5* and *CLDN5* showed no significant change when treated across all serum conditions for all participants and remained non-significant even with the removal of outlier data points identified running a ROUT test (Q=1%). (**Figure 17C**, *CD36*, $p = 0.5179$; *CDH5*, $p = 0.2413$; *CLDN5*, $p = 0.2088$; one-way ANOVA mixed-effects analysis).

Interestingly, 24h serum treatment altered gene expression compared to baseline for *CDH5* and *CLDN5*. Compared to HAMEC basal gene expression (after starvation), serum collected 3h post-exercise significantly decreased *CDH5* gene expression (**Supplementary Figure 2**, HAMEC vs 3h, $p = 0.0498$) Interestingly, *CLDH5* expression decreased at every time point of serum collection (**Supplementary Figure 2**, HAMEC vs pre, $p = <0.0134$; HAMEC vs post, $p = <0.0431$;

HAMEC vs 3h, p = <0.0320) compared to starved HAMEC conditions. There was no difference in time of serum collection for other genes compared to HAMEC baseline.



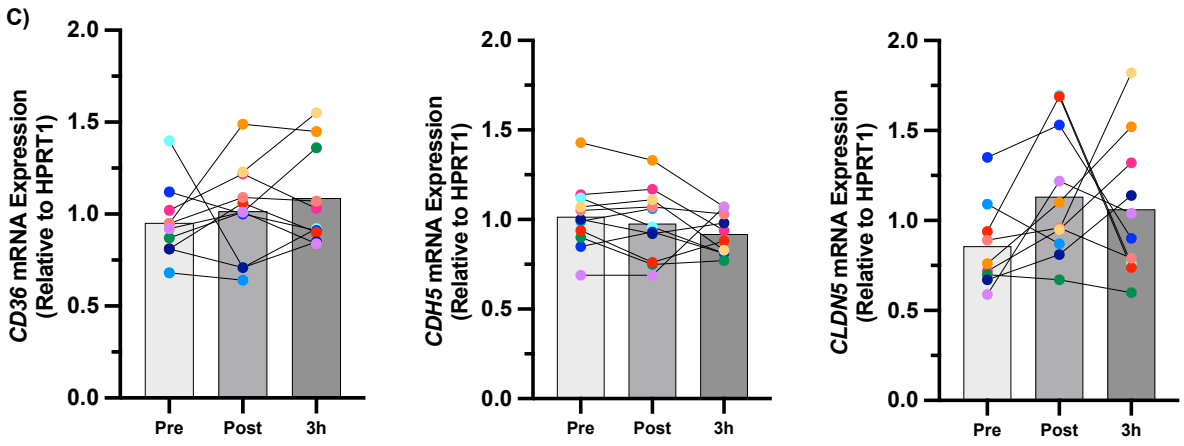


Figure 17. Effect of 24h Serum Treatment on HAMEC *FABP4, CPT1A, CD36, CDH5* and *CLDN5* Gene Expression

Gene expression in HAMEC treated with either 100ng/mL recombinant $VEGF_{165}$ or 10% serum (pre-, post-, 3h post-exercise for all participants) for 24h. **A)** *FABP4* gene expression (y=axis) in HAMEC alone or HAMEC treated with 100ng/mL recombinant $VEGF_{165}$ (x-axis). Bars represent mean ± SD gene expression. An unpaired t-test was conducted to determine the difference between both groups. Significant difference between groups: $^{**}p \leq 0.005$. No significance differences were found between conditions for all other genes. **B)** and **C)** *FABP4* **(B)**, *CPT1A* **(B)**, *CD36* **(C)**, *CDH5* **(C)**, and *CLDN5* **(C)** gene expression (y=axis) in HAMEC treated with 10% serum (x-axis) all participants (depicted by different colour circles). Bars represent mean ± SD gene expression. A one-way ANOVA mixed-effects analysis followed by a Fisher's LSD multiple comparisons test was conducted. Significant difference between groups: $^{*}p \leq 0.05$ for *FABP4* and *CPT1A* **(B)**. No significant differences were found between groups for all other genes **(C)**.

6.0. DISCUSSION

It was hypothesized that serum collected after an acute bout of exercise at maxFOR would impact HAMEC behavior by promoting key angiogenic processes (migration and proliferation) and increasing expression of genes related to FFA transport and endothelium permeability. The results from our study partially support our original hypothesis. Briefly, our results surprisingly demonstrated reduced migratory capacity with no change observed in HAMEC proliferation. Yet, *FABP4* gene expression increased after serum treatment, although *CPT1A* expression showed a concurrent decrease. Genes known to regulate that vascular permeability did not change across treatments with different serum samples.

All the women in our cohort reached their individual maxFOR. Although the range of workloads to obtain maxFOR was broad, they all fell within the expected range (~50 – 70% VO_2 max) when expressed as percentage of VO_2 max. Exercising 45 minutes at a workload that elicited maxFOR significantly increased serum FFA concentration. Interestingly, serum FFA concentration pre-exercise, but not post- or 3h post-exercise, was negatively correlated with SMI in our cohort. As our study did not focus on serum composition before and after the exercise bout, we used the collected serum to assess potential behavioral changes in our HAMEC cell model. HAMEC treated with serum collected 3h post-exercise showed significant decreased migratory capacity compared to HAMEC treated with serum collected pre-exercise. When HAMEC were treated with serum for a 24h period, time of serum collection and all serum conditions did not show any significant change in HAMEC proliferation compared to baseline levels. Interestingly, when HAMEC were treated with serum for a longer period (48h), time of serum collection had no significant effect on proliferation, but all serum conditions showed significant increased proliferation compared to HAMEC basal proliferation. For all behavioural assessments, great

inter-individual variability was found between participants. To help uncover underlying mechanisms behind altered HAMEC behaviour after serum treatment, we assessed the expression of genes related to different cellular processes in our serum-treated EC model: FFA transport, FFA oxidation and cell permeability. Overall, the expression of *FABP4,* a gene coding for a protein key to intracellular FFA transport, increased in cells treated with serum collected 3h post- exercise at maxFOR, compared to the pre-exercise condition. Interestingly, *CPT1A* gene expression, a gene key to FFA oxidation, decreased in cells treated with serum collected 3h post-exercise compared to the post-exercise condition. For all other genes, time of serum collection had no significant impact on expression. To assess the impact of overall serum treatment on HAMEC gene expression, we compared HAMEC basal expression to HAMEC that underwent serum treatment. Overall, *CDH5* gene expression was lower in HAMEC treated with serum collected 3h post-exercise compared to basal expression levels. Furthermore, *CLDN5* expression decreased for every serum condition compared to the HAMEC control. For this study, we hypothesized that exercise at a workload elicited maxFOR would shift the composition of the serum post-exercising at the maxFOR towards a pro-angiogenic profile, altering AT and EC behaviour. It was originally hypothesized that serum treatment would alter HAMEC cellular behaviour. While our findings support the notion that serum collected post-exercise can shift HAMEC gene expression, further investigation is required to elucidate whether it is sufficient to impact their angiogenic capacity and behaviour.

Achten and colleagues sought to find the exercise intensity that elicited maxFOR by studying a wide range of exercise intensities, introducing the term fatmax or maxFOR. The authors reported that maximal fat oxidation rate, or maxFOR, was found between 55 ± 3 and 72 ± 4 %VO_2 max and this workload varied between individuals (Achten et al., 2002). Despite this

characterization, most early studies utilized the same percent of VO_2 max for every participant, undermining inter-individual variability in maxFOR (Lanzi et al., 2014; Lyngsø et al., 2002; Moro et al., 2004; Moro, Pillard, et al., 2008; Simonsen et al., 2004; Stallknecht et al., 2007; D. Thompson et al., 2012). A great strength in our study is the use of a graded exercise test, similar to Achten and colleagues, to measure FOR during incremental exercise and determine the intensity that elicited maxFOR for every subject. In our cohort, the range of exercise intensities eliciting maxFOR was between 40.9 - 69.5 %VO_2 max. Although this range slightly differs from what has been previously reported (Achten et al., 2002), this can be due to the difference in subjects used (healthy, moderately trained men- mean age 28 years vs young healthy women- mean age 23 years). Furthermore, other studies have reported a maxFOR range similar to our cohort (Bagley et al., 2016; Bogdanis et al., 2008; Fletcher et al., 2017). The cohort recruited in our study presents another novelty to the field. To date, most studies examining maxFOR in women have used older and/or obese women or have investigated the effects of repeated bouts of exercise at maxFOR over weeks of training, rather than an acute bout exercise performed at maxFOR (Besnier et al., 2015; L. Cao et al., 2019; Jiang et al., 2020; Tan et al., 2012, 2014, 2018; Zeng et al., 2021). Our study aimed to help characterize the response to an acute bout of exercise performed at the maxFOR in young and healthy females, where only one other study used a similar cohort to assess the effects of two separate daytime schedules on a bout of exercise performed at the maxFOR (Robles-González et al., 2022). In our cohort, the mean maxFOR was $0.41 \pm 0.11 g \cdot min^{-1}$, with a range of $0.25 – 0.59 g \cdot min^{-1}$. During the graded exercise test, every participant reached their maxFOR at a different exercise intensity. This intensity was defined as that individual's maxFOR workload. The maxFOR range obtained in our study aligns with previous literature, validating our measurement protocol (Blaize et al., 2014; Fletcher et al., 2017; Gutiérrez-Hellín et al., 2022; Varillas-Delgado

et al., 2023). There are known sex differences in whole-body FOR during exercise, where women tend to show greater FOR than men (Chávez-Guevara et al., 2020; Chenevière et al., 2011; Maunder et al., 2018). The great inter-individual and inter-studies variabilities in estimating maxFOR can be attributed to sex (Chenevière et al., 2011; Venables et al., 2005), training status (Lima-Silva et al., 2010), nutritional habits (Arkinstall et al., 2001; Brun et al., 2022; Jeukendrup & Wallis, 2005; Romijn et al., 1993) and exercise modality used to measure maxFOR (Achten et al., 2003; Capostagno & Bosch, 2010; Maunder et al., 2018).

The Fitness Registry and the Importance of Exercise: A National Database (FRIEND) is a database of cardiopulmonary exercise tests used to enhance the value of CRF in the clinical setting, the public and to inform national policy. The database was put together, in parallel with a scientific statement from the American Heart Association, to help determine normative values of CRF and serve as the primary resource for researchers, clinicians and health policy makers (Kaminsky et al., 2015; Peterman et al., 2020). Studies included in the FRIEND registry were stringently reviewed for quality control and laboratories had to meet certain cardiopulmonary testing criteria to be included: valid and reliable calibration and testing procedures, experienced personnel qualified to conduct exercise test and averaging readings during the last 30 to 60 seconds of the cardiopulmonary test (Kaminsky et al., 2015). In our study, the VO_2 max testing protocol adhered to these guidelines, validating our exercise testing and VO_2 max determination for our cohort. Furthermore, all participants met the criteria to determine that VO_2 max has been reached (Keiller & Gordon, 2018) **1)** No further increase in VO_2 with increased workload; **2)** Participant was unable to continue; **3)** RER > 1.0; **4)** HR > 90% age predicted HR (**Equation 1:** *age predicted HR = 220 – age*) (Howley et al., 1995).

Regular endurance training favors the increase in the maxFOR. This has been demonstrated by Bircher and Knechtle, where endurance-trained athletes were reported to have higher maxFOR compared to sedentary subjects with obesity and maxFOR was highly correlated with training status (athlete vs person with obesity) (Bircher & Knechtle, 2004). In our cohort, a strong positive relationship exists between maxFOR and VO_2 max, where females with greater CRF had higher maxFOR. This relationship exists due to the adaptions in IMTG content, mitochondrial protein changes and hormonal regulation that come with endurance training (Purdom et al., 2018). Endurance training influences the ability of the muscle to liberate FFA via the improved activity of Hormone-Sensitive Lipase (HSL) (Van Loon, 2004) and LPL (Moro, Bajpeyi, et al., 2008). Furthermore, there is great reliance on IMTG during submaximal exercise, due to the stimulation of β-adrenergic receptor saturation (Mora-Rodriguez et al., 2001), and the ability of higher respiratory capacity to enhance type I fiber (slow-twitch and oxidative) IMTG almost 3-fold more than type II (fast-twitch and glycolytic) fibers (Shaw et al., 2010). Limiting factors in fat oxidation during exercise include the ability of the cell to transport FFA and the rate at which FFA are oxidized in the mitochondria. Interestingly, mitochondrial enzymes citrate synthase and β-hydroxy acyl-CoA dehydrogenase were found to be increased by 40% and 35%, in highly trained and moderately trained individuals, respectively (Nordby et al., 2006). Increasing these enzyme levels is associated with increased rates of the Krebs cycle and thus greater FFA oxidation, highlighting the benefits of increased fat oxidation observed with exercise training. These findings help reinforce the accuracy of our VO_2 max testing and validate the attainment of VO_2 max and maxFOR in our participants.

In our cohort a higher VO_2 max was also associated with greater EE during exercise workload performed at the maxFOR. This aligns with previous findings from Ando and colleagues,

where a significant association was found between VO_2 max and EE using a longitudinal inpatient study with a sample size of 357 individuals (Ando et al., 2019). These effects may be mediated through changes in body composition as it has been reported that the main determinants of EE are body size, body composition and food intake (Westerterp, 2017). Thus, it is suggested that individuals participating in regular intensity-based training that increases CRF (i.e. VO_2 max) are also indirectly affecting body composition via the increase in mass of metabolically active tissue (i.e. skeletal muscle) (Ramana et al., 2004). In our study, the workload performed at maxFOR varied between participants. Some subjects performed exercise at their maxFOR at a higher exercise intensity than others. Despite this variability, the EE was correlated to participants' VO_2 max, rather than exercise workload that elicited maxFOR.

Most studies investigating plasma FFA levels report concentrations after several bouts of exercise (Frandsen et al., 2019, 2021; Helge et al., 2001; S. Lee et al., 2017; Martin 3rd et al., 1993). Our study aimed to characterize the response after a single bout of exercise performed at the maxFOR. A study by Frandsen and colleagues investigated the effects of either fasting or a standardized meal on peak FOR in women during four exercise graded tests, separated by bed rest (Frandsen et al., 2021). Interestingly, the authors reported that plasma FFA were closely related to the maxFOR, although this relationship was not significant in our cohort. This may be attributed to the endurance-trained status of all participants in the study, as our cohort did not all have the same training status. The females in our study showed significantly elevated plasma FFA concentrations immediately post- exercise at the maxFOR, suggesting an increase in fat oxidation in our cohort, although it has been suggested that failure to remove lactic acid from the serum during exercise may result in falsely elevated levels of plasma FFA (Friedberg et al., 1960; Trout et al., 1960). The authors also noted similar plasma FFA levels (~200umol/L) during the first

exercise graded test, similar to the pre- exercise condition in our study (Frandsen et al., 2021). As observed in our cohort, Iwayama and colleagues reported significantly increased plasma FFA post-exercise at 60% VO_2 max (60 min) in a fasting state in young healthy men (Iwayama et al., 2020). Part of the increase in FFA observed immediately post-exercise may be attributed to the utilization of IMTG during exercise. Steffensen and colleagues reported a 25% decrease in the IMTG in the vastus lateralis of females at the end of a 90-minute exercise bout at 50% VO_2 max, regardless of training status (Steffensen et al., 2002). Iwayama *et al.* also reported a change in the FFA composition post-exercise, a detail our study did not investigate. It was noted that the increase in total FFA concentration post-exercise was due to an increase in saturated fatty acid (SFA), monounsaturated fatty acid (MUFA) and polyunsaturated fatty acid (PUFA). The change in FFA concentration and composition observed post-exercise may underline the mechanism upregulating FFA oxidation in the skeletal muscle during and after exercise (Iwayama et al., 2020). It has been reported that the expression of several genes is influenced by plasma FFA levels and their composition. In peripheral blood mononuclear cells, the expression of 25 genes related to fat metabolism is influenced by the ratio of SFA to PUFA (Larsen et al., 2018). Three hours post-exercise at the maxFOR, the females in our study showed a significant decrease in plasma FFA concentration, although levels were slightly elevated compared to pre-exercise. Interestingly, a multitude of studies show a sharp rise in plasma FAA during the recovery period after a moderate-intensity exercise bout (30 min - 2h) (Friedberg et al., 1963; Rodahi et al., 1964; Tunstall et al., 2007).

One of the main objectives of our study was to investigate the impact of serum collected from participants on HAMEC gene expression and cellular behaviour. Recovery serum (3h post-maxFOR) significantly reduced migratory capacity. This observation is concomitant to an

elevation in *FABP4* mRNA expression, a key observation in our study as this change in gene expression is crucial to FFA metabolism. Increased *FABP4* expression may support more efficient transport of FFA through the microvasculature. *FABP4* is highly expressed in the endothelium and its expression can support overall endothelial function (Harjes et al., 2014; Hotamisligil & Bernlohr, 2015). Chaperones, such as FABP4, are necessary for the transport of FFA in the endothelium (Liu & Dai, 2022). FABP4 is expressed intracellularly in EC and is regulated by VEGF, contributing to EC proliferation during angiogenesis and displays pro-angiogenic function (Elmasri et al., 2009; Ghelfi et al., 2013). In our HAMEC cellular model, 24h serum treatment induced an increased gene expression of *FABP4* during the recovery period (3h post-maxFOR), compared to pre-exercise serum, suggesting that recovery from exercise performed at the maxFOR may be promoting FFA transport in EC. The increase in FFA concentration observed immediately post-exercise in the serum of our cohort may be activating the expression of *FABP4* in HAMEC and may be helping to promote FFA uptake into the cell (Scifres et al., 2011). Interestingly, it has been demonstrated that stalk cells depend on FFA oxidation for dNTP synthesis during angiogenesis (Draoui et al., 2017; Schoors et al., 2015). Activation of stalk-determining Notch pathway has the capacity to promote *FABP4* expression. Harjes and colleagues described a reduced expression in *FABP4* expression in HUVEC with inhibition of Notch signaling (Harjes et al., 2014). These findings suggest that increased *FABP4* expression can support the acquisition of a stalk phenotype in EC. Conversely, FFA oxidation is also essential for quiescent EC (i.e. phalanx cells). Despite low overall metabolic activity during quiescence, phalanx cells upregulate FFA oxidation as a mechanism to protect against oxidative stress to support redox homeostasis (Kalucka et al., 2018). Despite the potential role of *FABP4* expression in EC proliferation, there was minimal impact of serum treatment on HAMEC proliferation between conditions in our study. Rather, an

increase in *FABP4* gene expression with no concurrent increase in proliferation might support EC quiescence, rather than acquisition of stalk cell identity. Furthermore, an increase in *FABP4* expression, with minimal impact on proliferation, may indicate an increased capacity of phalanx EC to maintain redox homeostasis. This may suggest a role of the serum in promoting EC stabilization. The increase in *FABP4* expression in HAMEC is also interesting, as multiple organs rely on FFA metabolism to sustain proper function including the heart, skeletal muscle and AT (Liu & Dai, 2022). The role of EC metabolism to support AT function has been well-described (AlZaim et al., 2023). For instance, the metabolism and transport of FFA by EC is important for ATP generation, transcriptional regulation and membrane synthesis in the AT. Increased expression of EC *FABP4* plays a crucial role in supporting insulin-induced lipolysis in adipocytes (Inouye et al., 2023). These results highlight the importance of EC *FABP4* expression to support overall AT function.

Overall, exercise performed at the maxFOR may allow greater transport of FFA from the AT, due to increased *FABP4* expression, while also ensuring the proper release of FFA into circulation to be used as fuel during exercise. Interestingly, the increase observed in *FABP4* gene expression was accompanied by a decrease in *CPT1A* expression. This was specifically seen when comparing cells treated with serum collected immediately post-exercise versus 3h post-maxFOR. CPT1A is the rate-limiting enzyme of FFA oxidation. It has been reported that CPT1A deficiency impaired angiogenesis *in vivo*, in line with reduced migratory capacity observed with 3h post-maxFOR serum treatment (Schoors et al., 2015). The differing gene expression levels of *FABP4* and *CPT1A* may suggest that the time immediately following a bout of exercise performed at the maxFOR may be most beneficial for FFA metabolism, as this is the time point where both *FABP4* and *CPT1A* expression are increased. During the immediate recovery period, EC may still have an

increased ability to uptake FFA, but their capacity to oxidize these substrates in the mitochondria become limited. Our results question whether the serum collected post- exercising at the maxFOR support a greater capacity to transport FFA through the AT endothelium while having minimal impact of angiogenic capacity. Rather, our results may support the maintenance of healthy quiescent cells or an increase in EC permeability to better support the exchange of nutrients in a fasted state. Despite observed changes in genes expression it is difficult to draw conclusions without further investigations, such as assessing HAMEC behaviour with serum treatment when genes of interest are completely knocked out.

Our study presented a multitude of strengths, helping develop the knowledge around the impact of exercise at the maxFOR. Firstly, maxFOR was determined for each all subjects, ensuring that every participant exercised at their individual maxFOR workload, maximizing benefits and responses to this type of exercise. Secondly, our study was the first to assess the effects of an acute bout of exercise performed at the maxFOR in young and healthy females, helping to characterize the expected, initial and strongest response to a bout of this exercise. Finally, the recruitment of female participants allowed us to address the underrepresentation of this cohort and characterize a response to exercise performed at the maxFOR in females. This is important as there are known sex differences in the response to exercise and in the behaviour of EC. Despite the multiple strengths within our study, there are still some limitations. Due to the smaller sample size and potential difference in training statuses of participants recruited, great inter-individual variability was present in all variables measured. For certain measures, significance was present with the use of uncorrected multiple tests. These types of tests may increase the rate of false positive findings. In our study, these tests were a useful starting point to guide future directions and experiments. The current study lacked the collection of blood before participants began fasting. Without this

comparison, it is difficult to eliminate the effects of prolonged fasting on overall gene expression and cellular behaviour. It was also difficult to draw conclusions regarding the AT microenvironment due to absence of AT biopsies and the effects of shear stress on the capillaries were not measured or considered due to the lack of blood flow in our cellular model. It has also been suggested that the cyQUANT® MTT Cell Proliferation Assay presents some limitations, despite being a well-known and used assay to measure proliferation (Akter et al., 2019; Jones et al., 2001; Sriwilaijaroen et al., 2004). The assay relies on the metabolic activity of cells which may cause an over estimation of cell proliferation occurring, for instance in cases where cell oxidative capacity is increased. Furthermore, all HAMEC cells were starved for 18h before serum treatment and subsequent gene expression measures. This means that serum-treated cellular gene expression was compared to HAMEC starved for an overall period of 36h, which may have greatly impacted gene expression in the control condition. Follow-up experiments should include comparing serum treated gene expression to HAMEC basal gene expression in normal culture conditions. Future directions include measuring gene expression related to stalk versus tip EC identity, assessing metabolic activity of EC with serum treatment, assessing the impact of serum treatment on tube formation using an *ex vivo* 3D tube formation assay and investigating serum composition before and after a bout of exercise performed at the maxFOR. If possible, further recruitment should be conducted to allow proper statistical tests to be conducted and assess true effect of serum treatment.

7.0. CONCLUSION

The workload that elicits the maxFOR varied between individuals, but every participant reached their maxFOR. Despite the differences in workload, an acute bout of exercise performed at the maxFOR increased the concentration of FFA in the blood. Overall, exercise performed at the maxFOR may help stabilize EC in the AT and prime AT capillaries for efficient FFA transport

and uptake. This response may be important in the maintenance of capillary health in the AT, mitigating EC metabolism in response to AT expansion or shrinkage.

8.0. SUPPLEMENTARY TABLES AND FIGURES

Supplementary Table 1. Workload corresponding to the maximal fat oxidation rate for each participant on a treadmill. Exercise performed during second laboratory visit (MPH- miles per hour).

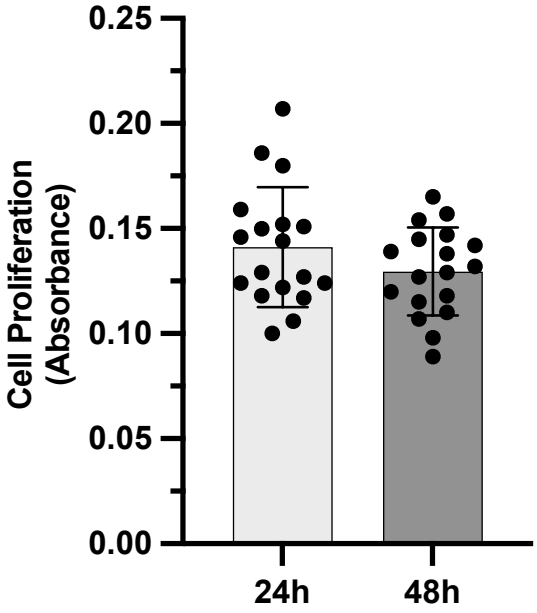
Participant ID	MaxFOR Workload (mph, %incline)
1	3.2, 5
2	3.2, 3
3	2.8, 3
4	3.4, 7
5	3.2, 7
6	3.6, 5
7	3.4, 7
8	3.2, 9
9	3.2, 7
10	3.2, 5
11	3.2, 9

Supplementary Table 2. Volume of water consumed (mL) by participants. Water consumption was monitored between the blood draw immediately post-exercise and the blood draw 3h post-exercise.

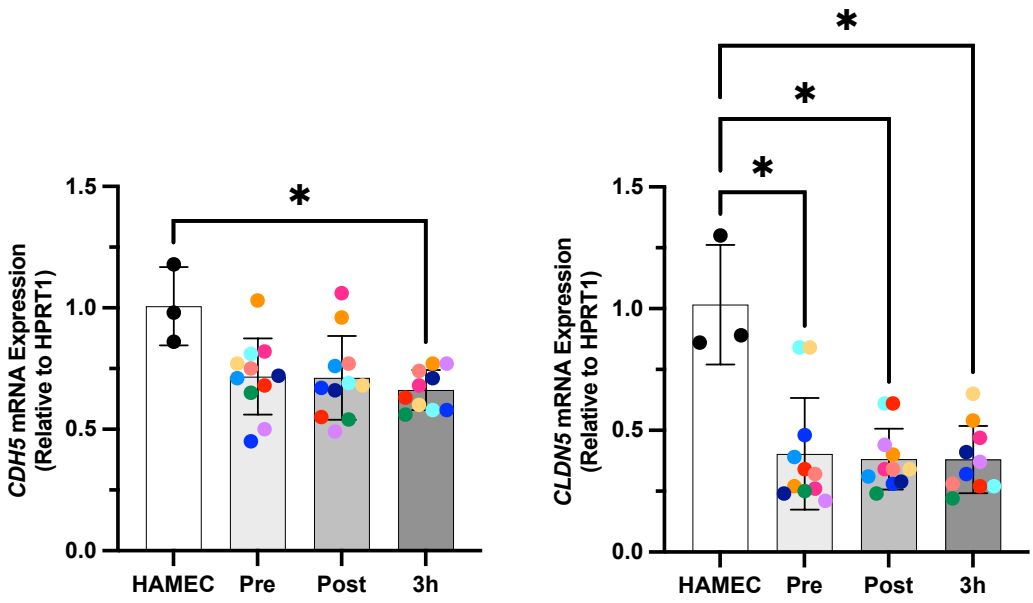
Participant ID	Water Consumed (mL)
1	660
2	150
3	500
4	100
5	200
6	250
7	800
8	250
9	200
10	250
11	1000
AVERAGE	396

Supplementary Table 3. Blood draw and exercise start/stop timepoints for all participants. All exercise was conducted between 8-10am. Exercise start/end times include 10-minute warm-up period. Blood draw 3h post-exercise was not collected for one participant due to complications during blood draw (indicated as NA).

Participant ID	Pre-Exercise Blood Draw Time	Exercise Start Time	Exercise End Time	Post-Exercise Blood Draw Time	3h Post-Exercise Blood Draw Time
1	8:42am	9:20am	10:30am	10:40am	NA
2	9:15am	9:30am	10:15am	10:30am	1:20pm
3	9:10am	9:25am	10:10am	10:18am	1:25pm
4	9:14am	9:40am	10:33am	10:40am	1:30pm
5	9:11am	9:35am	10:30am	10:40am	1:30pm
6	8:40am	9:05am	9:58am	10:02am	12:54pm
7	9:29am	10:10am	11:04am	11:06am	2:00pm
8	8:45am	8:55am	9:56am	10:03am	12:53pm
9	8:30am	8:58am	9:50am	10:00am	12:45pm
10	9:46am	10:07am	10:57am	11:02am	1:10pm
11	9:30am	9:55am	10:50am	10:55am	1:50pm



Supplementary Figure 1. 24h vs 48h Basal HAMEC Proliferation Assessed via MT
Wells platted with collagen were used to determine background absorbance and were subtracted from all absorbance readings. Wells with HAMEC only were used to determine baseline proliferation of cells. Cell proliferation (absorbance at 540nm; y-axis) for wells containing only HAMEC cells (x-axis) at both 24h and 48h. Bars represent the mean ± SD absorbance for each condition. An unpaired t-test was conducted to determine the difference between both groups.



Supplementary Figure 2. Effect of Serum Treatment on *CDH5* and *CLDN5* Gene Expression in Relation to Baseline HAMEC Gene Expression

Gene expression in HAMEC treated with 10% serum (pre-, post-, 3h post-exercise for all participants depicted by different colour circles; x-axis) for either 1h or 24h. Bars represent mean ± SD. All gene expression is expressed relative to *HPRT* (y-axis). A Kruskal-Wallis test was used to analyze difference between groups followed by Dunn's multiple comparison test. *$p \leq 0.05$ compared to HAMEC.

9.0. APPENDICES

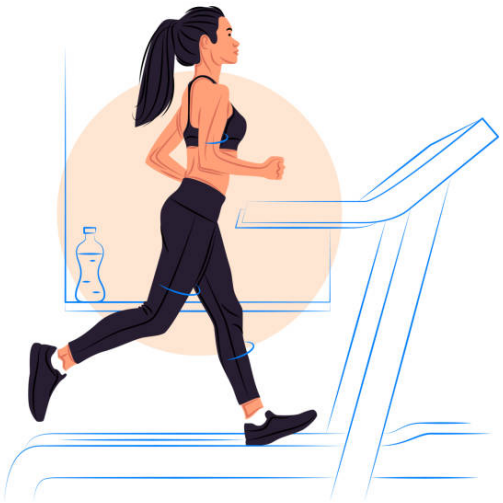
Appendix A - Approve Recruitment Poster

FEMALE VOLUNTEERS NEEDED FOR AN EXERCISE RESEARCH STUDY!!

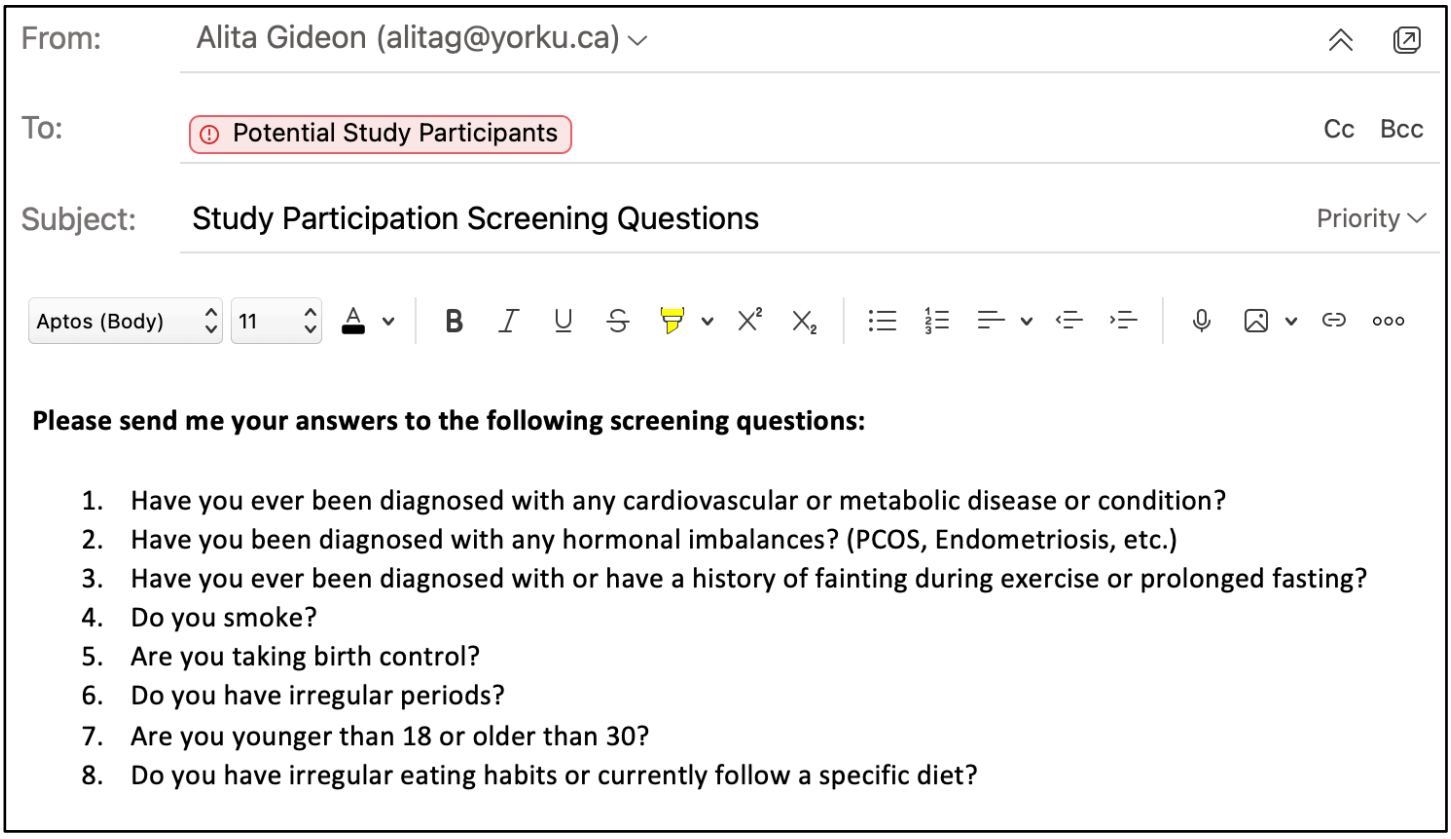
- Are you between the ages of 18 and 30?

- Are you curious to learn about your aerobic fitness capacity and other fitness-related assessments (i.e. body fat %, BMI and maximal fat oxidation rate)?

If you are interested, please email alitag@yorku.ca for further information!



Appendix B - Participant Screening Questions (sent via email)



From: Alita Gideon (alitag@yorku.ca)

To: Potential Study Participants Cc Bcc

Subject: Study Participation Screening Questions Priority

Aptos (Body) 11

Please send me your answers to the following screening questions:

1. Have you ever been diagnosed with any cardiovascular or metabolic disease or condition?
2. Have you been diagnosed with any hormonal imbalances? (PCOS, Endometriosis, etc.)
3. Have you ever been diagnosed with or have a history of fainting during exercise or prolonged fasting?
4. Do you smoke?
5. Are you taking birth control?
6. Do you have irregular periods?
7. Are you younger than 18 or older than 30?
8. Do you have irregular eating habits or currently follow a specific diet?

Appendix C - Informed Consent Form

Informed Consent Form

Please ensure to fully read and understand this consent form before signing (5 pages total). If you have any questions or need clarification, do not hesitate to ask.

Study Name: The effects of acute exercise on angiogenic capacity of adipose tissue microvascular endothelial cells

Researchers:
1) Alita Gideon, MSc student in Kinesiology and Health Sciences, York University
E-mail address: alitag@yorku.ca, Telephone: 647-888-6804

2) Dr. Emilie Roudier, Associate Professor in Kinesiology and Health Sciences, York University
E-mail address: eroudier@yorku.ca, Telephone: 416-736-2100 ext. 44312

Purpose of the Research:
The main purpose of this research is to assess the effects of acute exercise on the angiogenic capacity of endothelial cells found in the adipose tissue. The second purpose of this research is to address the gap in the field that involves female participants. To date, almost all studies of this nature has been conducted in males.

What You Will Be Asked to do in the Research:
You will be required to visit the laboratory on two occasions: The duration of each visit will range between 2 – 4 hours (start-finish). You will be required to exercise on both days. Day 1 will be used to determine your maximal fat oxidation rate (FOR) and your maximum aerobic capacity (VO2 max). On Day 2, you will exercise at your maximum FOR for 45 minutes (with a 10-minute warm-up) and have your blood drawn. Day 1 and Day 2 are not to be completed on 2 consecutive days. There must be a minimum of 48h between both days, with both days falling in the first 5 days of your menstrual cycle.

All measurements and exercise sessions will be performed in the Human Performance Laboratory (Rooms 120, 123 and 126 Norman Bethune College) at York University and the tests plus exercise sessions will be administered and monitored by qualified exercise professionals (Certified Exercise Physiologists).

Prior to each experimental day you will arrive to the laboratory following a 16 hour fast. Water consumption is permitted (no caffeine or alcohol). You will also refrain from exercise 24h before each laboratory visit. You will also be asked to keep a food log 24h prior to your first visit. You will be required to follow this food log 24h prior to your second visit. All required information will be communicated to you via email. Your second visit will be scheduled at the end of your first visit.

Day 1

Initial screening and fitness assessment: You will come to the laboratory for the initial visit to undergo the screening and select fitness assessments. You will undergo the "best practice" evidence-based pre-physical activity participation screening process which includes the completion of the PAR-Q+ (and if necessary the ePARmed-X+ accessed from www.eparmedx.com) and you will have your resting or pre-exercise Blood Pressure (BP) and Heart Rate (HR) assessed using the BpTRU. All participants whose BP is less than 160/90 mmHg or have received clearance using the ePARmed-X+ or from their physician related to the blood pressure will undergo the experimental proceedures. You will also be asked to declare the first day of your last menstrual cycle and your physical activity levels.

Body composition. Body composition will be assessed using traditional techniques: height (m) will be measured using a wall-mounted stadiometer (Fitness Precision, Toronto, ON) to the nearest 0.5 cm. Body mass (kg- to the nearest 0.1 kg- wearing light clothing and without shoes) and percent body fat will be assessed using a bioelectric impedance instrument (InBody 270 scale and skinfold calipers). BMI (kg/m^2) will be calculated from height and body mass. Waist circumference (cm) will be measured using the National Institute of Health (WHO, 2008) landmark using anthropometric tape. Sum of five skinfolds (mm) is to be derived from the sum of the biceps, triceps, subscapula, iliac crest and medial calf skinfold landmarks using a Harpenden caliper (Baty International, West Sussex, UK). All measurements will be completed by an exercise professional (Certified Exercise Physiologist) of the same gender if requested.

Hemoglobin and hematocrit content: Your hemoglobin and hematocrit content will be assessed. This will be done by lightly pricking the top of your index finger and adding a few drops of blood into a microcuvette that will be placed into a HemoCue 201+ machine for analysis.

FAT Oxidation plus Maximal O_2 consumption. Your maximal Fat Oxidation Rate (FOR) and VO2 max will be determined. You will undergo a continous ramp graded exercise test (GXT) on the treadmill. The initial workloads will be 3-minute in duration and the demand of the workloads will progressively increase from light (slow) to moderate to vigorous intensity until your maximum FOR is reached. During the exercise, you will wear a mouthpiece or a nose plug plus mouth mask (your choice) so that your expired air can be collected and analyzed until the maximum FOR is reached. Once the maximum FOR is determined you will undergo additional workloads for the determination of your aerobic fitness (VO_2max). Following completion of the test you will go through a cool down period on the treadmill. You will wear a chest strap for the determination of your heart rate during the whole duration of the exercise.

Day 2

Max FOR: You will exercise at max FOR for 45 minutes (with a 10-minute warm-up period). You will wear a chest strap for the determination of your heart rate during the whole duration of the exercise. During the FOR exercise you will wear a mouthpiece or a nose plug plus mouth mask (your choice) so that your expired air can be collected and analyzed to ensure you are truly exercising at your FOR.

Blood draw: You will provide pre- and post- exercise blood samples (3 blood draws: immediately beofre and after exercise and 3h after exercise). You must remain fasted until all

blood draws are complete. You will be monitored during this time, and dextrose sugar pills will be available in case you feel like fainting. For the blood collection, blood will be drawn into biomarker specific vacutainers from the antecubital vein (a needle will be inserted into your arm vein and a small amount of blood will be collected in small tubes) by a trained phlebotomist using sterile vacutainers. The blood drawn procedure will be carried out in accordance with York University Biosafety "best practice" standards. Up to 60mL of blood will be drawn (3 tubes of blood per blood draw). Blood will be used to isolate the serum and measure angiogenic factors. Biomedical waste (e.g. Blood, blood vials and saliva) will be placed in impervious, leak- and tear- resistant yellow biohazard waste bags. Once sealed, these bags will then be placed in a leak-proof container and sealed with strapping tape. The box will then be labelled with the laboratory Principal Investigator's name, date and contact information using permanent marker and then will be safely transported to a Farquharson Science Store for appropriate disposal. Any sharps (i.e. needles) will be discarded into a sharps container and labelled with the laboratory Principal Investigator's name, date and contact information using permanent marker and then will be safely transported to a Farquharson Science Store for appropriate disposal.

Risks and Discomforts:
Being physically active can have risks, especially to those who are not regularly active. The risks of an incremental-to-maximum effort aerobic fitness test include abnormal heartbeat or blood pressure, muscle cramps, potential muscle strain or injury, muscle soreness persisting a few days, light headedness, fatigue and in very rare instances, heart attack. These risks are minimized through pre-screening using the PAR-Q+ form, ePARmed-X+ form and assessment by a qualified exercise physiologist. In addition, providing an adequate warm-up and cool-down period during the exercise session will decrease any risks. If at any time, you wish to stop the aerobic fitness test, you may let the testers know and the test will be stopped. Every possible precaution will be taken to avoid an adverse event. In the event of an emergency, standard first-aid emergency procedures will be initiated as per the York University Medical Emergency Procedures.
There is also a possible risk of bruising at the needle site from taking blood samples, and an extremely small risk of infection with the blood draw. Pressure will be applied after each draw to minimize the risk of bruising. Proper sanitation and disinfection practices will be applied to minimize risk of infection. All possible precautions will be taken to avoid such problems. In the event of an emergency, York University's Security Services and 911 will be notified as per York University's Emergency procedure. Along with the study investigators, the Security Services personnel are trained in First Aid and CPR and have access to First Aid Kits and Automated External Defibrillators (AEDs). They will attend the scene and help the person experiencing a medical emergency until emergency responders arrive.

Benefits of the Research and Benefits to You:
You will find out what your aerobic fitness status is and peak exercise heart rates. VO_2max is the single most reliable predictor of morbidity and premature mortality. You will learn what you could do to maintain or improve your aerobic fitness. You will also find out at which intensity you burn the most fat. This could be of interest if you are looking to find the exercise intensity to help in weight management.

Voluntary Participation and Withdrawal:
Your participation in the study is completely voluntary and you may choose to stop participating at any time. Your decision not to volunteer will not influence the relationship you may have with the researchers or study staff or the nature of your relationship with York University either now, or in the future.

Confidentiality:
All information you supply during the research will be held in confidence and unless you specifically indicate your consent, your name will not appear in any report or publication of the research. The data will be collected by hand and then transferred to an electronic format. Your data will be safely stored in a locked facility and only research staff will have access to this information, for the duration of the study. Please note that at the end of the study, data will be removed and shredded from the locked facility and anonymized identified data may be deposited into one or more publicly accessible scientific repositories, such as York University Dataverse, an institutional research data repository, managed by York University Libraries and provided by Scholars Portal on behalf of the Ontario Council of University Libraries (OCUL), through which researchers from around the world will have access to these data for future research, through a CC BY-NC 4.0 DEED standard data sharing license. York University Dataverse does NOT accept content that contains confidential or sensitive information. Dataverse can be used to share de-identified and non-confidential data only. Contributors are required to remove, replace, or redact such information from datasets prior to upload. Scholars Portal makes backup copies of the uploaded data regularly in the event of a server or system malfunction, malicious attack, or other technical issues. The data collected may also be used for future research purposes, presentations, and statistical analysis, but results will only be reported in an anonymized group format. Such projects will still undergo ethics review by the HPRC, our institutional REB. Any secondary use of anonymized data by the research team will be treated with the same degree of confidentiality and anonymity as in the original research project. Confidentiality will be provided to the fullest extent possible by law.

Questions About the Research?
If you have any questions about the research in general or about your role in the study, please feel free to contact the researchers Emilie Roudier, PhD Associate Professor (e-mail: eroudier@yorku.ca) or Alita Gideon MSc student (e-mail address: alitag@yorku.ca) or the Graduate Program in Kinesiology and Health Science, 341 Norman Bethune College, York University, (telephone: 416-736-5728, e-mail: kahs@yorku.ca).
This research has been reviewed and approved by the Human Participants Review Sub-Committee, York University's Ethics Review Board and conforms to the standards of the Canadian Tri-Council Research Ethics guidelines. If you have any questions about this process, or about your rights as a participant in the study, please contact the Office of Research Ethics, Kaneff Tower, York University (e-mail: ore@yorku.ca)

Legal Rights and Signatures:

I_____consent to participate in "The effects of acute exercise on angiogenic capacity of adipose tissue microvascular endothelial cells" conducted by Alita Gideon, and Dr. Emilie Roudier, PhD. I have understood the nature of this project and wish to participate. I am not waiving any of my legal rights by signing this form. My signature below indicates my consent.

_____ _____

Participant Signature Date

_____ _____

Principle Investigator Signature Date

I_____consent to have my data anonymously posted to the York University Dataserve repository through which researchers from around the world will have access to these data for future research, through a CC BY-NC 4.0 DEED standard data sharing license. I am not waiving any of my legal rights by signing this form. My signature below indicates my consent.

_____ _____

Participant Signature Date

_____ _____

Principle Investigator Signature Date

Appendix D - The Physical Activity Readiness Questionnaire (2018 Par-Q+)

PAR-Q+

The Physical Activity Readiness Questionnaire for Everyone

The health benefits of regular physical activity are clear; more people should engage in physical activity every day of the week. Participating in physical activity is very safe for MOST people. This questionnaire will tell you whether it is necessary for you to seek further advice from your doctor OR a qualified exercise professional before becoming more physically active.

GENERAL HEALTH QUESTIONS

Please read the 7 questions below carefully and answer each one honestly: check YES or NO.	YES	NO
1) Has your doctor ever said that you have a heart condition ☐ **OR** high blood pressure ☐?	☐	☐
2) Do you feel pain in your chest at rest, during your daily activities of living, **OR** when you do physical activity?	☐	☐
3) Do you lose balance because of dizziness **OR** have you lost consciousness in the last 12 months? Please answer **NO** if your dizziness was associated with over-breathing (including during vigorous exercise).	☐	☐
4) Have you ever been diagnosed with another chronic medical condition (other than heart disease or high blood pressure)? **PLEASE LIST CONDITION(S) HERE:** _____	☐	☐
5) Are you currently taking prescribed medications for a chronic medical condition? **PLEASE LIST CONDITION(S) AND MEDICATIONS HERE:** _____	☐	☐
6) Do you currently have (or have had within the past 12 months) a bone, joint, or soft tissue (muscle, ligament, or tendon) problem that could be made worse by becoming more physically active? Please answer **NO** if you had a problem in the past, but it **does not limit your current ability** to be physically active. **PLEASE LIST CONDITION(S) HERE:** _____	☐	☐
7) Has your doctor ever said that you should only do medically supervised physical activity?	☐	☐

If you answered NO to all of the questions above, you are cleared for physical activity.
Please sign the PARTICIPANT DECLARATION. You do not need to complete Pages 2 and 3.

- Start becoming much more physically active – start slowly and build up gradually.
- Follow Global Physical Activity Guidelines for your age (https://www.who.int/publications/i/item/9789240015128).
- You may take part in a health and fitness appraisal.
- If you are over the age of 45 yr and NOT accustomed to regular vigorous to maximal effort exercise, consult a qualified exercise professional before engaging in this intensity of exercise.
- If you have any further questions, contact a qualified exercise professional.

PARTICIPANT DECLARATION

If you are less than the legal age required for consent or require the assent of a care provider, your parent, guardian or care provider must also sign this form.

I, the undersigned, have read, understood to my full satisfaction and completed this questionnaire. I acknowledge that this physical activity clearance is valid for a maximum of 12 months from the date it is completed and becomes invalid if my condition changes. I also acknowledge that the community/fitness center may retain a copy of this form for its records. In these instances, it will maintain the confidentiality of the same, complying with applicable law.

NAME _____ DATE _____

SIGNATURE _____ WITNESS _____

SIGNATURE OF PARENT/GUARDIAN/CARE PROVIDER _____

If you answered YES to one or more of the questions above, COMPLETE PAGES 2 AND 3.

Delay becoming more active if:

- You are currently experiencing a temporary illness, such as a cold or fever. It is best to wait until you feel better.
- You are pregnant. In this case, talk with your health care practitioner, physician, qualified exercise professional, and/or complete the ePARmed-X+ at www.eparmedx.com before becoming more physically active.
- Your health changes. Answer the questions on Pages 2 and 3 of this document and/or talk to your health care practitioner, physician, or qualified exercise professional before proceeding with any physical activity program.

 1 / 4
01-11-2023

APPENDIX E – HAMEC Donor Information

From: sales@sciencellonline.com <sales@sciencellonline.com>
Sent: Thursday, September 21, 2023 7:36:19 PM
To: Alita Gideon <alita123@yorku.ca>
Subject: FW: Question regarding HAMEC (#7200)

Dear Alita

Thank you for providing further details.
The donor information for Cat # 7200 Lot # 19427 is female.

Please do not hesitate to reach out if I can be of further assistance with this or any other matter.

Sincerely,

Alison McDonald
Manager Sales & Customer Service

1610 Faraday Avenue l Carlsbad, CA 92008
(760) 602-8549 x 237 | sciencellonline.com

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