ABSTRACT

Title of Dissertation: **EFFECTS OF EXERCISE AND INFLAMMATION ON CIRCULATING MICROPARTICLES**

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Circulating microparticles (MPs), a subset of extracellular vesicles, have been implicated as novel biomarkers connected to vascular dysfunction. As such, they may contribute to atherosclerosis, hypertension, and other conditions leading to cardiovascular disease. MPs are involved in cell-to-cell communication in response to apoptosis and activation of the immune and inflammatory response, transferring their contents to nearby cells and effectively spreading each condition. The objective of this dissertation was to explore how circulating MP number and function are affected by stimuli such as diet and exercise. Our first study examined how post-prandial inflammation caused by a high-fat meal affects circulating MP number and function in young, healthy adults. We determined that a high fitness level may have a protective effect against the inflammatory load posed by a high-fat meal. The second study determined the effects of acute high-intensity interval aerobic exercise versus acute moderate intensity continuous aerobic exercise on circulating MP number and function in overweight versus lean recreationally

active adults. We found that MPs and arterial stiffness in overweight individuals are differentially impacted by the type of acute exercise. Our findings suggest that overweight individuals undergo a greater inflammatory response following high-intensity exercise compared to lean. The third study investigated the effects of a 6-month aerobic exercise training program on circulating MP counts and function in previously sedentary older adults. While we found no effect of the exercise training program on MPs, we provide insight into how improvements in cardiovascular fitness as well as higher exercise intensities may be needed to see changes in MP number and function following aerobic exercise training in older adults. For the first time, we have shown that both dietary inflammation and acute exercise can significantly impact MP function. Furthermore, we have shown that fitness status and body composition play important roles in determining MP number and function after each stimulus. Our findings provide novel insight into how MPs contribute to various types of inflammation as well as how they may be used as biomarkers to measure the progression of cardiovascular disease.

EFFECTS OF EXERCISE AND INFLAMMATION ON CIRCULATING MICROPARTICLES

by

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Dedication

This dissertation is dedicated to my wife, Kelsey. We met not long after I started this degree and you have been my biggest supporter over the past 5 years. You have been one of the brightest constants through all the ups and downs, and this would not have been possible without you.

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List of Abbreviations

ADLs – Activities of daily living

Aix – Augmentation index

APC – Activated protein C

AUC – Area under the curve

BCL-2 – B-cell lymphoma 2 protein

BMI – Body mass index

DBP – Diastolic blood pressure

EGM2 – Endothelial growth medium 2

ELISA – Enzyme-linked immunosorbent assay

eNOS – Endothelial nitric oxide synthase

ERK1/2 – Extracellular signal-regulated kinase 1/2

ET – Exercise training

EVs – Extracellular vesicles

FBS – Fetal bovine serum

FC – Flexibility control

FMD – Flow-mediated dilation

HCAECs – Human coronary artery endothelial cells

HGF – Hepatocyte growth factor

HIIT – High-intensity interval training

HUVECs – Human umbilical vein endothelial cells

ICAM-1 – Intercellular adhesion molecule-1

IRB – Institutional review board

LDL – Low-density lipoprotein

KLF2 – Krüppel-like factor 2

MAPK – Mitogen activated protein kinase

MICT – Moderate-intensity continuous training

MLC-P – Phosphorylated myosin light chain

MPs – Microparticles

MSNA – Muscle sympathetic nerve activity

NF-κB – Nuclear factor-kappa B

NIHem – Noninvasive hemodynamic workstation

NO – Nitric oxide

PAR-1 – Protease-activated receptor-1

PBS – Phosphate-buffered saline

PE - Phycoerythrin

PECAM-1 – Platelet endothelial cell adhesion molecule-1

PerCP – Peridinin-chlorophyll-protein

PCOS – Polycystic ovary syndrome

PPO – Peak power output

RER – Respiratory exchange ratio

ROS – Reactive oxygen species

RPE – Rate of perceived exertion

RPM – Revolutions per minute

SBP – Systolic blood pressure

SPRED-1 – Sprouty-related EVH1 domain-containing protein 1

TF – Tissue factor

TNF-α – Tumor necrosis factor-alpha

VE-cadherin – Vascular endothelial cadherin

VEGF – Vascular endothelial growth factor

VCAM-1 – Vascular cell adhesion molecule-1

Chapter 1: Introduction

Vascular health and exercise are tightly intertwined. It is therefore important to expand our understanding of their related mechanisms to better address chronic diseases stemming from vascular dysfunction, such as atherosclerosis and cardiovascular disease. On a molecular level, it is well-known that extracellular vesicles (EVs) are involved in cell-to-cell communication following acute exercise or other inflammatory stimuli.[1] One subset of EVs known as microparticles (MPs) and/or microvesicles has gained attention due to strong connections with vascular function.[2–4] MPs can be broken down further into their respective subtypes stemming from different phenotypic changes in their parent cells.[2] The influence that exercise has on MPs and how they respond following inflammatory stimuli both require further exploration. Specifically, there are gaps in the literature regarding the effects of both acute and chronic exercise on circulating MP number, as well as how an individual's training status or body composition influences how MPs respond to exercise. This bring up questions about which intensities of exercise are best suited to induce reductions in MP number and inflammatory function and how these changes may impact cardiovascular health. The role of a high-fat meal on circulating MP release is also largely unexplored, creating further questions about MP function and postprandial inflammation.[5] Indeed, this may stimulate future research, specifically regarding how exercise-induced MPs may bring about changes in individuals who are at risk of developing vascular disease.

Microparticles

Basic Physiology:

MPs are a class of EVs that are blebbed (shed) from the plasma membrane of parent cells in response to cellular activation or apoptosis.[2,6] They differ in size and function from other EVs such as exosomes (40-120 nm) and apoptotic bodies (500-4000 nm). MPs typically have diameters of approximately 100-1000 nm and have been shown to carry many different types of cargo, including cell adhesion molecules and other proteins derived from their parent cells.[2,7,8] Some of these proteins that are exposed on the surface are typically utilized as markers in microparticle isolation techniques. The main function of MPs is to transfer their cargo to other cells, causing them to adopt the same phenotype as the parent cell from which they originated.[2,6]

The other EV subsets include exosomes and apoptotic bodies. Exosomes are the smallest subset, and their stimulus of release is the fusion of endosomal compartments with the plasma membranes of target cells during cellular respiration and activation of the immune response.[2,9] They are thought to be mainly involved in cell-to-cell communication.[2,9] Apoptotic bodies, the largest subset of EVs, are specific to the regulated mechanism of cell death known as apoptosis. They are blebbed from the plasma membrane during this process and, like MPs and exosomes, are also involved in cell-to-cell communication.[2] However, apoptotic bodies differ from the other EV subsets because they are involved in cell clearance.[2] While apoptosis is a necessary immunological process to clear out dysfunctional cells throughout the body, it is important to note that aberrant apoptosis is considered to be indicative of an inflammatory state.

Apoptotic cell disassembly is the process during which the EV subsets are released. It occurs in three steps, starting with blebbing from the membrane of the apoptotic cell. The process is initiated via the cleavage of caspase, an important factor in the apoptotic cascade.[10]

Following blebbing from the plasma membrane, the apoptotic membrane will begin to protrude from the cell in various shapes, such as microtubule spikes and apoptopodia.[2] Finally, the protruding shapes will break off, discharging apoptotic bodies away from the cell where they will participate in cell-to-cell communication along with the earlier released exosomes and MPs.[2,8] The release of EVs is a direct result of destabilization of the plasma membrane and the cytoskeleton, which is common in cell activation and apoptosis.[6]

Mechanisms of Endothelial MP formation:

Endothelial MPs blebbed from endothelial cells share the same basic characteristics as other MPs regarding their mechanism of release. However, there are nuances specific to the endothelium that influence their formation. Previous literature has demonstrated the release of endothelial MPs in cell culture in response to a variety of inflammatory stimuli including tumor necrosis factor-alpha (TNF-α), lipopolysaccharides, reactive oxygen species (ROS), thrombin, and other cytokines.[11] Each of these factors tie into different pathways within the endothelium that potentiate endothelial activation and apoptosis. Therefore, endothelial MPs are connected to activation and apoptosis in that they are a type of MP, but also because they stem from an inflammatory environment. Together, these are the two main characteristics of endothelial MPs. However, an in-depth look at how endothelial MPs are formed in the endothelium uncovers a fascinating amalgamation of inflammatory pathways.

Indeed, it appears that the presence of thrombin is one part of the early endothelial MP response.[3,6] As a seminal piece of the procoagulant cascade, thrombin plays a role in clotting in response to tissue damage with the ultimate goal of producing fibrin.[3] This pathway is normally most active in response to external tissue damage, but it is also thought to be associated with cardiovascular disease risk factors related to thromboembolism.[12,13] According to the authors

who uncovered this pro-endothelial MP pathway, thrombin binds to protease-activated receptor-1 (PAR-1) on endothelial cells, which has widespread effects in the cell that work together in the process of generating endothelial MPs.[13] The binding of thrombin to PAR-1 increases intracellular Ca^{2+}, causing the membrane and cytoskeletal instability typically seen in apoptotic cell disassembly.[6,13,14] The binding of thrombin to PAR-1 also results in increased cleaved caspase, feeding into this mechanism by phosphorylating myosin light chain (MLC-P), causing contraction of the cell.[10,13,14] These interactions contribute to the release of endothelial MPs expressing phosphatidylserine on their outer membranes.[2,6,15] In addition, the binding of thrombin to PAR-1 has been shown to increase nuclear factor-kappa B (NF-κB), a transcription factor that feeds into downstream pro-inflammatory effects that are part of the late phase of this mechanism (Figure 1).[6] All of the changes taking place prior to NF-κB activation are considered part of the "early" phase of thrombin-induced endothelial MP formation (Figure 1).[3,6,13] Following NF-κB activation comes the "late" phase of the pathway, which generates endothelial MPs slightly different from those of the early phase. In the late phase, NF-κB activation produces endothelial MPs externalizing both phosphatidylserine as well as tissue factor (TF) on their outer membranes. Surface phospholipids such as phosphatidylserine on endothelial MPs act to bind TF as well as other procoagulant molecules.[6,15] TF is a very important piece of endothelial MP function, as it feeds directly into the extrinsic coagulation pathway by converting factor X to factor Xa when von Willebrand factor (a glycoprotein involved in platelet function) is available.[3,6] This in turn allows for the conversion of prothrombin to thrombin, which once again binds to the PAR-1 receptor on the cell surface and signals through NF-κB and Rho-kinase. Rho-kinase has been found to undergo a high rate of transcription in the presence of thrombin.[13]

These two factors further potentiate the coagulation cascade leading to upregulated thrombin and subsequent endothelial MP release.[3,6,13]

TNF-α also plays a role in endothelial MP release via binding to the cell and acting through p38 mitogen activated protein kinase (MAPK).[3,6,13] However, there is some debate as to how endothelial MPs are generated through this mechanism, and whether the presence of thrombin is necessary to potentiate TNF-α-mediated endothelial MP release or if TNF-α works on its own.[3,6] The presence of thrombin is also thought to upregulate the release of other proinflammatory cytokines via NF-κB, such as IL-1, IL-6, and IL-8 which are part of late phase endothelial MP formation.[3,6,13,16] The presence of these cytokines signals further inflammatory responses and indicates the environment present in endothelial cells when they are actively blebbing endothelial MPs.

Oxidative stress also plays an important role in endothelial MP formation, stemming from multiple pathways involved in endothelial MP release. TNF-α can induce the expression of TF on endothelial MPs that will be blebbed from endothelial cells.[17] In addition, the presence of TF is associated with an increase in procoagulant activity as well as apoptosis within the endothelium, signaling ROS production.[18] The redox balance within the cell is thought to determine the production of TF, further connecting ROS to TF and the production of endothelial MPs via TNF-α.[17] As such, Szotowski and colleagues reported that ROS production acts as a second messenger in endothelial MP formation via connections to TF production as well as NF-κB, which plays a vital role in both early and late phase endothelial MP formation.[6,17] A redox imbalance within the cell may therefore lead to increased endothelial MP formation, as well as procoagulant activity.

Mechanisms of endothelial MP uptake and clearance:

Although the formation of endothelial MPs is an important process to study, the mechanisms of endothelial MP uptake and clearance are vital to gain a complete understanding of their role in the body. Uptake of endothelial MPs by their target cells is performed largely through endocytosis, specifically phagocytosis. This is dependent on the presence of specific surface proteins on the MPs themselves.[19] Surface proteins bind to scavenger receptors on the target cell surface, which recognize the external MP markers and bind them for subsequent processing of their cargo.[19,20] Phosphatidylserine is thought to be one of the most potent MP surface markers involved with uptake, as blocking of the marker with annexin-V leads to a significant decrease in uptake. If they are not taken up and their cargo not processed by target cells, their phenotype will not spread.[619,21] The balance between endothelial MP release and uptake can be disrupted because of aberrant endothelial cell apoptosis or activation. Indeed, an increase in the amount of endothelial MPs in circulation has been observed in patients with coronary artery disease.[22] This suggests that with excessive inflammation comes an increase in circulating endothelial MPs, leading to an imbalance between formation and uptake.[6] More research is needed on the precise mechanisms involved.

While endothelial MP uptake is an important mechanism related to their function within the body, it is equally as important to consider the number in circulation as well as their function. The number of MPs in circulation can indicate that a large amount is being produced by parent cells in response to some amount of stimulus.[6] It is also well known that MP function is largely determined by their stimulus of release, which suggests that studying MP function is imperative.[6,19] It is important to consider MP number, uptake, and function to better understand the roles that this EV subset plays within the body. While endothelial MP number can be thought

of as a biomarker, it is also important to consider that they can contribute to the spread of inflammation as well. Endothelial MPs are known to instigate a procoagulant pathway via interaction with tissue factor (TF).[3] This suggests they are involved with the formation of thrombin which feeds into the immune response within endothelial cells as well as apoptosis.[3,6] While endothelial MP numbers are often considered biomarkers of endothelial activation and apoptosis, they also play an important role in the spread of inflammation within the endothelium.

Endothelial MP surface markers and quantification:

The analysis of MPs first requires that they are identified via size (100-1000 nm) using standardized sizing beads in a flow cytometer.[23] However, there are also markers commonly present on the surface of endothelial MPs that must be used to identify specific subsets related to either endothelial cell activation or apoptosis. Indeed, the vast majority of circulating MPs present phosphatidylserine, a phospholipid that is externalized following apoptosis and cell activation. The presence of phosphatidylserine allows them to easily bind procoagulant factors such as TF later on.[15] Because of this, annexin-V, which binds to phosphatidylserine, is commonly used to identify the presence of phosphatidylserine when quantifying total MPs in flow cytometry. Although the presence of phosphatidylserine on all MPs has been up for debate, it is widely considered to be a classic marker.[2,6,15]

Endothelial MPs will externalize other markers such as e-selectin (CD62E) and platelet endothelial cell adhesion molecule-1 (PECAM-1) (CD31) depending on the stimulus of their release. E-selectin is an endothelial-specific marker expressed when the cells are activated by cytokines, contributing to the inflammatory response.[24] Therefore, annexin-V+/CD62E+ endothelial MPs are characterized as having been blebbed in response to endothelial cell activation.[6,24] E-selectin is expressed when endothelial cells are activated via TNF-α and IL-1β,

and it is further promoted by NF-κB.[25] This explains why it is expressed on endothelial MPs blebbed from activated endothelial cells, as those factors are all present in the inflammatory response that generates endothelial MPs. In general, e-selectin functions to decrease leukocyte rolling, thereby directing immune cells to focus on specific areas.[25] PECAM-1, another classic endothelial MP marker, is commonly associated with those generated through apoptosis.[26] However, because this marker (CD31) is also present on platelet-derived MPs, it is typically co-stained in flow cytometry as CD31+/CD42b-, as CD42b is a platelet-specific marker.[3,6,26] As such, platelet MPs have been commonly stained as Annexin-V+CD42b+. PECAM-1 is an adhesion molecule typically associated with regulation of leukocyte movement into and out of endothelial cells. In addition, it has been implicated in the upkeep of endothelial cell junctions.[27] Its association with endothelial MPs generated via apoptosis is due to this role. Indeed, endothelial MPs have been linked to the impairment of cytoskeletal homeostasis in part due to enhanced PECAM-1 expression induced via TNF-α.[28] In addition to the two classic markers, previous studies have also considered vascular endothelial cadherin (VE-cadherin), CD144, as an endothelial-specific MP marker.[2,3,6] However, because of its specificity, it does not always provide adequate counts/results when quantified using flow cytometry.[3,6,23] While identifying surface markers on endothelial MPs is immensely important in their analysis, it is also crucial in understanding their functional capacities as well as the stimuli resulting in their release.

Endothelial MP Contents and Related Functions:

In addition to their classic surface markers, endothelial MPs also contain cargo in the form of proteins, cell adhesion molecules, and nuclear material such as miRNA.[6] It is from their contents that we can characterize their function and not just their stimulus of release. It is generally accepted that endothelial MPs are blebbed from activated and apoptotic cells.[2,3,6] Their

stimulus of release can be used to characterize them as effectors and markers of endothelial dysfunction, as elevated circulating endothelial MP levels have been associated with a variety of cardiovascular disease risk factors, such as obesity, type 2 diabetes mellitus, and atherogenesis.[2,12,29,30] In addition, a significant increase in circulating endothelial MPs relative to levels seen in healthy individuals have been associated with endothelial dysfunction, highlighting the importance of endothelial MP clearance once they enter the bloodstream.[4,6] It is thought that MPs transfer their cargo to target cells by first binding to receptors on the cells. They are then either taken up into the cell via endocytosis or fuse with the cell membrane to transfer their cargo.[31–33] Therefore, it would appear that transfer of their contents is largely dependent on their interaction with the target cell. This is the main mechanism by which MPs act as cell-to-cell communicators, effectively spreading the state of their parent cell to another.[33]

The contents that they deliver to adjacent or nearby endothelial cells contribute heavily to their function within the body. Jansen et al. demonstrated that miR-126 and miR-199a contained within apoptotic endothelial MPs could be used to predict cardiovascular events and risk among patients with coronary artery disease.[34] miR-126 is known to suppress Sprouty-related EVH1 domain-containing protein 1 (SPRED1), and its suppression of this pathway appears to be impaired with coronary artery disease.[34,35] This leads to a downregulation of miR-126-containing endothelial MPs. In addition, transport of miR-34a contained within endothelial MPs results in a greater expression of VCAM-1 and ICAM-1, upregulating the inflammatory response.[36–38] It is thought that miR-34a is transferred to target cells, acting on the B-cell lymphoma 2 protein (BCL-2), which regulates endothelial cell apoptosis.[38] Indeed, endothelial MPs act as transporters for miRNA, delivering them to target cells and effectively propagating the

phenotype of their release of stimulus. This further demonstrates the important role that endothelial MPs play in the spread of inflammatory phenotypes from cell to cell.

The role of endothelial MPs in atherosclerosis:

Endothelial MPs are thought to play an important part in atherogenesis and atherosclerosis, which hinges on their role as purveyors of inflammation via cell-to-cell transfer. Even though atherogenesis is frequently associated with lipids and the accumulation of atherosclerotic plaques, endothelial MPs play a critical role in both. As mentioned above, endothelial cell apoptosis is a normal occurrence in atherogenesis and atherosclerosis.[2,39] Because of this, endothelial MP release tends to accompany atherogenesis and contribute to atherosclerosis. While an increase in circulating endothelial MP number is indicative of target cell surface receptor saturation, an increase in uptake may still occur as many surface receptors would be occupied.[19] In contrast, a normal balance between MP formation and uptake would likely not overwhelm surface receptors.[6,19] This suggests that a high number of endothelial MPs in circulation may not necessarily indicate decreased MP uptake. During atherogenesis, a decrease in nitric oxide (NO) bioavailability combined with the subsequent increase in ROS leads to endothelial MP release via endothelial cell apoptosis.[2] The decrease in NO bioavailability comes in part through low shear stress, which is seen in areas primed for atherogenesis such as bifurcations.[2,40] Downstream from atherosclerotic plaques, there is a decrease in shear stress due to turbulent flow. Endothelial cells sustain damage from this type of flow, rupturing nearby atherosclerotic plaques.[41] Following the apoptotic and inflammatory milieu released from this action, the coagulation cascade is quickly upregulated to fix the issue, which then involves apoptotic endothelial MPs with phosphatidylserine and TF on their surface contributing to the procoagulant/prothrombotic cascade.[2,3] It has been theorized that endothelial

MPs expressing TF will transfer it to nearby platelets, thereby contributing to the procoagulant cascade.[42] Furthermore, endothelial MPs displaying intercellular adhesion molecule-1 (ICAM-1), e-selectin, and PECAM-1 add to the prothrombotic environment in atherogenesis.[2,3] Apoptotic endothelial MPs contribute even further to atherogenesis by carrying oxidized phospholipids to target cells, causing subsequent atherogenic effects on monocytes and contributing to foam cell generation.[43] This is an interesting mechanism as it mirrors the function of oxidized low-density lipoprotein in classic atherosclerosis. In a clinical sense, increased circulating endothelial MPs have been associated with acute coronary syndromes because of their prothrombotic properties.[44] Indeed, endothelial MPs have been tightly associated with atherogenesis because of their apoptotic mechanism of release and subsequent role in the coagulation cascade.

Interestingly, researchers have posed a newer idea of endothelial MP functions being attributed to their mechanism of release. Historically, studies have mostly focused on how endothelial MPs from activated and apoptotic endothelial cells contribute to chronic disease. However, it is interesting to note that many believe the stimulus of release largely determines endothelial MP function. Indeed, following a proteomic analysis of endothelial MPs from various stimuli, approximately one third of their proteins come from their stimulus of release.[45] Contributing to that point, endothelial MPs stimulated by activated protein C (APC), an anticoagulant, were found to have inhibited factor Va on their surface, effectively downregulating the prothrombotic pathway.[46,47] If factor Va is inhibited, then factor Xa, which is activated by TF, will not assist in the conversion of prothrombin to thrombin.[3]

Hergenreider et al. examined an antiatherogenic effect of endothelial MPs in response to shear stress. The authors showed that endothelial MPs generated through high shear stress *in vitro* contained miR-143/145 and miR-126. miR-126 has already been seen as a regulator of

SPRED1, the mechanism of which is suppressed in cardiovascular disease and leads to increased proliferation and adhesion.[34,35] The miR-143/145 cluster is considered to be antiatherogenic as well, decreasing atherosclerotic lesions in a mouse aorta.[40] It is also necessary for vascular smooth muscle cell function. Expression of miR-143/145 in endothelial MPs was found to be dependent on Krüppel-like factor 2 (KLF2), a transcription factor expressed on endothelial cells when exposed to shear stress.[40] A more recent study has also shown that KLF2 exerts its antiatherogenic effects by inhibiting endothelial bone morphogenetic proteins and others leading to vascular calcification.[48] Although this does not directly involve endothelial MPs, it amplifies the importance of increased shear stress and blood flow in endothelial health. If shear stress mechanisms can conformationally change endothelial MP function, this begs the question of how exercise may affect them.

Indeed, others have explored the relationship between endothelial MPs and their mechanism of release. Silva and colleagues recently reported that heart failure patients who had disturbed blood flow (i.e. cyclic flow) exhibited a significant increase in CD31+/42b- endothelial MPs ($P = 0.03$) as well as a decrease in endothelial function measured via flow-mediated dilation (FMD) ($8.50\% \pm 1.41$ vs. $4.64\% \pm 1.42$, $P = 0.01$) after occlusion.[49] Disturbance of blood flow is a common factor in endothelial dysfunction, suggesting that a lack of shear stress leads to increased apoptotic endothelial MPs in circulation. This strengthens the relationship between endothelial MPs and their stimulus of release.

Effects of MPs and ROS on Premature Vascular Aging:

Age-related oxidative stress is a major component of endothelial cell senescence/dysfunction, leading to decreases in cell division and increased apoptotic signaling.[50] These age-related phenotypic changes lead to increased arterial stiffness and are significant in

the enhanced risk of cardiovascular disease seen with aging. An abundance of ROS is known to disrupt cellular structure and cause inflammation within the cell. Specifically, NO reacting with superoxide leads to impaired NO bioavailability and therefore a decrease in NO-mediated vasodilation.[51,52] In addition, NO reacts with superoxide to produce peroxynitrite, which moves into endothelial cells and contributes to apoptotic signaling, as well as upregulation of adhesion receptors.[53] The latter significantly enhances the inflammatory response directed at the cell. Peroxynitrite also disrupts the ionic balance of the cell membrane, contributing to permeability issues and allowing inflammatory molecules to enter the cell.[53]

The role of endothelial MPs in endothelial cell ROS production has been studied throughout the literature. As previously mentioned, enhanced ROS production feeds into the procoagulant mechanism of endothelial MP formation via upregulation of TF.[17] However, ROS produced from endothelial cell uptake of endothelial MPs have been linked to premature vascular aging in rat aortic rings.[54] The same study found that the endothelial dysfunction-induced rise in endothelial MPs led to diminished angiogenesis and decreased nitric oxide.[54] Human umbilical vein endothelial cells (HUVECs) incubated with endothelial MPs *in vitro* have also exhibited enhanced ROS production, providing further evidence of this mechanism.[55] Another study found that after 4 hours of exposure to endothelial MPs, cultured mouse aortic cells produced a significantly greater amount of ROS compared to controls.[56] In addition, ROS carried into the cells via endothelial MPs decreased endothelial nitric oxide synthase (eNOS) activity and nitric oxide. The authors speculate that endothelial MPs may act in a paracrine fashion, effectively controlling redox signaling within the cell.[56] This study found that endothelial MPs stained with annexin-V contain ROS-producing NADPH subunits such as Nox1, Nox2, and Nox4.[56,57] They also showed that endothelial MPs increase phosphorylation of

the extracellular signal-regulated kinase 1/2 (ERK1/2) pathway and the tyrosine kinase Src in endothelial cells.[56] While the ERK1/2 pathway is known to regulate many cellular processes, it can induce the stress response as well as cellular apoptosis which both lead to ROS production.[56,58] Furthermore, phosphorylation of Src family kinases induces Nox1-mediated production of intracellular ROS. This occurs when the NADPH subunits convert oxygen to superoxide. Each of these mechanisms contribute to ROS production and activity of each appears to be increased when endothelial MPs are incubated with endothelial cells.[56] The ROS produced under these conditions, superoxide anion, contributes to a decline in NO-dependent vasodilation and subsequent vascular function.[56] This occurs because superoxide reacts with NO to produce peroxynitrite, which upregulates apoptotic signaling within the cell and increasing expression of cell adhesion molecules.[51–53] Both outcomes increase the immune response directed at the cell, heightening the inflammatory response.[53] According to Burger and colleagues, endothelial MP-derived ROS enhances endothelial cell dysfunction and leads to a marked increase in endothelial cell ROS production.[56] The immergence of these mechanisms introduces new questions, such as how lifestyle factors known to influence cardiovascular disease risk including exercise, training status, and dietary inflammation affect the ability of endothelial MPs to influence endothelial cell ROS production *in vitro*.

Microparticles and Exercise

Microparticles and Acute Exercise:

Although changes in circulating endothelial MP counts and function in response to chronic disease have been previously studied, their response to exercise has been less well documented. Indeed, there have been a small number of studies that focused on bouts of acute

exercise, but even fewer have examined changes in endothelial MPs in response to exercise training. Regarding the former, Serviente et al. reported that a 30-minute bout of moderate intensity treadmill exercise at 60% of VO_{2peak} significantly reduced total MPs by 45%, as well as CD62E+ endothelial MPs by 34% and CD31+/42b- endothelial MPs by 44% in healthy women aged 40-65 years 30 minutes after exercise.[59] This study, while hoping to find novel insights into how the combination of menopause and activity status affect endothelial MP counts, highlighted the important role of exercise in endothelial dysfunction through the lens of circulating MP counts. A moderate intensity acute bout led to a significant decrease in endothelial MP counts at 30 minutes post-exercise compared to baseline, showing that moderate intensity exercise can affect circulating MPs .[59] Furthermore, endothelial MP reductions were seen in participants regardless of fitness or menopausal status.[59] While this implies increased endothelial MP uptake, it is important to note that acute exercise can increase expression of superoxide dismutase mRNA. Because superoxide dismutase scavenges ROS and acts as a defense against oxidative stress, it appears that acute exercise may lead to increased superoxide dismutase expression.[60] This suggests some mitigation of ROS production and inflammation related to MP uptake.

An acute bout of moderate intensity continuous aerobic exercise has also been shown to significantly reduce circulating CD62E+ endothelial MPs by 18% following exercise in healthy active individuals aged 18-40 years.[61] While there were no significant reductions at individual time points, there was a decrease from baseline when compared to all post-exercise time points combined. Interestingly, there was no significant effect of acute high-intensity interval training (HIIT) on this MP population.[61] The reasons for this are unclear, but the higher intensity of exercise may have fed back into the signaling pathway that produce endothelial MPs, effectively canceling out any MP count reductions. This suggests that acute exercise-based changes in

endothelial MPs may be dependent on the intensity of exercise performed. A similar study comparing acute high-intensity continuous exercise consisting of 20 minutes of cycling right above ventilatory threshold to high-intensity interval exercise in overweight/obese individuals aged 19-27 years found comparable results. CD62E+ endothelial MPs were significantly increased by 40% 18 hours post-exercise in females following only the interval exercise compared to a control visit, indicating increased endothelial cell activation.[29] However, both CD31+/42b- and CD2E+ endothelial MPs were reduced by 46% and 24%, respectively in males 18 hours after interval training compared to the control visit. This highlights a potential sex difference when studying endothelial MPs and acute exercise in sedentary overweight/obese individuals. The results of this study also suggest that endothelial MPs, while affected by exercise, may act differently depending on the body composition of the individual. Indeed, both sex and fitness status may play a role in the balance between the release and uptake of endothelial MPs by endothelial cells.[29]

Wahl et al. attempted to address this using three acute aerobic exercise bouts on a cycle ergometer, covering a wide range of exercise intensities and volumes. The goal of the study was to examine the effects of acute exercise on CD31+/42b- endothelial MPs and known angiogenic growth factors in male triathletes 21-28 years.[62] Participants completed 130 minutes at 55% of their peak power output (PPO), 4x4 minutes at 95% PPO, and 4x30 seconds at an all-out intensity.[62] The trained individuals displayed significant decreases of 40%, 50%, and 69% in apoptotic CD31+/42b- endothelial MPs 60 minutes after the 4x4 min, high-volume training, and 4x30 bouts compared to baseline, respectively. It is important to note that the study did not include untrained participants, which may limit comparisons with less-fit individuals. There were also significant increases of 9% and 11% in serum-based vascular endothelial growth factor

(VEGF) immediately after the 4x4 and 4x30 bouts, respectively, compared to baseline. Values returned to baseline 30 minutes post-exercise. Hepatocyte growth factor (HGF) also increased 180 minutes after the 4x4 and 4x30 bouts by 12% and 33%, respectively, compared to baseline.[62] The decrease in apoptotic endothelial MPs measured 60 minutes after all three exercise bouts again suggests that there may be fewer in circulation due to increased MP clearance. To this point, the authors reported that endothelial MP uptake by cultured human coronary artery endothelial cells (HCAECs) doubled when previously exposed to conditioned serum from 3 hours after exercise compared to baseline serum.[62] Cellular apoptotic activity was measured via caspase-3 enzyme-linked immunosorbent assay (ELISA). HCAECs incubated with endothelial MPs and conditioned serum from the 4x4 180-minutes post-exercise timepoint exhibited decreased apoptotic activity compared to cells exposed to baseline endothelial MPs and serum.[62] These data, along with the increased angiogenic growth factors post-exercise support an interesting scenario where acute high-intensity aerobic exercise may functionally alter endothelial MPs to protect against apoptosis or at least increase endothelial MP uptake thereby decreasing the amount in circulation. This theory may explain decreases in endothelial MPs post-exercise reported in some of the abovementioned studies.[29,59,61,62] Modifications to endothelial MPs and their uptake may differ based on intensity of the exercise performed, as well as the fitness status of participants.[29,59,62] However, more insight is needed into how different forms of acute aerobic exercise affect individuals based on body composition and fitness status.

Microparticles and Exercise Training:

Endothelial MP count changes following exercise training have received very little attention in the literature. However, a few studies present data relevant to this issue. Rafiei et al. reported a significant 7% decrease in CD62E+ endothelial MPs following two weeks of HIIT

and a decrease of 6% following moderate intensity continuous training workouts in overweight/obese women aged 38-62 years who were at an increased risk of type 2 diabetes mellitus.[63] Post-training samples were taken ~72 hours following the last session, highlighting an interesting effect from training and teasing out the acute response.[63] As both exercise intensities led to a decrease in endothelial MPs, it appears that the cumulative effect of training decreases circulating endothelial MP counts regardless of intensity. This is in contrast to their responses to acute exercise, where endothelial MP counts in at least one study decreased with moderate intensity exercise but were unchanged by high intensity exercise.[61] The CD31+/42b- subset was not significantly changed in either the overweight/obese group or the age-matched healthy control group.[63] The overweight/obese group did have greater baseline amounts of the apoptotic endothelial MP subset in circulation compared to the control group. This suggests that the overweight/obese participants had some form of chronic low-grade inflammation that the healthy controls did not.

Another study utilized a 6-month aerobic exercise intervention in sedentary African Americans aged 40-75 years and reported that CD62E+ endothelial MPs significantly decreased by 48%, as well as IL-6 by 12%.[64] In addition, FMD, a non-invasive measure of endothelial function, increased significantly (6% ± 1.0 vs. 10% ± 0.5).[64] Interestingly, the authors found no correlation between the increase in FMD and the decreases in both endothelial MPs and IL-6. However, others have reported decreased FMD along with increased endothelial MP numbers in individuals with heart failure.[49] As previously mentioned, Silva and colleagues utilized middle-aged adults with heart failure when observing this relationship.[49] Because Babbitt, et al. studied healthy older adults, there may not have been a as much room for improvement or responsiveness in the two measurements compared to the heart failure patients. Following a brief

review of the existing body of knowledge on endothelial MPs and exercise training, circulating CD62E+ endothelial MPs are indeed decreased with various types of aerobic exercise training in at least two different populations.[63,64] The CD31+/42b- subset was not analyzed in Babbitt et al., but it did decrease in the healthy control group in Rafiei et al. while remaining unchanged in the overweight/obese group.[63,64] This provides insight into exercise-related changes in both apoptotic and activated endothelial MPs due to training, but more research is needed to elucidate more specific mechanistic findings.

Microparticles and High-fat Meal

High-fat Meal and Vascular Inflammation:

The high-fat meal is a well-established method to bring about acute inflammation in both human and animal studies. Indeed, cardiovascular disease risk can be influenced by hypertriglyceridemia, of which postprandial lipemia is a potent model.[65] Endothelial dysfunction is known to be enhanced by aberrant ROS production.[65,66] As such, a high-fat meal is an adequate stimulus to study acute endothelial dysfunction and its accompanying mechanisms as it is a potent stimulator of both oxidative stress and upregulated cytokine activity.[65] For more than 60 years, mechanisms that lead to atherosclerosis following postprandial lipemia have been thoroughly explored. Previous studies have reported significant increases in IL-6 following a high-fat meal as well as high-fat diet.[67,68] In addition, a high-fat diet has been associated with enhanced production of NF-κB and TNF-α, as well as IL-1.[68] Each of these contributes to both the formation and release of pro-coagulant endothelial MPs from endothelial cells, suggesting that high-fat meals may influence this MP subset.[3] As such, a high-fat meal may be in part priming cells to bleb endothelial MPs due to the upregulation of certain inflammatory factors.

Effects of High-fat Meal on Microparticles:

The effect of high-fat meal-induced inflammation on MP concentrations has been previously studied in the literature.[5,65,67,69] However, MP isolation techniques and sample detection have significantly improved since much of this research was completed. In addition, endothelial MP function following a high-fat meal has yet to be explored. As endothelial MPs and other MP subsets are considered proinflammatory and procoagulant in their function, it stands to reason that they would be affected by the acute inflammation typically seen following a high-fat meal. Over the last 15 years there has been a limited number of studies on this subject. According to Tushuizen and colleagues, two consecutive high-fat meals led to a significant 43% increase in area under the curve (AUC) for total MPs between the high-fat meal visit and a control visit, indicating a rise in total MPs throughout the high-fat meal.[65] Total MPs also exhibited a non-significant trend to increase starting at 2 hours following the meals, then increased again at hours 6 and 8 following a brief plateau. It is important to note that the MP staining panel was lacking a CD42b- stain, meaning platelets were not removed from analysis and quantification. Endothelial MPs were also not included in the published data. This study utilized healthy male adults, highlighting that the inflammatory effects of high-fat meals are not limited to populations with underlying conditions.[65] In addition to the MP changes, researchers observed impaired FMD after administering the second high-fat meal, potentially exposing a connection between endothelial function and total MPs.[65]

Similarly, Ferreira and colleagues observed significant increases of 64% and 59% in CD31+42b- (apoptotic) endothelial MPs (lacking a stain for annexin-V+) following a high-fat meal compared to a control group at hours 1 and 3 compared to baseline, respectively.[69] This study utilized healthy males and females and did not report any sex differences in MP count

responses to the high-fat meal. Interestingly, this study also found that increases in apoptotic endothelial MPs correlated with increased serum triglycerides.[69] This shows that there is an increase in endothelial cell apoptosis, further strengthening the connection between a high-fat meal and endothelial inflammation. A similar study looking at the effect of prior exercise on CD31+42b- endothelial MPs following a high-fat meal found that they increased regardless of prior exercise in recreationally active young men.[67] This occurred post-prandially compared to baseline at 2, 4, and 6 hours, respectively with significant increases of ~114%. This represents what is occurring regarding endothelial MP formation and uptake following the high-fat meal, showing that the rate of blebbing from parent endothelial cells may be outpacing the rate at which endothelial MPs are taken up into target cells. The authors also observed significant increases in IL-6 and leukocytes following the high-fat meal which mirrored the increase in endothelial MPs.[67] Interestingly, a second study focusing on the same factors saw an effect of prior exercise but not the high-fat meal on both activated and apoptotic endothelial MPs.[5] However, Strohacker and colleagues were able to see an effect of prior exercise, observing a 47% increase in apoptotic endothelial MPs in the meal-only group but no increase in those who had completed exercise beforehand.[70]

Indeed, it appears that total MPs and endothelial MPs are affected by inflammation brought on by a high-fat meal. Increases in MPs seem to correlate with a rise in triglycerides, as well as a decrease in vascular function measured via FMD. As such, this is a relevant area warranting further study. There has been a very limited number of studies on the topic. Knowledge in this area needs to be expanded upon, especially regarding how a high-fat meal affects MP function. It is well-known that a high-fat meal induces oxidative stress via ROS.[65] In addition, endothelial MPs tend to increase following a high-fat meal, and they have also been

shown to induce a rise in endothelial cell ROS production.[54,67,69] Therefore, this brings up an interesting question about how the high-fat meal might affect endothelial MP function regarding their ability to induce endothelial cell ROS production. After a brief review of possible connections, there is some evidence that the high-fat meal may prime endothelial MPs to induce enhanced ROS production in endothelial cells. The ability of MPs generated through a high-fat meal to bring about subsequent inflammation and oxidative stress *in vitro* requires more exploration.

Conclusion

While the roles of MPs have received some previous attention, there are many gaps in the literature that require further exploration regarding how they are affected by lifestyle factors associated with cardiovascular disease risk. The basic physiology of MPs shows their relevance to inflammation and potential roles in vascular function. Furthermore, both acute and chronic bouts of exercise decrease MP counts in a variety of populations, highlighting potential therapeutic mechanisms. Finally, the role of inflammation brought about from a high-fat meal appears to correlate with changes in MP counts, cementing this as an appropriate model to study the effects of dietary inflammation on MPs. However, there are still gaps in our knowledge of MPs and how they function in response to various stimuli. Indeed, gaps identified throughout the above review include: i) how does a high-fat meal affect activated and apoptotic endothelial MPs, as well as total MPs in healthy young adults? ii) how do different intensities of acute aerobic exercise affect circulating MP counts in overweight vs. lean healthy adults? iii) how are circulating MP counts affected by exercise training in older adults? iv) how do endothelial MPs generated from a high-fat meal, acute exercise, and aerobic exercise training affect endothelial cell ROS production *in vitro*?

Chapter 2: Effects of a high-fat meal on circulating microparticle quantity and function

Introduction

A traditional western diet high in animal-based saturated fats has been repeatedly linked to the progression of cardiovascular disease.[5,71,72] The postprandial rise in low-density lipoprotein (LDL) cholesterol as well as repeated exposure to high amounts of lipids leads to endothelial cell inflammation and subsequent oxidative stress.[5,69,72] One product of oxidative stress, reactive oxygen species (ROS), is known to be detrimental to vascular function. This can subsequently lead to the development of various conditions contributing to cardiovascular disease, such as atherosclerosis, hypertension, and heart failure.[5,72] As cardiovascular disease is the leading cause of death in many western countries, it is increasingly important to broaden our understanding of its contributing mechanisms.

A high-fat meal is a well-known stimulus for inducing an acute rise in postprandial lipemia through increased serum triglycerides.[5,65,67] Because of this, it is an excellent model for studying diet-induced vascular inflammation and the inflammatory mechanisms that will eventually give rise to atherosclerosis. One important connection between the high-fat meal and cardiovascular disease is an acute onset of endothelial dysfunction brought on by oxidative stress.[65,66] This has been examined by multiple studies, finding a significant decrease in postprandial flow-mediated dilation (FMD).[65,73] Following consumption of a high-fat meal, the body is flooded with triglycerides. Lipid-laden cells attempt to clear this via oxidative metabolism, producing large amounts of ROS in the process.[74,75] Within endothelial cells, ROS can scavenge nitric oxide (NO) and degrade a crucial enzyme known as endothelial nitric oxide

synthase (eNOS). This has deleterious effects on vascular function, which largely requires NO to perform vasodilation.[74,75] Chronic diet-induced vascular dysfunction via these pathways allows for the development of atherosclerosis. Although this process may not occur in every endothelial cell following a high-fat meal, the inflammatory environment may be communicated to other cells via extracellular vesicles.

Annexin-V+ MPs and their subpopulations are procoagulant extracellular vesicles released by endothelial and other "parent" cells in response to stimuli including inflammation, apoptosis, cellular damage, and exercise.[65,67,69] Microparticles are membrane-bound vesicles that are mainly mechanisms of cell-to-cell communication through continual release and uptake by parent and target cells, respectively. In this way they are thought to spread phenotypic changes dependent on their stimulus of release.[6,7,65] The effect of high-fat meal-induced inflammation on circulating MP concentrations has been previously studied. For example, Ferreira and colleagues observed significant increases of 64% and 59% in the number of CD31+42b- (apoptotic) endothelial MPs following a high-fat meal compared to a control group at hours 1 and 3 compared to baseline, respectively.[69] The changes in MPs correlated with a rise in serum triglycerides, making an important connection between the high-fat meal stimulus and endothelial MPs. This study utilized healthy adults, highlighting that the inflammatory effects of the meal are not limited to populations already at risk for cardiovascular disease.[65,69]

Interestingly, Jenkins and colleagues studied endurance-trained males and found no difference in endothelial MP counts at 4 hours following a high-fat meal.[5] This introduces a novel question of whether fitness status can impact postprandial MP counts. Other previous studies have utilized healthy adults, but none have investigated fitness-related differences.[5,69] A study from Ramirez-Velez suggested that just 12 weeks of aerobic exercise training diminished

postprandial declines in endothelial function, suggesting that a higher fitness status may improve an individual's ability to process the acute inflammatory stimulus.[76] Prior exercise has been shown to decrease intracellular ROS production following a high-fat meal, but the chronic effect of exercise training status has not been addressed.[5] Similarly, shear stress is known to regulate the release of MPs and it could be theorized that the MPs of high-fit individuals who are regularly exposed to shear stress may react to a high-fat meal differently than those of a lower fitness status.[77] While postprandial MP counts may be differentially affected based on fitness status, changes in MP function following a high-fat meal have yet to be explored in any population. As MPs are functionally proinflammatory, and given how fitness status affects the postprandial response to inflammation, it stands to reason that MPs would be affected by the acute inflammation triggered by the high-fat meal.[65] In addition, MP function may be differentially affected by fitness status.

The purpose of this study was to determine the effects of a high-fat meal on circulating MP counts as well as their ability to stimulate endothelial cell oxidative stress *in vitro*. We hypothesized that (1) the number of circulating MPs will be significantly increased following a high-fat meal compared to baseline samples, and (2) that human umbilical-vein endothelial cells (HUVECs) incubated with MPs collected following a high-fat meal will exhibit increased ROS production compared to baseline samples. We further investigated the exploratory hypothesis that participants of a higher fitness status may exhibit attenuated postprandial MP responses and endothelial cell ROS production compared with lower-fit participants.

Methods

Ethical Approval:

All human subject research performed in this study received prior approval from the University of Maryland Institutional Review Board (IRB). A signed informed consent was obtained from each participant stating that the study conformed to the standards set by the latest revision of the Declaration of Helsinki.

Participants:

A total of 20 participants (10 males and 10 females) were recruited for the study. Participants were healthy, non-smokers, and 18-35 years of age, with body mass indexes (BMI) <30 kg/m^2. Participants had blood pressures <140/90 mmHg and were free from any heart, lung, or metabolic disease, as well as diabetes mellitus. All female participants were non-pregnant with regular menstrual cycles. Pregnancy status was confirmed through pre-screening questionnaire. Female participants were all tested during the early follicular phase or placebo pill phase for those taking oral contraceptives. All participants were free from sickness at least 2 weeks prior to their study visits.

VO_{2peak} Test:

Participants completed a VO_{2peak} exercise test on a cycle ergometer (Parvo Medics, Salt Lake City) to establish cardiovascular fitness level during their first study visit. This consisted of a 3-minute warm-up, followed by a maximal exercise test with an increase of 1 kg in resistance after each 2-minute stage. Heart rate and rate of perceived exertion were recorded at the end of each stage. Participants were instructed to maintain a range of 70-80 rpm during the test, and the test was terminated once they could not maintain this. Participants completed a 3-minute cool-

down once the test had ended. Oxygen consumption was measured throughout the test (Parvo Medics, Salt Lake City, UT) and the highest oxygen consumption value achieved during the test was considered as a VO_{2peak}. This was used to classify participants' aerobic fitness levels when analyzing data. Participants were split into a high-fit group and a lower-fit group based on whether they scored above or below the 50^{th} percentile for VO_{2max} testing based on age and sex according to the American College of Sports Medicine classification.[78]

High-fat Meal:

At least 24 hours following their first visit, participants returned to complete the study. They underwent a baseline blood draw, consumed a high-fat meal, and underwent subsequent blood draws at 2 and 4 hours postprandially. Blood was collected using acid citrate dextrose vacutainer tubes (Becton Dickinson, Franklin Lakes, NJ). Participants consumed the high-fat meal within 10 minutes. It consisted of heavy whipping cream, chocolate syrup, and non-fat powdered milk normalized to the subject's body surface area. The meal was made up of 85.3% fat, 11.3% carbohydrates, and 3.4% protein. A 386g serving contained 1362 kcal. The meal size was normalized to the participant's body surface area (386g per $2m^2$ body surface area). Our laboratory has used this high-fat meal formula for previous studies.[5]

Microparticle Isolation:

Plasma samples from each time point were used to isolate endothelial MPs via sequential centrifugation. Briefly, 500µL aliquots from each time point were centrifuged at 1500xg for 20 minutes at 20°C to obtain platelet poor plasma. The top two-thirds of the supernatant were transferred to a new microcentrifuge tube and centrifuged again at 1500xg for 20 minutes at 20°C to obtain platelet free plasma. 100µL of each sample was incubated with fluorescent

monoclonal antibodies for quantification of each MP population, including annexin-V+ anti-annexin-V-violet 450 (v450) (BD, Franklin Lakes, NJ, USA, Cat#560508, RRID: Ab_2869356) which binds to phosphatidylserine, CD31+ anti-CD31-allophycocyanin (APC) (ThermoFisher Scientific, Waltham, MA, USA, Cat#47031942, RRID: Ab_10730582) for PECAM-1, CD42b-anti-CD42b-peridinin-chlorophyll-protein (PerCP) (ThermoFisher Scientific, Waltham, MA, USA, Cat#46042942, RRID: Ab_2762458) to remove platelets and CD62E+ anti-CD62E-phycoerythrin (PE) (BD, Franklin Lakes, NJ, USA, Cat#551145, RRID: Ab_394072) for e-selectin. MP sub-populations including Annexin-V+/CD31+/CD42b- and Annexin-V+/CD62E+ as well as Annexin-V+ for total MPs and Annexin-V+CD42b+ for PMPs were quantified via flow cytometry. Standardized 900 nm and 1000 nm nanobead NIST traceable particle size standards (Polysciences, Warrington, PA, USA) and CountBright absolute counting beads (ThermoFisher Scientific, Waltham, MA, USA) were used for proper quantification of MPs. Data analysis was performed using FlowJo V10.1rs (FlowJo, LLC, Ashland, OR, USA).

Endothelial Cell Culture:

Passage 3-6 pooled male and female human umbilical vein endothelial cells (HUVECs) were seeded in T75 flasks with 15 mL of endothelial growth medium 2 (EGM2), then passed into separate 96-well plates with 200 µL of EGM2 with 2% fetal bovine serum (FBS) per well once they had reached ~80% confluence. Once they had once again reached ~80% confluence HUVECs were ready for subsequent experiments.

Microparticle Incubation and Intracellular ROS Quantification:

Five thousand MPs were isolated from each time point of each sample using the previously mentioned MP isolation technique, then added to each well of a flat-bottomed 96-well

plate. This amount is similar to amounts previously utilized to quantify ROS production in endothelial cells.[56] HUVECs were incubated with MPs for 1 hour.

Following incubation with MPs, Cell ROX Green Reagent (Life Technologies, Carlsbad, CA, USA) was added to the cells at a concentration of 5 μM and incubated for 30 minutes at 37ºC. Following incubation, cell medium was removed, and each well was washed two times with 1x phosphate-buffered saline (PBS). Endothelial cell ROS production was quantified using plate fluorescence (Synergy H1 Hybrid Reader; Biotek, Winooski, VT, USA). Microscopic images of each well were captured following CellROX incubation using a Nikon Eclipse Ti-U microscope with DS-Ri2 camera (Nikon Instruments, Melville, NY, USA). Cell counts within each well were obtained using NIS-Elements AR 5.21.03 imaging software (Nikon Instruments, Melville, NY, USA). To compare ROS fluorescence among sample wells, fluorescence was normalized to HUVEC count of each well. To obtain the ROS-producing capacity of total MPs, the total MP count originally obtained via flow-cytometry was divided by 5000 (the number of MPs added to each well). This was then multiplied by the ROS/cell count obtained from microscope images.

Statistical Analysis:

Following completion of data collection, a Shapiro-Wilk test of normality showed a non-normal distribution of microparticle count and ROS data ($P < 0.05$). This was not resolved with log transformation; therefore, Wilcoxon signed-rank tests were used to analyze any differences between paired MP counts among time points. The Mann-Whitney U test was used to conduct exploratory analyses between independent high- and lower-fit groups at specific timepoints. Unpaired t-tests were used to analyze any differences in participant characteristics between high-fit and lower-fit groups. Statistical significance was accepted at $P < 0.05$.

Results

Participant Characteristics:

Participant characteristics are summarized in Table 1. There was a significant difference in VO_{2peak} between the high-fit and lower-fit groups ($P < 0.0001$). There were no significant differences in other participant characteristics between groups.

Table 1. Participant Characteristics

	Total	High-fit	Low-fit
Age	24 ± 1.00	25 ± 1.93	23 ± 1.01
Males	10	5	5
Females	10	2	8
Weight (kg)	66.66 ± 2.46	69.2 ± 3.81	65.30 ± 3.23
BMI (kg/m^2)	22.56 ± 0.63	22.53 ± 0.84	22.58 ± 0.89
BSA (m^2)	1.76 ± 0.05	1.84 ± 0.07	1.72 ± 0.06
HFM Kcal	1311.88 ± 33.65	1376.47 ± 50.73	1277.10 ± 42.17
VO_{2peak}	43.59 ± 2.55	53.03 ± 2.67*	33.29 ± 2.03*
Max HR	184.82 ± 2.73	184.50 ± 4.97	185.14 ± 2.74

Data are means ± SEM. BMI, Body mass index; BSA, Body surface area; HFM Kcal, High-fat meal kilocalories; Max HR, Maximal heart rate. *$p < 0.0001$

Circulating total microparticle counts following a high-fat meal:

There were no significant differences in total MP counts between baseline, 2h post- and 4h post-high-fat meal ($P > 0.05$), shown in Figure 1. We also conducted exploratory analyses based on fitness status. After splitting participants into high-fit and lower-fit groups, total MP number was numerically but not statistically higher at 2h and decreased significantly at 4h such that counts were similar to baseline in the high-fit group ($P < 0.05$). There were no significant effects of the high-fat meal on total MP counts in the lower fit group (Figure 2).

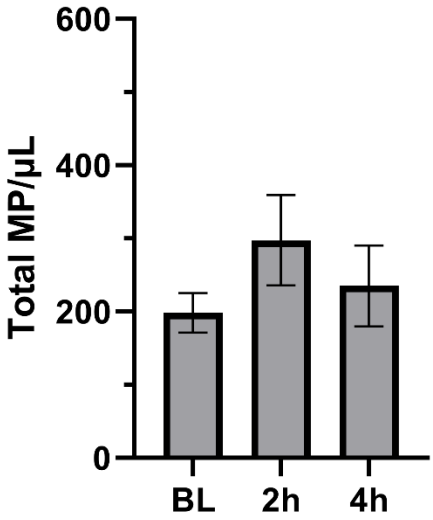

Figure 1. Total MP counts in response to a high-fat meal between baseline (BL), 2-hour post (2h), and 4-hour post (4h) time points. Mean ± SEM, n=20.

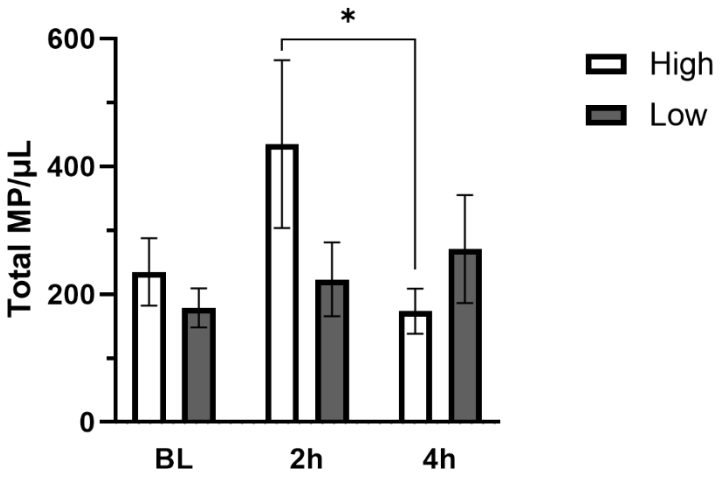

Figure 2. High-fit vs. lower-fit total MP counts in response to a high-fat meal between baseline (BL), 2-hour post (2h), and 4-hour post (4h) time points. Mean ± SEM, High-fit = 7, Lower-fit = 13.

ROS production after MP incubation:

Following incubation with MPs from the three time points, there was a significant increase in HUVEC ROS production from the BL to 4h time points among all participants'

samples ($P < 0.05$), shown in Figure 3. For our exploratory aim based on differences in fitness status, the lower-fit group exhibited significant increases in HUVEC ROS production from BL to 2h and BL to 4h, respectively ($P < 0.05$ for both). This is shown in Figure 4. There were no significant changes in HUVEC ROS production among the high-fit group.

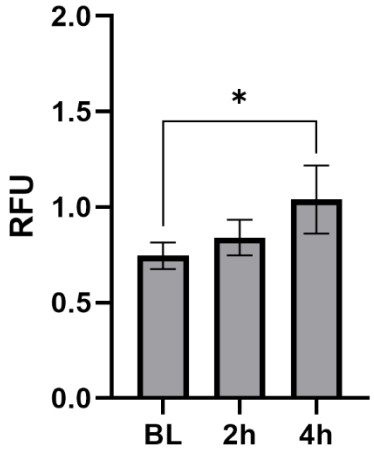

Figure 3. HUVEC ROS production following 1-hour incubation with MPs isolated from high-fat meal BL, 2h, and 4h timepoints. Mean ± SEM, n = 20. *p < 0.05

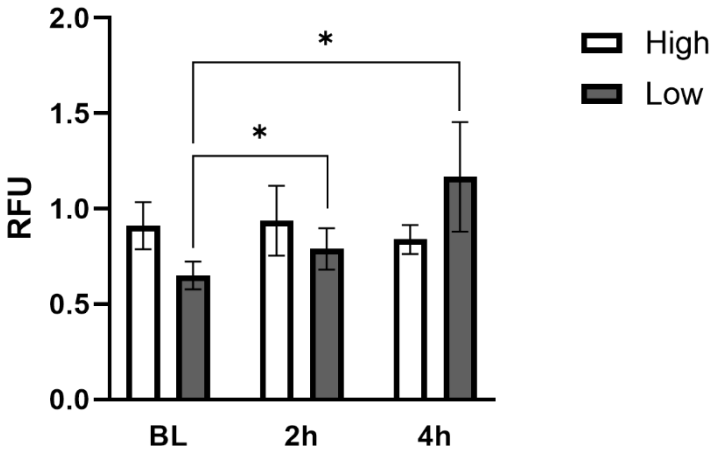

Figure 4. High-fit vs. lower-fit HUVEC ROS production in response to 1-hour incubation with MPs isolated from high-fat meal BL, 2h, and 4h timepoints. Mean ± SEM, High-fit = 7, Lower-fit = 13. *p < 0.05.

ROS-producing capacity of total MPs:

When HUVECs were incubated with MPs from the BL, 2h and 4h time points, there was tendency (P = 0.064) for ROS-producing capacity of total MPs to increase from BL to 2h in the entire group, shown in Figure 5. This appeared to be driven by the high-fit group, as ROS-producing capacity of total MPs was significantly higher in the high-fit compared with lower-fit groups at the 2h timepoint (Figure 6, P < 0.05). There was a subsequent decrease in ROS-producing capacity of total MPs in the high-fit group from 2h to 4h (P < 0.05), also shown in Figure 6.

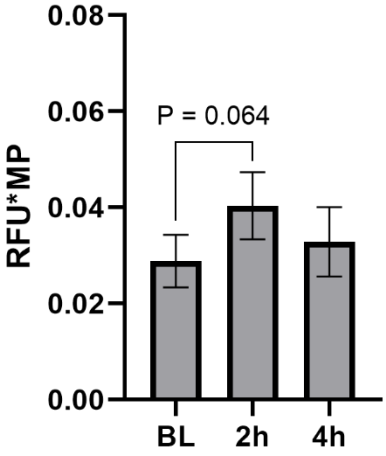

Figure 5. ROS-producing capacity of total MPs between high-fat meal BL, 2h, and 4h timepoints. Mean ± SEM, n = 20.

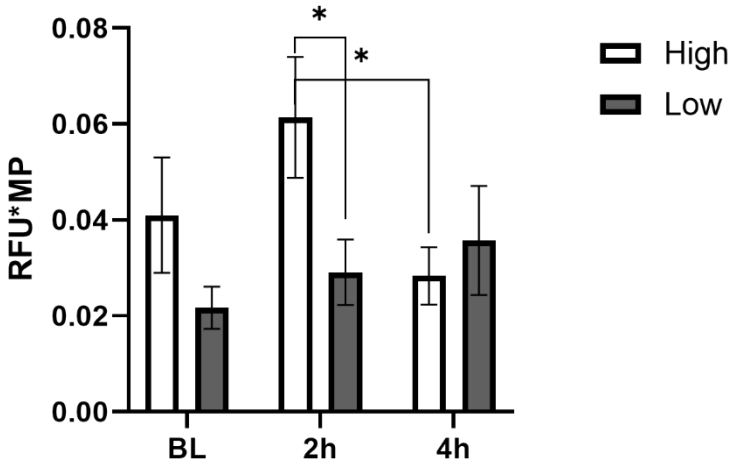

Figure 6. High-fit vs. lower-fit ROS-producing capacity of total MPs between high-fat meal BL, 2h, and 4h timepoints. Mean ± SEM, High-fit = 7, Lower-fit = 13. *p < 0.05.

Endothelial MPs:

While endothelial MP counts generally followed the same numeric trends as total MP counts in response to the high-fat meal, there were no statistically significant differences in

apoptotic CD31+/CD42b- endothelial MPs among high-fat meal time points (Figure 7A, P > 0.05). In addition, there were no significant differences detected in response to the high-fat meal among activated CD62e+ endothelial MPs (Figure 8A, P > 0.05). There were no significant differences between high- and lower-fit groups for either subset of endothelial MPs, although they did follow the same trends as total MPs (Figures 7B and 8B, P > 0.05 for both).

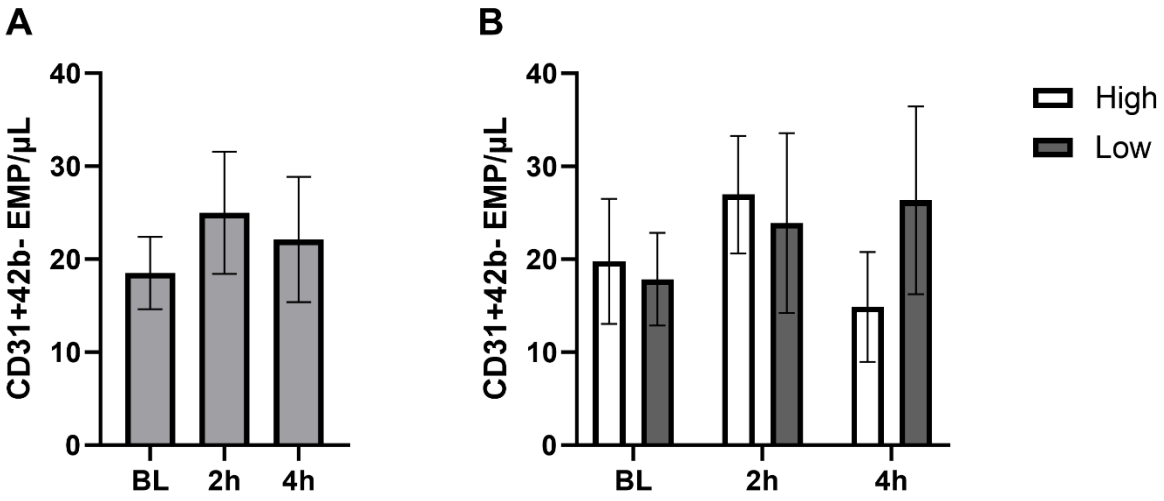

Figure 7. Circulating apoptotic CD31+/CD42b- endothelial MP counts between high-fat meal BL, 2h, and 4h timepoints (A). High-fit vs. lower-fit circulating apoptotic CD31+42b- endothelial MP counts between high-fat meal BL, 2h, and 4h timepoints (B). Mean ± SEM, *n* = 20, High-fit = 7, Lower-fit = 13.

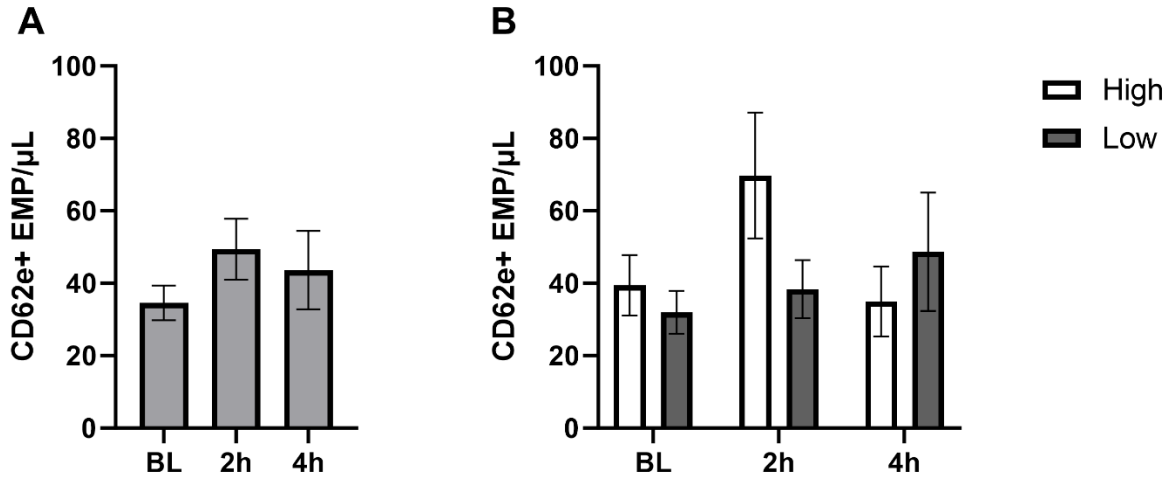

Figure 8. Circulating activated CD62e+ endothelial MP counts between high-fat meal BL, 2h, and 4h time points (A). High-fit vs. lower-fit circulating activated CD62e+ endothelial MP counts between high-fat meal BL, 2h, and 4h timepoints (B). Mean ± SEM, n = 20, High-fit = 7, Lower-fit = 13.

Discussion

In response to consuming a high-fat meal, we found that MP number was not different between time points among all participants. However, postprandial HUVEC ROS production was significantly increased when all participants were analyzed together. Our exploratory analysis of differences in fitness status indicated MPs from young individuals with relatively low fitness levels induce higher amounts of ROS in endothelial cells, whereas MPs from high-fit individuals do not. Additionally, there appears to be a rise in MP counts at the 2h timepoint in high-fit individuals that resolves by 4h. This initial increase in MP number in the high-fit group seems to drive the apparent rise in the ability of total MPs to produce ROS, which also resolves by 4h. To our knowledge, this is the first study demonstrating differences in how a high-fat meal affects MP number and function, even in young healthy adults. While these subjects did not have signs of cardiovascular disease, individuals with a lower fitness status were differentially

affected by a high-fat meal, potentially indicating a greater susceptibility to diet-induced inflammation.

Over the past two decades, at least four studies have reported significant changes in either total or endothelial MP counts following one or more high-fat meals, while also assessing the effects of prior exercise.[5,65,67,69] However, none have examined MP function via ROS or ROS-producing capacity of total MPs. We observed a near-significant increase in the ROS-producing capacity of total MPs following the high-fat meal in the entire group, but our exploratory analyses indicated that this was predominately shown in the high-fit group at the 2h time point. The initial increase returned to a level near BL at the 4h timepoint, implying a relatively short duration of the response. Jenkins and colleagues reported a significant increase in intracellular ROS in circulating CD31+/CD14-/CD34- cells (which included circulating endothelial cells) postprandially at four hours among their participants with an average VO_{2max} of 48 mL/kg/min[5], which was similar to our high-fit group. Because increases in intracellular ROS in circulating CD31+/CD14-/CD34- cannot be solely attributable to circulating MPs, and because of the *in vivo* vs. *in vitro* natures of the present and former studies, the results are not directly comparable. However, both studies support the notion that a high-fat meal increases ROS in endothelial cells, with the present study indicating that this may be at least partially attributable to MPs. A longer postprandial time course with sampling of endothelial cells and MPs at multiple time points may be needed to deduce the temporal response in high-fit individuals and whether they truly "recover" faster from the inflammatory stimulus. There is previous evidence that our high-fat meal can induce elevated postprandial IL-6 levels in circulation.[79] Brandauer and colleagues found that the high-fat meal led to increases in this cytokine in a group of physically active men who were similar in characteristics to the men in our study.[79] While the present study did not

measure levels of inflammatory cytokines after the high-fat meal, we can theorize that a similar induction of proinflammatory cytokines occurred in our participants.

In the lower-fit group, MPs postprandially led to increased endothelial cell ROS production. Both total MP counts and ROS producing capacity of total MPs tended to increase from BL to 4h, but neither were significant. Although we observed robust results in HUVEC ROS production in response to MP incubation among the lower-fit group, this group may display a slower rise in ROS-producing capacity of total MPs that only a longer postprandial time course could reveal. Few studies have examined changes in MP subsets following consumption of a high-fat meal, and to our knowledge only one has specifically reported results for total MPs. Tushuizen and colleagues showed a significant increase in total MP counts postprandially over the span of 8 hours in recreationally active adult males compared to a fasted control visit.[65] That study included two consecutive high-fat meals versus only one in the present study. This serves as a key difference between the two, as a much larger inflammatory stimulus was given to the participants. However, studying the consumption of two consecutive high-fat meals may not be as applicable to the dietary habits of the public.

Circulating total MP counts in the lower-fit group showed only a tendency to increase from BL to 4h, without statistically significant changes. Lower-fit individuals did exhibit a significant increase in endothelial cell ROS production from BL to 2h and BL to 4h, suggesting that MP cargo may have been altered regardless of seeing no change in MP number. The rise in endothelial cell ROS among all participants appears to be driven by the postprandial MPs of this lower-fit group. Therefore, MPs of lower-fit individuals appear to functionally become more inflammatory following a high-fat meal, regardless of there being no change in circulating MP counts. While this may suggest that MP number is not relevant, it is important to note that MP

number can often be indicative of endothelial cell activation and apoptosis.[6] MP quantification is also important to determine their origins and stimulus of release. In addition, MP numbers in the present study were needed to determine MP counts that were added to endothelial cells for measurement of ROS production. Although we did not see changes in MP number in the lower-fit group, their counts are still an important factor, and they are required to study MP function.

The high-fit group's circulating MP counts showed a numerical increase post-prandially from BL to 2h. Similar to the response shown in the ROS-producing capacity of total MPs, they significantly recovered by the 4h timepoint. Although the high-fit group exhibited a spike in circulating MP counts, it is important to note that endothelial cell ROS production caused by MP incubation remained unchanged following the high-fat meal. This may indicate a protective effect of their higher fitness status, diminishing detrimental changes in MP function following the high-fat meal. This possible mechanism has never been addressed in the literature.

Antunes and colleagues provided some rationale for a protective effect of fitness status, as the authors observed elevated IL-6 concentrations in high-fit individuals (mean VO_{2max} 58 mL/kg/min) post-exercise compared to lower-fit participants.[80] IL-6 can have both inflammatory and anti-inflammatory functions based on its stimulus of release.[80] The authors postulated that elevated IL-6 in fit individuals leads to the release of anti-inflammatory cytokines, introducing a protective mechanism mediated by fitness status. IL-6 is also known to upregulate lipolysis, which may have played a role in the present study.[81,82] These ideas have been previously discussed, and add an interesting connection to our study as elevated IL-6 is known to stimulate the release of MPs.[3,81,82] High-fit individuals in the present study experienced a slight postprandial rise in MP counts and ROS-producing capacity of total MPs which may have served as a stimulus that ultimately protected them from the inflammatory high-fat meal. There are

numerous other studies highlighting the benefits of regular aerobic exercise training on vascular function and related biomarkers.[63,64,83] Focusing on the results of the present study, our data suggest that circulating MPs of high-fit individuals may quickly recover from the inflammatory effects of a high-fat meal.

The lower-fit group's fitness status may amplify the proinflammatory effects that the high-fat meal has on their MPs. While this notion has not been directly explored, previous studies do offer supporting evidence. Interestingly, Baugh and colleagues report that endurance-trained individuals show increased skeletal muscle fat oxidation following a 5-day high-fat diet compared to sedentary individuals.[84] This may offer some explanation relevant to the present study regarding why MPs from lower-fit individuals elicited increased HUVEC ROS production compared to their high-fit counterparts. The high lipid load introduced by the high-fat meal causes increased oxidative stress and subsequent ROS production. However, if the high-fit individuals were able to metabolize this load faster than the lower-fit individuals, as suggested by Baugh and colleagues, they would seemingly escape much of the inflammation caused by the high-fat meal.[84] Therefore, MPs from lower-fit individuals may be more likely to induce elevated HUVEC ROS production caused by the high lipid load compared to high-fit individuals. A more recent study found a similar result in male rats following a 6-week high-fat diet paired with exercise training. The authors highlighted that exercise-trained rats were able to maintain their skeletal muscle fatty acid oxidation compared to those who only consumed the high-fat diet.[85] The authors postulated that exercise training protected those rats from an inflammatory fatty acid buildup caused by the high-fat diet.[85] Therefore, high-fit individuals may be protected from fatty acid buildup and subsequent inflammation caused by a high-fat diet. The idea of increased fat oxidation in high-fit individuals may serve as another possible explanation relevant to the present

study. Our data on lower-fit individuals suggests that they may be more susceptible to the proinflammatory effects of a high-fat meal, displayed via MP function. In contrast, increased aerobic fitness may be protective due to characteristics leading to increased fat metabolism.

Endothelial MPs:

While the numeric trends among endothelial MPs mirrored those of total MPs, there were no significant differences observed within the activated or apoptotic populations. The CD31+42b- (apoptotic) endothelial MPs were numerically higher in the lower-fit individuals at 2h and 4h compared to baseline. A longer time course following the high-fat meal may have led to significant differences in this population. This was indeed the case in a study by Harrison and colleagues, which observed a significant increase in endothelial MPs at the 6-hour postprandial timepoint compared to baseline.[67] However, a study from Ferreira and colleagues did report a significant increase in endothelial MPs at 3 hours postprandially compared to baseline. In contrast, Jenkins and colleagues did not observe significant differences in endothelial MP counts at four hours following a high-fat meal, with or without prior exercise.[5] Although there is some discrepancy between our results and those of previous studies, it is important to note that endothelial MP isolation methods continue to improve. Notably, the addition of annexin-V staining as a pan-MP marker is now commonplace. Annexin-V is known to bind phosphatidylserine externalized by apoptotic cells, which is one of the main mechanisms by which MPs are released.[6,86] None of the previous studies examining changes in endothelial MPs following a high-fat meal included this stain, which is important for distinguishing between MPs and cells or cellular debris.[5,65,67,69] Our results may display a "cleaner" endothelial MP count free from debris, which could have inflated numbers from previous studies.

Strengths and limitations of the study:

This study is the first to observe changes in MP function following a high-fat meal. Others have examined circulating total and endothelial MP counts in response to a high-fat meal, but none previously have assessed function or ROS-producing capacity. As such, our findings may allow future studies to look deeper into mechanisms behind postprandial changes in MP function. The present study also utilized updated methods for isolating and reporting of MPs compared to previous high-fat meal studies. The combination of Annexin-V with other endothelial-specific stains allows for a more accurate count via flow cytometry that is free from other cells or debris. Previous studies lacked a stain for Annexin-V, which is now considered to be a classic marker for MPs.[5,6,69,86]

The present study was not without limitations. Due to our relatively small sample size, the exploratory analysis of group differences based on fitness status was likely underpowered. Therefore, future studies should include larger sample sizes when studying differential effects of fitness status on MPs with postprandial inflammation. In addition, some previous studies have continued collecting blood samples longer than four hours following a high-fat meal. Indeed, two previous studies have found significant changes in vascular function and endothelial MP counts among recreationally active individuals post-prandially at 6 hours.[65,67] The lower-fit individuals in the present study may have undergone a delayed response in this regard and could have displayed increased MP counts with a longer time course. Also, data from the present study were non-normally distributed. This may be attributed to variations between participants' MP counts obtained via flow cytometry. Trends within the data were relatively consistent across the cohort, but the variance did impact our statistical analysis approach. The extremely small size of MPs may play a role in this issue, as MP quantification techniques can still be improved. Finally, we

were unable to use only endothelial MPs when incubating MPs with HUVECs and instead utilized total MPs for those experiments. As such, we cannot rule out the inclusion of other MP subsets as well as the influence this may have had on our results. Our approach may not specifically address the question of how *endothelial* MPs affect HUVEC ROS production following a high-fat meal. However, the utilization of isolated endothelial MPs for this experiment would pose logistical issues as so few are available in each 10 mL blood sample. However, in using total MPs for this experiment, we created an environment that was perhaps more indicative of what occurs in circulation. Indeed, our data may provide a more complete picture of how MP function is affected by a high-fat meal.

Conclusions:

In this study, we have demonstrated that lower-fit individuals undergo a significant change in circulating MP function following a high-fat meal compared to their high-fit counterparts. High-fit individuals appear to be somewhat protected from the proinflammatory changes that accompany a high-fat meal, but this requires further research. Functional changes occurred in both groups regardless of any changes in circulating MP counts, which may indicate that MP function could be determined by fitness status as well as other factors. As such, these mechanistic insights help broaden our understanding of how MPs are affected by diet and exercise along with other inflammatory perturbations. Our findings may also contribute to the use of MPs as a novel biomarker of vascular inflammation and accompanying disease.

Chapter 3: The effect of different aerobic exercise intensities on circulating microparticle counts and function

Introduction

It is well-known that habitual aerobic exercise plays a role in preventing the onset and progression of cardiovascular disease. This stems from the inherent benefits aerobic exercise can have on the vasculature.[87,88] In contrast, a decline in vascular health leads to the emergence of conditions associated with the progression of cardiovascular disease, such as atherosclerosis and hypertension.[61,72,87] Overweight/obese individuals are at greater risk of developing these conditions due to chronic systemic inflammation.[89] Consequently, overweight/obese individuals experience increased aortic stiffness and higher systolic blood pressures than lean.[90–92] There is growing evidence that high-intensity interval training (HIIT) workouts can elicit benefits to vascular health that last long after the cessation of exercise.[88] Furthermore, HIIT has been shown to be beneficial for overweight/obese individuals as it helps them build aerobic fitness while decreasing percent body fat.[93] However, overweight and obese individuals are also known to be in a state of low-grade inflammation, displaying chronically elevated IL-6 and TNF-α.[94] HIIT workouts can lead to a similar response, increasing IL-6 and TNF-α.[95] As such, overweight and obese individuals performing HIIT workout may be exposed to a greater amount of inflammation compared to lean individuals. It is therefore important to explore the mechanisms through which this occurs in both overweight and lean individuals.

Although it is quite clear how aerobic exercise leads to vascular health benefits, some of the mechanisms through which this happens remain unclear. The continuous release/uptake of microparticles (MPs) by cells throughout the vasculature may be involved in the influence that exercise can have on vascular health.[6] MPs are a subset of extracellular vesicles that is associated

with cell-to-cell communication.[6,29] Endothelial MPs specifically are known to be released by endothelial cells in response to cellular activation of the immune response as well as apoptosis. They are subsequently taken up by target cells and release various cargo such as endothelial-derived proteins, cell adhesion molecules, and miRNAs.[6,29] As such, it is thought that their function is determined by their stimulus of release. In the context of cardiovascular disease, levels of circulating endothelial MPs are associated with inflammation as well as functional changes within the endothelium.[6,62]

Endothelial MPs may also play a role in arterial stiffness. They are known to produce ROS in endothelial cells, which can lead to arterial stiffness.[3,56,96,97] Endothelial MPs contain NADPH subunits such as Nox1, Nox2, and Nox4 which in turn convert oxygen to superoxide, a type of reactive oxygen species (ROS).[56,57] They are known to transfer their cargo to target cells after they are taken up, meaning NADPH subunits would enter the cell and increase ROS production.[6,54] ROS then reacts with nitric oxide (NO) to produce peroxynitrite. Peroxynitrite can increase apoptotic signaling as well as cell adhesion molecule expression.[51,52] ROS is also known to increase collagen synthesis through activation of matrix metalloproteinase (MMP), which disrupts collagen metabolism leading to extracellular matrix remodeling and subsequent arterial stiffness.[97]

Endothelial MPs have also been implicated in the onset of atherosclerosis, as they feed into the thrombin-producing pathway which contributes to the inflammatory procoagulant response to atherogenic plaque buildup.[3,39] This in part is due to their expression of tissue factor, which helps convert factor X to factor Xa and in turn assists in the production of thrombin.[3] Thrombin plays a crucial role in the coagulation cascade in breaking fibrinogen into fibrin, which connects platelets allowing coagulation to occur.[3,98] As such, endothelial MPs are known

to be elevated in individuals with hypertension.[99,100] Increased coagulant activity, specifically related to fibrin production, has been associated with the development of hypertension due to greater plaque buildup.[101] In addition, elevated ROS production contributes to the development of hypertension via its role in collagen metabolism as well as its ability to stimulate vascular hypertrophy.[102] Both of these mechanisms suggest that endothelial MPs play a role in the development of hypertension and warrant further study of how they relate to systolic blood pressures.

An acute bout of aerobic exercise can lead to upregulation of anti-inflammatory pathways as well as many other benefits.[29,87,88] Acute exercise increases production of superoxide dismutase (SOD), which scavenges excess ROS.[60] While this is a well known mechanism, it is unclear how this is communicated throughout the vasculature. Indeed, there are numerous pathways that may be involved in the ability of exercise to promote vascular health. MPs may play a role in this regard. The relationship between circulating MPs and various intensities of acute aerobic exercise has received some attention in the literature. Indeed, Serviente and colleagues reported that after healthy adults performed 30 minutes of moderate-intensity aerobic exercise there was a 45% decrease in total circulating MPs, as well as a reduction in CD62e+ (activated) and CD31+/CD42b- (apoptotic) endothelial MPs by 34% and 44%, respectively.[59] This shows that even moderate intensity exercise can affect the MPs of healthy individuals. Wahl and colleagues also reported significant reductions in endothelial MPs and platelet MPs 60 minutes after various types of HIIT workouts in highly trained participants.[62] Others have observed reductions in endothelial MP counts following acute aerobic exercise in both overweight and lean adults with both moderate and high intensities.[29,61]

There is mounting evidence that acute aerobic exercise can have a significant effect on circulating MP counts. However, only one other study has assessed changes in MP function in response to exercise. Wahl and colleagues reported reductions in endothelial cell apoptotic activity following incubation with endothelial MPs and serum from both pre- and post-exercise time points[62]; however, this potential mechanism requires further exploration. Endothelial MPs are known to induce ROS production in endothelial cells, and this may have significant effects on arterial stiffness over time,[56] but the ability of exercise to ameliorate these harmful processes remains unclear. In addition, it is unknown how different types of exercise may affect these mechanisms in individuals with different body compositions. This may have significant implications on MPs being known as markers of the previously undetectable early onset of chronic low-grade inflammation leading to cardiovascular disease. As such, the purpose of this study was to determine how different energy-matched bouts of acute aerobic exercise affect circulating MP counts, their ability to stimulate endothelial oxidative stress *in vitro*, and central/peripheral blood pressures as well as arterial stiffness in overweight or lean, recreationally active, healthy adults. We hypothesized that (1) both HIIT and moderate-intensity continuous training (MICT) would result in decreased circulating MP counts, (2) that following HIIT, MICT, or both, overweight individuals would undergo a smaller decrease in circulating MP numbers from baseline compared with lean participants, and (3) that endothelial cells incubated with MPs isolated following both HIIT and MICT would exhibit decreased ROS production compared to samples collected after the control visit.

Methods

Ethical Approval:

All human subject research performed in this study received prior approval from the University of Maryland Institutional Review Board (IRB). Written informed consent was obtained from each participant, and all procedures conformed to the standards set by the latest revision of the Declaration of Helsinki.

Participants:

Twenty participants were recruited for the study, consisting of adult men and women aged 18-35 years. Ten participants were classified as overweight or obese (Class I) by a body mass index (BMI) range of 25.0-34.9 kg/m^2. The other 10 participants were classified as lean based on a BMI range of 18.0-24.9 kg/m^2. All participants were recreationally active. Participants were non-smokers with no history of cardiovascular disease or diabetes mellitus. Female participants were verified as not being pregnant but menstrual cycle was not monitored.

Study Design:

The study utilized a randomized, crossover design with a total of 4 visits each separated by at least one week. Visit 1 was approximately 60 minutes in duration. Study staff obtained informed consent and verified that participants had fasted overnight. Participants completed health history and physical activity questionnaires, as well as body composition measurements and a peak aerobic power test. During visits 2-4, participants performed either 30 minutes of acute aerobic exercise (HIIT or MICT) on a cycle ergometer, or a 30-minute bout of sitting to serve as a control. The order of these visits was randomized between participants. Blood samples

as well as radial augmentation index and carotid/radial blood pressures were collected before and twice (15 and 60 minutes) after exercise/sitting.

Body Composition:

Body composition was assessed via air displacement plethysmography (BOD POD, COSMED, Concord, California) to measure fat mass and fat-free mass. Participants sat in a body-sized capsule while wearing a swimming cap and bathing suit (or other tight-fitting clothing). The door to the capsule was shut for approximately 1 minute for each reading and 2-3 readings were taken. All measurements were administered by trained study staff.

Peak Aerobic Power:

During visit 1, participants also performed a graded exercise test on a cycle ergometer (Monark Exercise AB, Sverige, Sweden) to determine peak aerobic power. Participants' rating of perceived exertion (RPE: Borg 6-20 scale) and heart rates (chest-strap or wrist heart rate monitor) were monitored throughout. Following a 3-minute warm-up with a resistance of 1 kg, resistance increased by either 0.3 or 0.6 kg for each 2-minute stage. Participants were instructed to maintain 70-80 revolutions per minute (rpm) during the test. The test was terminated when the participant could no longer maintain the required speed. This point was determined via communication with the participant and by their physiological data. A brief 3-minute cool-down period followed at a resistance of 1 kg. The resistance against which participants were pedaling during their last stage was utilized in determining peak aerobic power. Peak aerobic power results from the test were used to determine loads for acute bouts of high-intensity interval training (HIIT) and moderate-intensity continuous training (MICT) in subsequent visits.

Acute Exercise:

The duration of each of these visits was approximately 120 minutes. Following a ≥8-hour, overnight fast, subjects came to the lab to complete either HIIT, MICT, or sitting in a randomized order determined at the time of enrollment. During each bout of exercise, participants' heart rate, rating of perceived exertion, and cycle ergometer power output were recorded. The acute bout of HIIT was performed on a cycle ergometer (Monark Exercise AB, Sverige, Sweden) and consisted of a 3-minute warm-up, followed by 10x 1-minute intervals at 90% of the participant's peak aerobic power with 2 minutes of active recovery (50% of peak aerobic power) between each. Participants then completed a 3-minute cool-down. Participants completed a single bout of MICT on a cycle ergometer (Monark Exercise AB, Sverige, Sweden). The session consisted of a 3-minute warm-up, followed by cycling at 60% of peak aerobic power for 30 minutes. A 3-minute cool-down followed. Blood draws and measures of vascular function were performed before and after this session. The sitting session required the participant to sit quietly in a chair for 30 minutes while refraining from making sudden movements or performing extraneous behavior. Blood draws and measures of vascular function were performed before and 15 and 60 minutes after the completion of each 30-minute session.

Blood Sampling:

Participants underwent blood sampling during visits 2-4. Participants had ~10mL drawn at baseline, 15 minutes after each condition, and 60 minutes after each condition. Blood was drawn by venipuncture using aseptic technique and collected in acid citrate dextrose tubes (BD Vacutainer). Plasma was isolated from the sample for laboratory assessments of MP concentration and function.

Measurements of Augmentation Index and Blood Pressure:

Participants' vascular health was evaluated via radial augmentation index as well as brachial, carotid, and radial blood pressures. Measurements were obtained while participants were laying in a supine position using a proprietary tonometer, which is part of a device called a Noninvasive hemodynamic workstation (NIHem) from Cardiovascular Engineering, Inc. (Norwood, MA, United States). Based on the obtained blood pressure waveforms, augmentation index (AIx) (a measurement of arterial wave reflection) was calculated. Augmentation index is a measurement of wave reflection that can be affected by arterial stiffness. Radial and brachial blood pressures are used to indicate pressures within the peripheral vasculature. Carotid blood pressure is typically utilized as a surrogate for central aortic blood pressure, which is considered to be a superior indicator of cardiovascular disease risk compared to brachial blood pressure.[103] Subsequently, using applanation tonometry, researchers held a tonometer that senses pulse waves on the participant's pulse at the right common carotid artery while the participant was supine. The tonometer was connected to the NIHem software and records the waveform of the pulse for offline analysis. This technique was repeated at the right common carotid artery and right radial artery, respectively. Measurements of vascular health were assessed at baseline, then again at 15 minutes and 60 minutes after each condition (HIIT, MICT, and sitting).

Microparticle Isolation:

Plasma samples from each time point were used to isolate EMPs via sequential centrifugation. Briefly, 500μL aliquots from each time point were centrifuged at 1500xg for 20 minutes at 20°C to obtain platelet poor plasma. The top two-thirds of the supernatant were then transferred to a new microcentrifuge tube and centrifuged again at 1500 x g for 20 minutes at

20°C to obtain platelet free plasma. 100µL of each sample was incubated with fluorescent monoclonal antibodies for quantification of each MP population, including annexin-V+ anti-annexin-V-violet 450 (v450) (BD, Franklin Lakes, NJ, USA, Cat#560508, RRID: Ab_2869356) which binds to phosphatidylserine, CD31+ anti-CD31-allophycocyanin (APC) (ThermoFisher Scientific, Waltham, MA, USA, Cat#47031942, RRID: Ab_10730582) for PECAM-1, CD42b-anti-CD42b-peridinin-chlorophyll-protein (PerCP) (ThermoFisher Scientific, Waltham, MA, USA, Cat#46042942, RRID: Ab_2762458) to remove platelets and CD62E+ anti-CD62E-phycoerythrin (PE) (BD, Franklin Lakes, NJ, USA, Cat#551145, RRID: Ab_394072) for e-selectin. MP sub-populations including Annexin-V+/CD31+/CD42b- and Annexin-V+/CD62E+ as well as Annexin-V+ for total MPs and Annexin-V+CD42b+ for PMPs were quantified via flow cytometry. Standardized 900 nm and 1000 nm nanobead NIST traceable particle size standards (Polysciences, Warrington, PA, USA) and CountBright absolute counting beads (ThermoFisher Scientific, Waltham, MA, USA) were used for proper quantification of MPs. Data analysis was performed using FlowJo V10.1rs (FlowJo, LLC, Ashland, OR, USA).

Endothelial Cell Culture, Microparticle Incubation, and Intracellular ROS Quantification:

Pooled male and female human umbilical vein endothelial cells (HUVECs) (Lonza, Basel, Switzerland) were seeded in T75 flasks with 15 mL of endothelial growth medium 2 (EGM2) (Lonza, Basel, Switzerland), then passed into separate 96-well plates with 200 µL of EGM2 with 2% fetal bovine serum (FBS) per well once they had reached ~80% confluence. Once the HUVECs had reached ~80% confluence, 5,000 MPs (isolated from each time point and condition of each sample using the previously mentioned MP isolation technique) were added to

each well. This amount is similar to amounts previously utilized to quantify ROS production in endothelial cells.[56] HUVECs were incubated with MPs for 1 hour.

Following incubation with MPs, Cell ROX Green Reagent (Life Technologies, Carlsbad, CA, USA) was added to the cells at a concentration of 5 μM and incubated for 30 minutes at 37ºC. Following incubation, cell medium was removed, and each well was washed two times with 1x phosphate-buffered saline (PBS). Endothelial cell ROS production was quantified using plate fluorescence (Synergy H1 Hybrid Reader; Biotek, Winooski, VT, USA). Microscopic images of each well were captured following CellROX incubation using a Nikon Eclipse Ti-U microscope with DS-Ri2 camera (Nikon Instruments, Melville, NY, USA). Cell counts within each well were obtained using NIS-Elements AR 5.21.03 imaging software (Nikon Instruments, Melville, NY, USA) to compare ROS fluorescence among wells.

Statistical Analysis:

Following completion of data collection, a Shapiro-Wilk test of normality showed a non-normal distribution of microparticle count and functional data ($P < 0.05$). This was not resolved with log transformation; therefore, Wilcoxon signed-rank tests were used to analyze any differences between paired MP counts among time points. The Mann-Whitney U test was used to test between independent conditions as well as overweight and lean groups at specific timepoints. Unpaired t-tests were used to analyze any differences in participant characteristics between overweight and lean groups. Statistical significance was accepted at $P < 0.05$.

Results

Participant Characteristics:

Participant characteristics are summarized in Table 2. Weight, BMI, resting brachial systolic blood pressure (SBP), and resting brachial diastolic blood pressure (DBP) were all significantly higher in the overweight group compared to the lean group ($P < 0.005$ for all). Resting carotid and radial SBPs were significantly higher in overweight participants when compared to lean ($P < 0.05$ for both). There were no significant differences between groups for other participant characteristics.

Table 2. Participant characteristics

	Total	Overweight	Lean
Age	23 ± 0.98	23 ± 1.56	24 ± 1.31
Males	10	5	5
Females	10	3	7
Weight (kg)	69.54 ± 2.49	79.19 ± 2.57	63.10 ± 2.38*
BMI (kg/m^2)	24.84 ± 0.80	28.12 ± 0.99	22.65 ± 0.60*
% Body fat	20.88 ± 1.89	23.16 ± 3.43	19.35 ± 2.19
Peak Aerobic Power (W)	172.25 ± 7.48	176.25 ± 10.80	169.58 ± 10.49
Resting Brachial SBP (mmHg)	114.70 ± 1.24	118.75 ± 1.03	112.00 ± 1.52*
Resting Brachial DBP (mmHg)	71.80 ± 1.32	77.25 ± 0.67	68.17 ± 1.34*
Resting Carotid SBP (mmHg)	108.72 ± 2.21	116.17 ± 3.09	104.65 ± 2.18†
Resting Carotid DBP (mmHg)	81.40 ± 1.74	84.17 ± 2.71	79.89 ± 2.19
Resting Radial SBP (mmHg)	117.83 ± 2.70	126.94 ± 4.17	112.86 ± 2.50†
Resting Radial DBP (mmHg)	74.46 ± 1.32	78.14 ± 0.98	72.45 ± 1.71
Resting Radial AIX (mmHg)	-30.67 ± 3.74	-34.22 ± 3.22	-28.73 ± 5.55

Data are means ± SEM. BMI, Body mass index; SBP, Systolic blood pressure; DBP, Diastolic blood pressure; AIX, Augmentation index; MPs, Microparticles. MP counts and vascular measurements are represented as the mean baseline value of the averages from each participant across all 3 study conditions. *$p < 0.005$ between overweight and lean groups. †$p < 0.05$ between overweight and lean groups.

Circulating total microparticle counts:

When all participants were analyzed together, there was a tendency for the HIIT workout to increase circulating total MP counts from baseline (BL) to 60 minutes post-exercise (60P) by

128% (Figure 9, P = 0.0547), but there was no effect of sitting or MICT. Additionally, total MP counts were 97% higher at the 60P time point after the HIIT workout compared to MICT (Figure 9, $P < 0.05$).

When participants were separated into overweight and lean groups (Figure 10), we observed a 146% increase in total MP counts in the lean group following the HIIT workout from BL to 60P and an increase of 86% from 15 minutes post-exercise (15P) to 60P ($P < 0.05$ for both), but no statistically significant changes after other conditions or in other groups. When total MP counts were compared between the overweight and lean groups, the BL values of the lean group appeared to be more variable across conditions and were significantly higher than those of the overweight group before the MICT and sitting sessions ($P < 0.05$ for both).

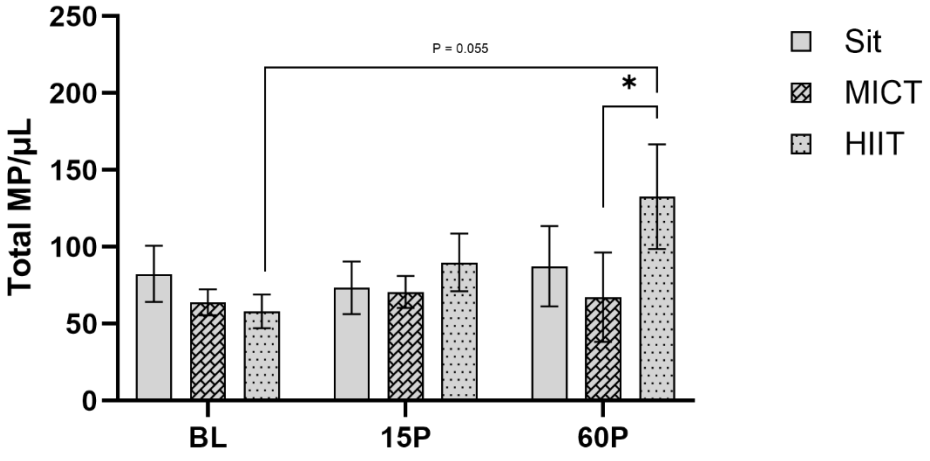

Figure 9. Total MP counts in response to high-intensity interval training (HIIT), moderate-intensity continuous training (MICT), and sitting (Sit) at baseline (BL), 15 minutes post-exercise (15P), and 60 minutes post exercise (60P) time points. Mean ± SEM, n=20. *p < 0.05.

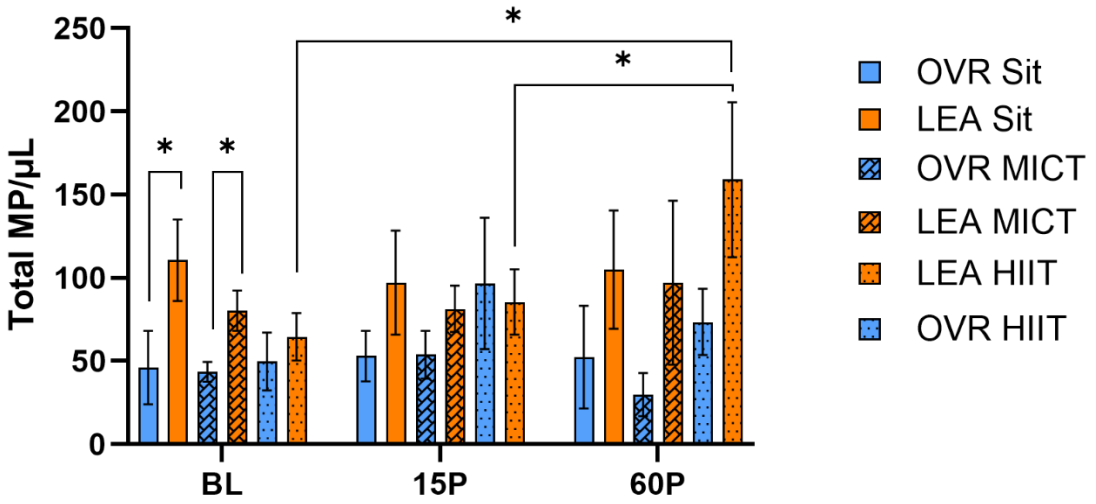

Figure 10. Overweight and lean group total MP counts in response to high-intensity interval training (HIIT), moderate-intensity continuous training (MICT), and sitting (Sit) at baseline (BL), 15 minutes post-exercise (15P), and 60 minutes post exercise (60P) time points. Mean ± SEM, n=20. *p < 0.05.

Circulating Platelet MP counts:

There was a significant effect of the HIIT workout to increase circulating platelet MP counts by 75% among all participants from BL to the 60P timepoint (Figure 11, $P < 0.05$). Additionally, platelet MP counts were 75% higher at the 60P time point after the HIIT workout compared to the MICT workout (Figure 11, $P < 0.05$). There were no other significant differences between exercise intensities or time points when all participants were analyzed together. When analyzing differences within and between the overweight and lean groups, platelet MP counts increased by 81% in the lean group following the HIIT workout from BL to 60P (Figure 12, $P < 0.05$). Additionally, platelet MP counts at BL in the lean group were 94% higher than those in the overweight group before the sitting session (Figure 12, $P < 0.05$).

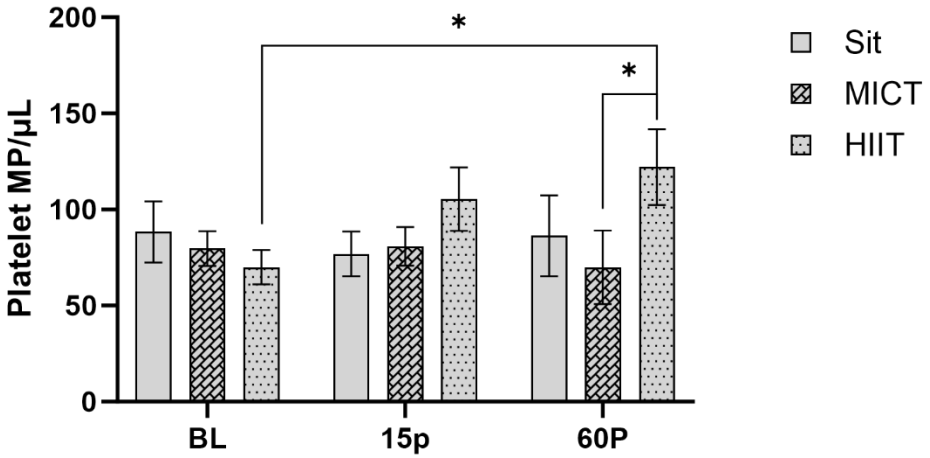

Figure 11. Platelet MP counts in response to high-intensity interval training (HIIT), moderate-intensity continuous training (MICT), and sitting (Sit) at baseline (BL), 15 minutes post-exercise (15P), and 60 minutes post exercise (60P) time points. Mean ± SEM, n=20. *p < 0.05.

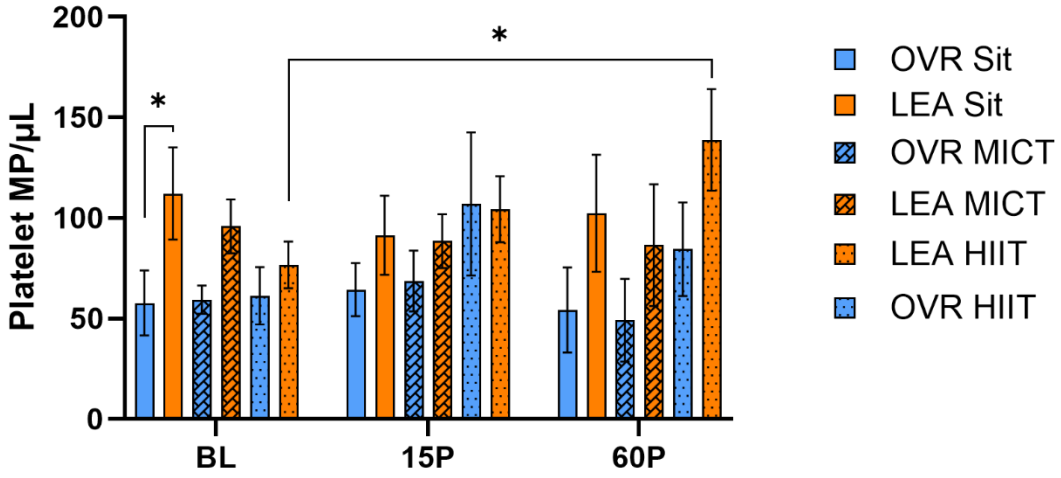

Figure 12. Overweight and lean group platelet MP counts in response to high-intensity interval training (HIIT), moderate-intensity continuous training (MICT), and sitting (Sit) at baseline (BL), 15 minutes post-exercise (15P), and 60 minutes post exercise (60P) time points. Mean ± SEM, n=20. *p < 0.05.

Circulating CD62e+ (activated) endothelial MPs:

CD62e **(**activated) endothelial MP counts were 97% higher at the 60P time point following the HIIT workout compared to the MICT workout (Figure 13, P < 0.05). There were

no other significant differences between exercise intensities or time points among total participants.

CD62e endothelial MP counts in the lean group were 80% higher at the 60P time point following the HIIT workout compared to the MICT workout (Figure 14, $P < 0.05$). Additionally, CD62e endothelial MP counts at BL in the lean group were 112% higher than those in the overweight group before the sitting session ($P < 0.05$).

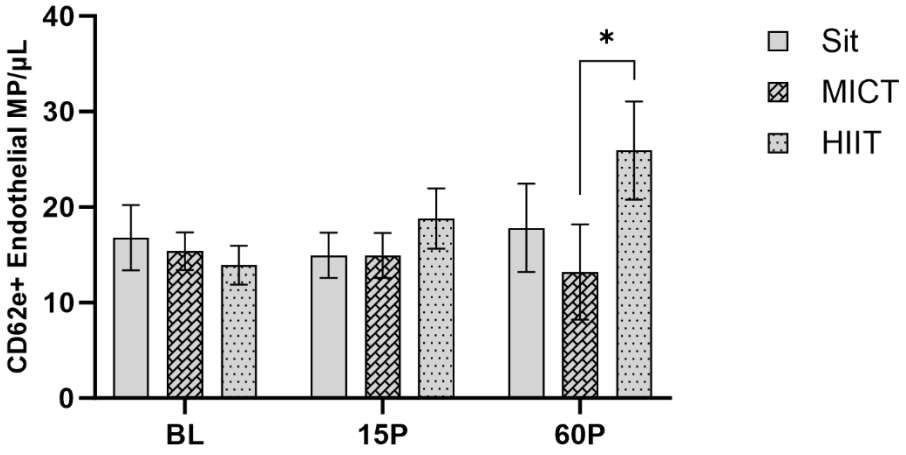

Figure 13. CD62e+ endothelial MP counts in response to high-intensity interval training (HIIT), moderate-intensity continuous training (MICT), and sitting (Sit) at baseline (BL), 15 minutes post-exercise (15P), and 60 minutes post exercise (60P) time points. Mean ± SEM, n=20. *p < 0.05.

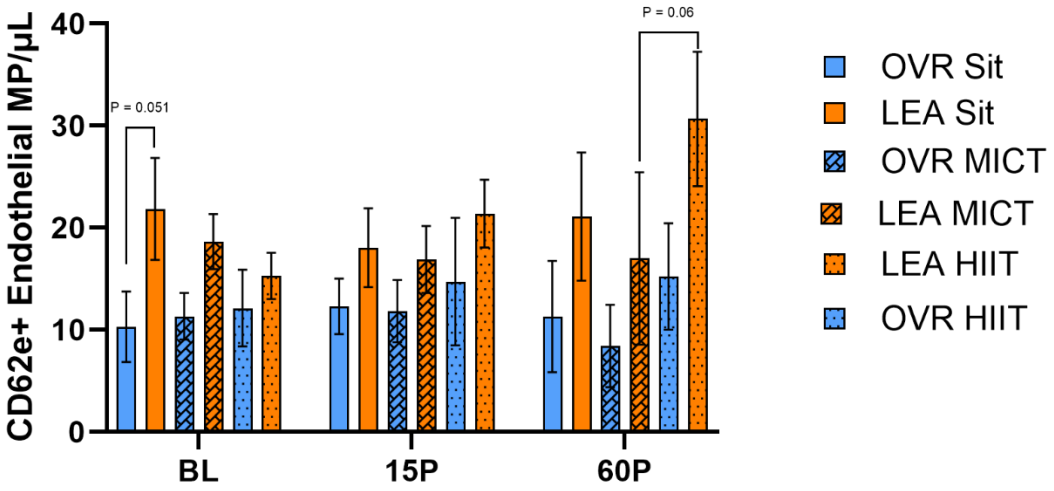

Figure 14. Overweight and lean group CD62e+ endothelial MP counts in response to high-intensity interval training (HIIT), moderate-intensity continuous training (MICT), and sitting (Sit) at baseline (BL), 15 minutes post-exercise (15P), and 60 minutes post-exercise (60P) time points. Mean ± SEM, n=20. *p < 0.05.

Circulating CD31+/CD42b- (apoptotic) endothelial MPs:

CD31+/CD42b- (apoptotic) endothelial MP counts were 103% higher 60 minutes after the HIIT workout compared to MICT (Figure 15, P < 0.05). There were no other significant differences between exercise intensities or time points among total participants.

In the lean group, the 54% increase in CD31+/CD42b- MP counts 60 minutes after the HIIT workout approached significance compared with the 15-minute timepoint (Figure 16, P = 0.0547). In addition, the lean group's average BL values were 103% higher than their overweight counterparts at the MICT session and 222% higher at the sitting session (P < 0.05 for both).

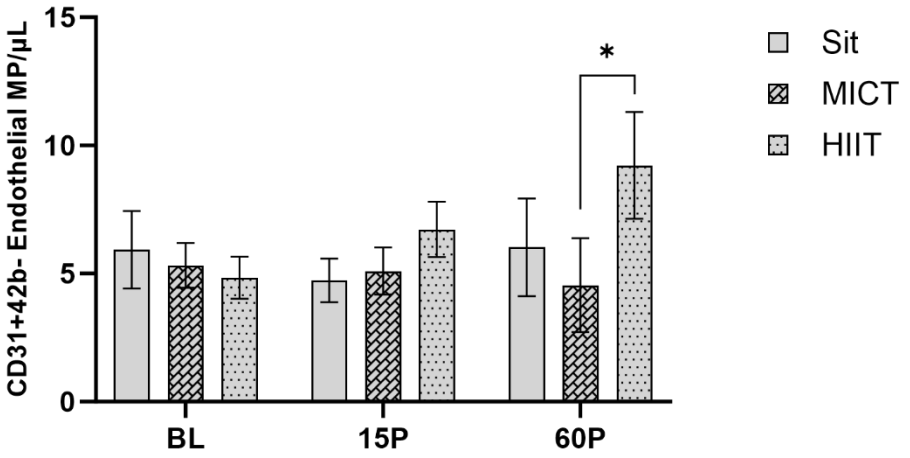

Figure 15. CD31+/CD42b- endothelial MP counts in response to high-intensity interval training (HIIT), moderate-intensity continuous training (MICT), and sitting (Sit) at baseline (BL), 15 minutes post-exercise (15P), and 60 minutes post-exercise (60P) time points. Mean ± SEM, n=20. *p < 0.05.

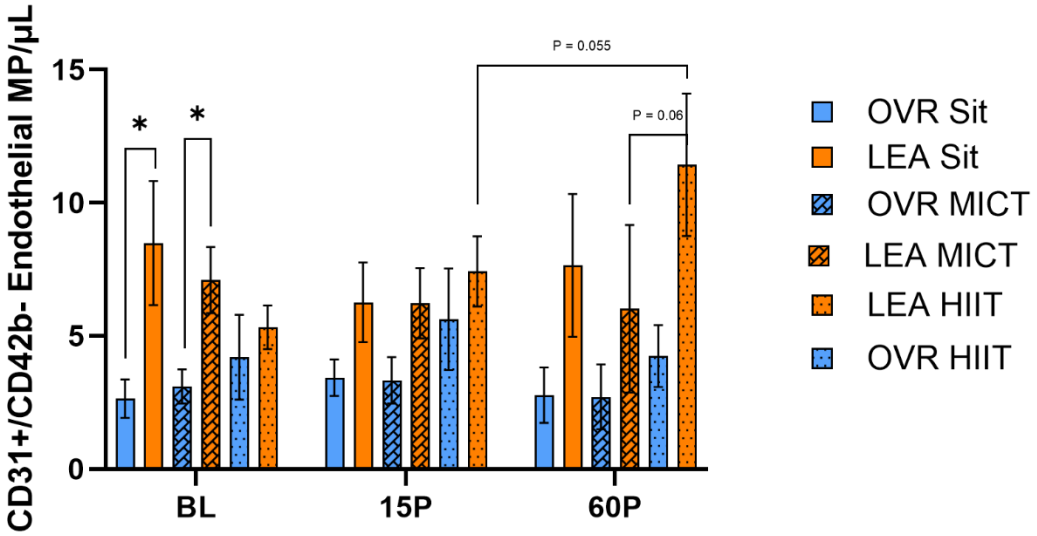

Figure 16. Overweight and lean group CD31+/CD42b- endothelial MP counts in response to high-intensity interval training (HIIT), moderate-intensity continuous training (MICT), and sitting (Sit) at baseline (BL), 15 minutes post-exercise (15P), and 60 minutes post- exercise (60P) time points. Mean ± SEM, n=20. *p < 0.05.

Endothelial Cell ROS production after MP incubation:

Following incubation with MPs from the three time points of each exercise intensity, there were no significant differences in HUVEC ROS production when including all participants (Figure 17). Fifteen minutes after the HIIT workout, incubation with MPs from overweight participants led to HUVEC ROS production that was 83% higher than sitting and 70% higher than the lean group at the same timepoint post-HIIT (Figure 18, $P < 0.05$ for both). Among lean participants, HUVEC ROS production decreased by 22% in response to the HIIT workout from BL to 15P ($P < 0.05$).

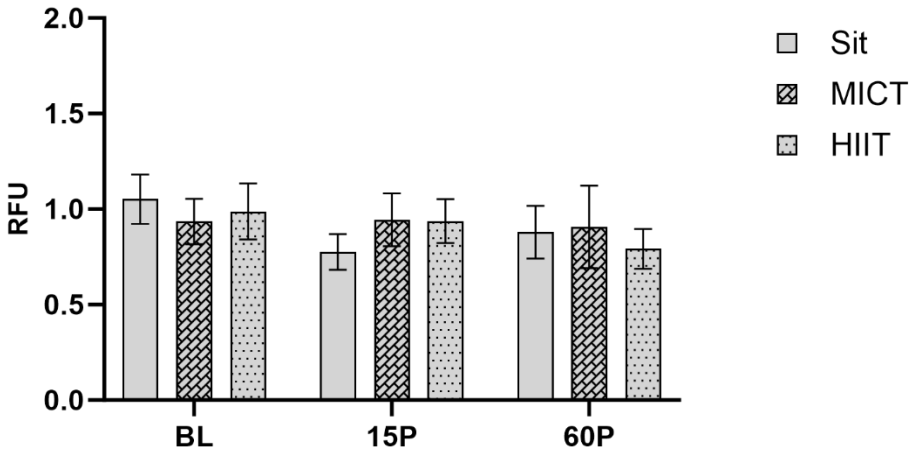

Figure 17. HUVEC ROS production following 1-hour incubation with MPs isolated from high-intensity interval training (HIIT), moderate-intensity continuous training (MICT), and sitting (Sit) at baseline (BL), 15 minutes post-exercise (15P), and 60 minutes post exercise (60P) time points. Mean ± SEM, n=20.

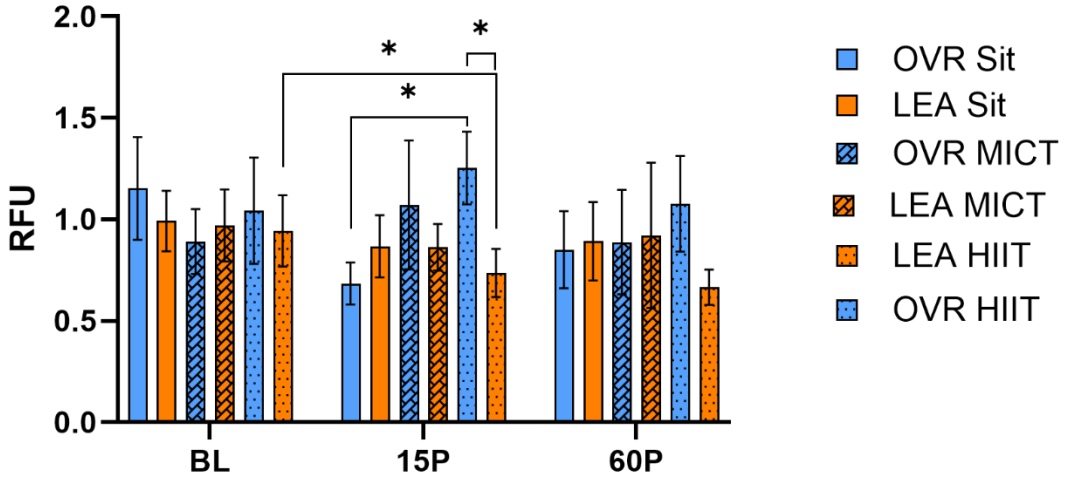

Figure 18. Overweight and lean group HUVEC ROS production following 1-hour incubation with MPs isolated from high-intensity interval training (HIIT), moderate-intensity continuous training (MICT), and sitting (Sit) at baseline (BL), 15 minutes post-exercise (15P), and 60 minutes post exercise (60P) time points. Mean ± SEM, n=20.

ROS-producing capacity of total MPs:

ROS-producing capacity of total MPs was calculated using previously obtained total MP counts combined with HUVEC ROS production data. While there were no significant differences in ROS-producing capacity between sessions or time points among total participants (Figure 19), there appeared to be differences between groups. Among overweight participants, ROS-producing capacity of total MPs approached a near-significant 131% increase following the HIIT workout from BL to 15P (Figure 20, P = 0.062). In addition, ROS-producing capacity following the HIIT workout was numerically higher at 15P compared to the sitting session (P = 0.0728).

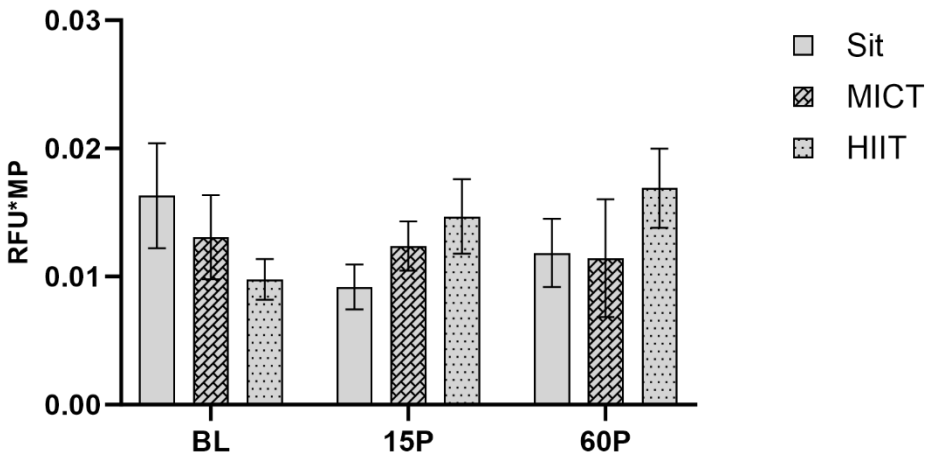

Figure 19. ROS-producing capacity of total MPs isolated from high-intensity interval training (HIIT), moderate-intensity continuous training (MICT), and sitting (Sit) at baseline (BL), 15 minutes post-exercise (15P), and 60 minutes post exercise (60P) time points. Mean ± SEM, n=20.

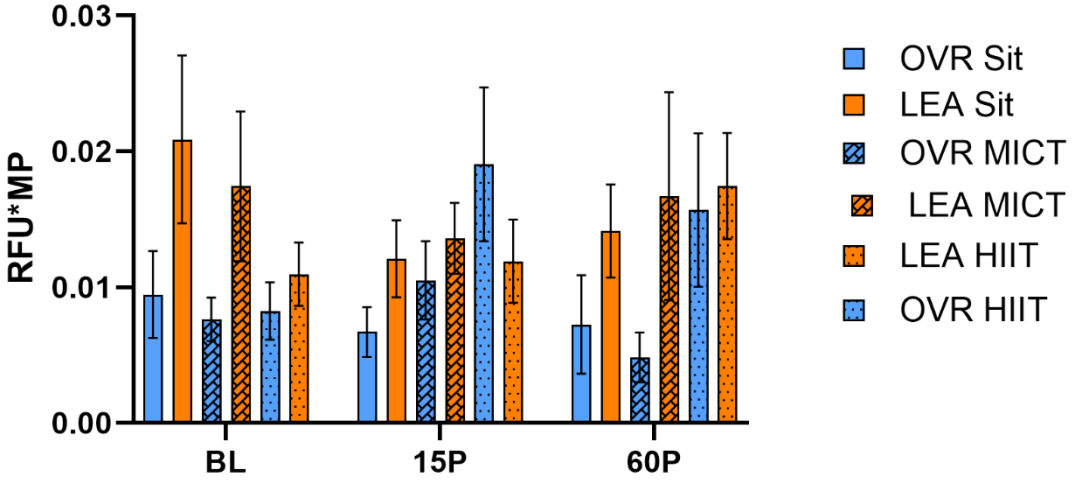

Figure 20. Overweight and lean group ROS-producing capacity of total MPs isolated from high-intensity interval training (HIIT), moderate-intensity continuous training (MICT), and sitting (Sit) at baseline (BL), 15 minutes post-exercise (15P), and 60 minutes post exercise (60P) time points. Mean ± SEM, n=20.

Measurements of Augmentation Index and Blood Pressure:

There were no significant effects of sitting, or either type of exercise on carotid SBP at 15 or 60 minutes after exercise among total participants (Figure 21). However, the overweight group experienced significantly higher carotid SBP 60 minutes following the HIIT workout compared to the lean group (Figure 22, $P < 0.05$). In addition, the overweight group showed higher carotid SBP 15 minutes and 60 minutes post-MICT compared to the lean group ($P < 0.05$ for both). There were no differences between overweight and lean participants in response to the sitting visit.

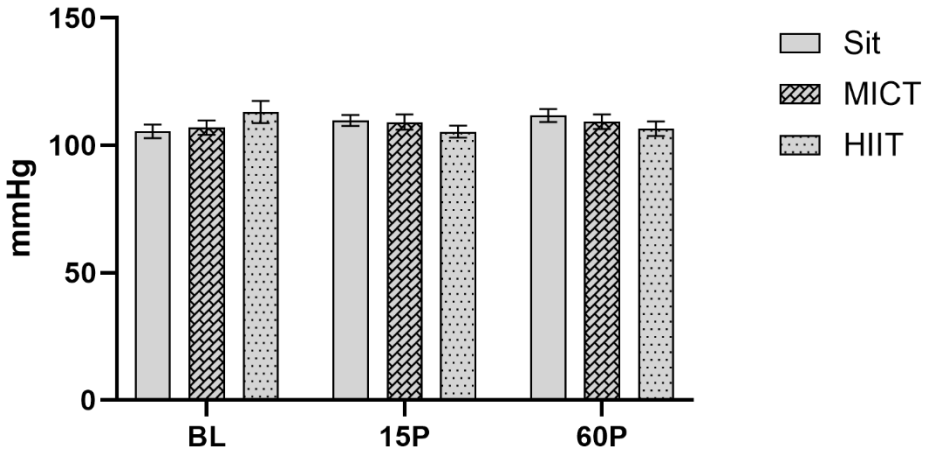

Figure 21. Carotid SBP in response to high-intensity interval training (HIIT), moderate-intensity continuous training (MICT), and sitting (Sit) at baseline (BL), 15 minutes post-exercise (15P), and 60 minutes post exercise (60P) time points. Mean ± SEM, n=20.

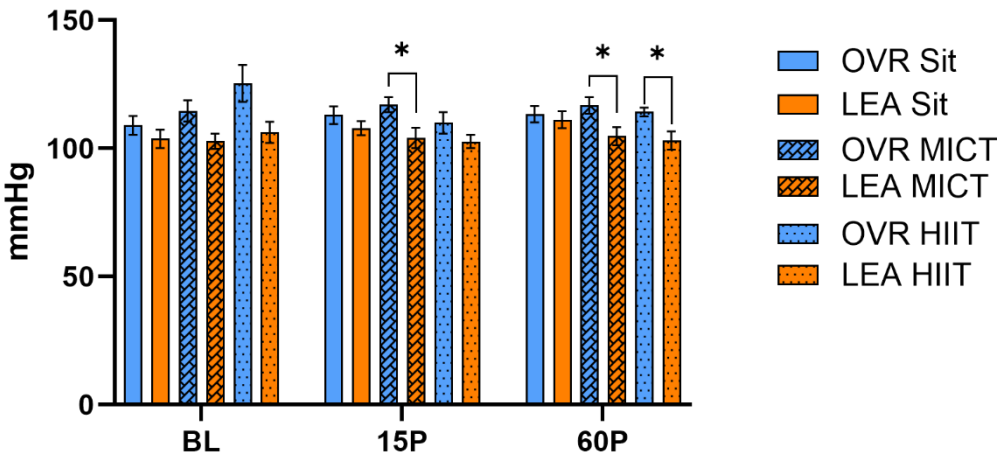

Figure 22. Overweight and lean group Carotid SBP in response to high-intensity interval training (HIIT), moderate-intensity continuous training (MICT), and sitting (Sit) at baseline (BL), 15 minutes post-exercise (15P), and 60 minutes post exercise (60P) time points. Mean ± SEM, n=8 for overweight, n=12 for lean.

At 15 minutes following the sitting visit, there was a significant increase in radial SBP among all participants (Figure 23, $P < 0.05$). This appeared to be driven by the lean group, but the interaction did not reach significance (Figure 24, $P = 0.059$).

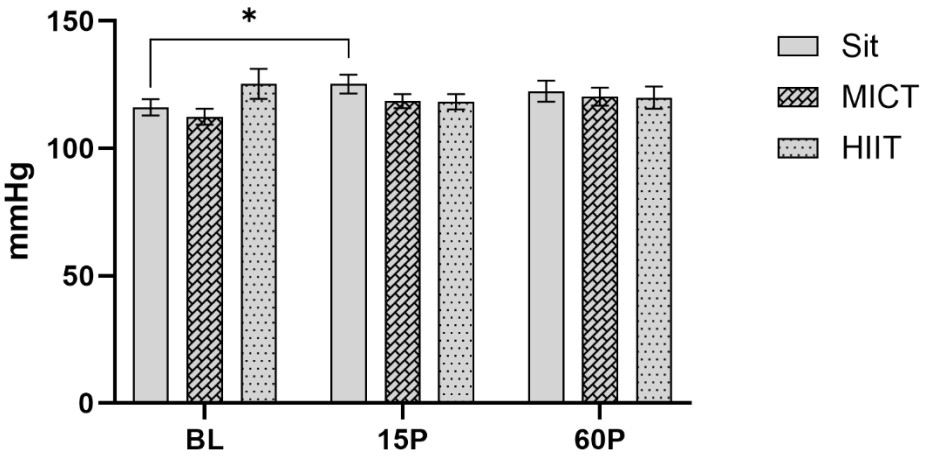

Figure 23. Radial SBP in response to high-intensity interval training (HIIT), moderate-intensity continuous training (MICT), and sitting (Sit) at baseline (BL), 15 minutes post-exercise (15P), and 60 minutes post exercise (60P) time points. Mean ± SEM, n=20.

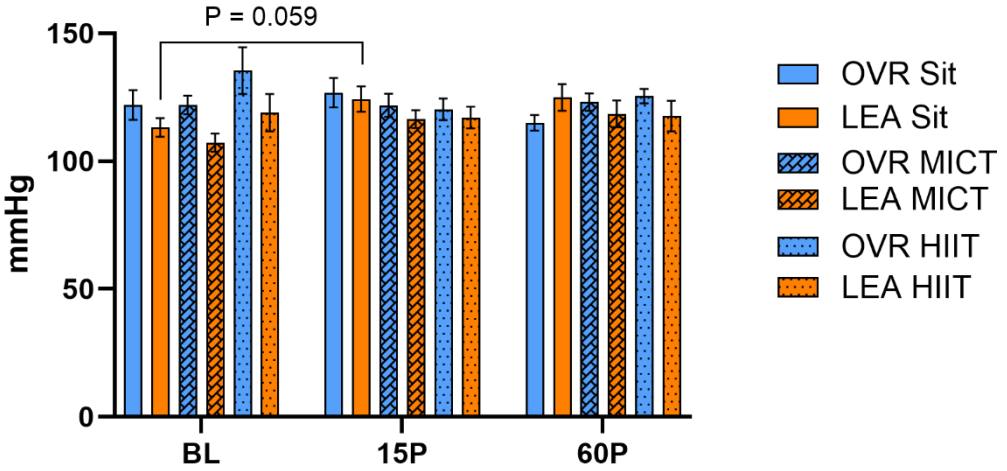

Figure 24. Overweight and lean group Radial SBP in response to high-intensity interval training (HIIT), moderate-intensity continuous training (MICT), and sitting (Sit) at baseline (BL), 15 minutes post-exercise (15P), and 60 minutes post exercise (60P) time points. Mean ± SEM, n=8 for overweight, n=12 for lean.

Among all participants, radial AIx was significantly lower at 15 minutes following the HIIT workout compared to MICT (Figure 25, $P < 0.05$). This difference appeared to be driven by the overweight group (Figure 26, $P < 0.05$).

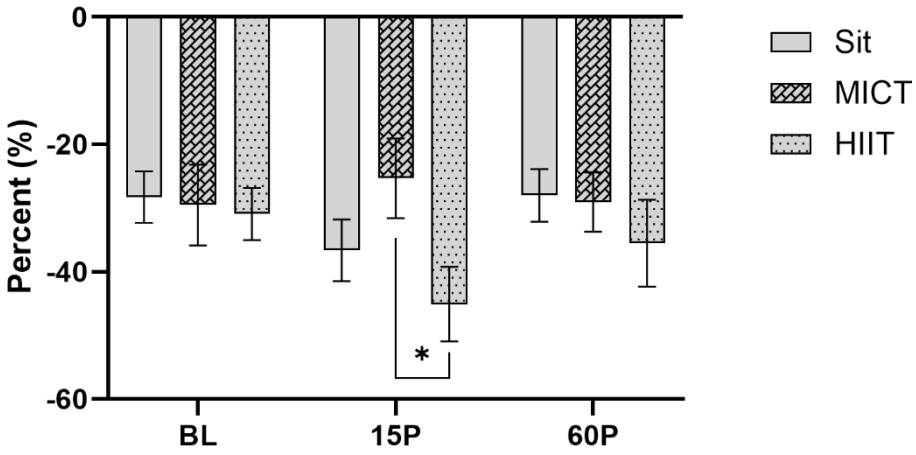

Figure 25. Radial AIx in response to high-intensity interval training (HIIT), moderate-intensity continuous training (MICT), and sitting (Sit) at baseline (BL), 15 minutes post-exercise (15P), and 60 minutes post exercise (60P) time points. Mean ± SEM, n=20.

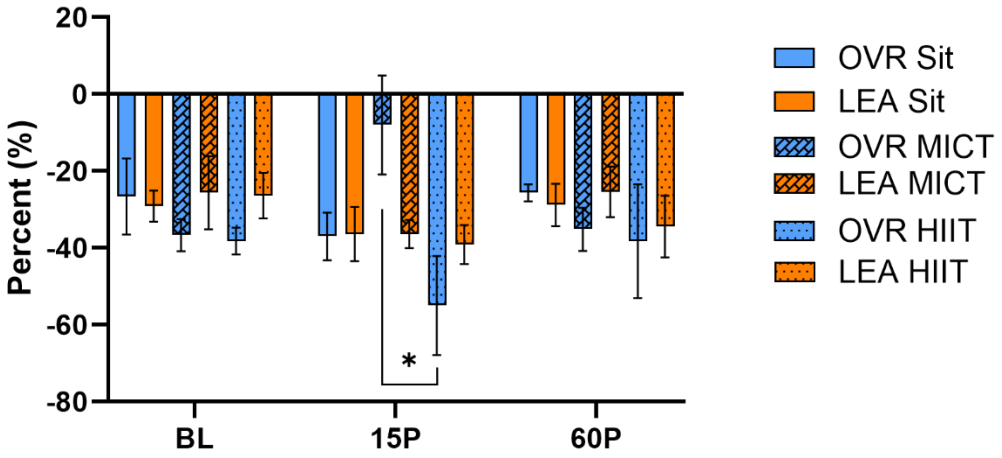

Figure 26. Overweight and lean group Radial AIx in response to high-intensity interval training (HIIT), moderate-intensity continuous training (MICT), and sitting (Sit) at baseline (BL), 15 minutes post-exercise (15P), and 60 minutes post exercise (60P) time points. Mean ± SEM, n=8 for overweight, n=12 for lean.

Discussion

Just 15 minutes after a HIIT workout, MPs from overweight individuals induced higher levels of ROS in endothelial cells compared to sitting. The higher concentrations of ROS occurred with no significant change in circulating MP counts, indicating different MP function responses to HIIT and sitting. While lean individuals showed an increase in MP counts 60 minutes after the HIIT workout among all subsets, this was offset by a decrease in endothelial cell ROS production per MP at 15 minutes, resulting in no difference in the ROS-producing capacity of total MPs. This suggests that both MP number and function should be considered regarding acute exercise, as one may be affected by acute exercise while the other is not. To our knowledge, this is the first study to assess changes in total MP function following different intensities of acute aerobic exercise. The HIIT workout appeared to induce changes in MP function more so than MICT or sitting. These changes were differentially affected by body

composition, with significantly elevated endothelial cell ROS in the overweight group and decreased endothelial cell ROS in the lean group following the HIIT workout. Young, overweight individuals may not necessarily display classical signs of chronic inflammation associated with cardiovascular disease; however, they may experience more oxidative stress following a HIIT workout compared to lean individuals.

MP-induced endothelial cell ROS:

To our knowledge, only one other study has assessed MP function following an acute bout of high-intensity exercise. Wahl and colleagues explored the effects of various high-intensity workouts on endothelial MP number and function in male triathletes.[62] Utilizing human coronary artery endothelial cells (HCAECs), the authors reported that endothelial cells incubated with post-exercise endothelial MPs and serum led to decreased apoptotic activity compared to pre-exercise endothelial MPs. It is important to note that their measurement of MP function differed from that of the present study, as Wahl and colleagues used caspase-3 activity as an indication of apoptosis.[62] In contrast, our experiments measured endothelial cell ROS production following incubation with isolated total MPs; therefore, results from the two studies may not be comparable. However, our measurements may provide an assessment of MP function that is more specific to acute changes in vascular function and oxidative stress after exercise.

Regardless of apparent methodological differences, our data on body composition-dependent changes in MP function following acute exercise are generally in agreement with Wahl and colleagues who studied a highly fit group of male triathletes (VO_{2max} 64 mL/kg/min) with an average BMI of 22 kg/m^2.[62] While our participants were only recreationally active, the lean group exhibited a similar BMI of 22.6 kg/m^2. The fit participants in the former study showed MP function responses similar to our lean group, as post-exercise MPs led to diminished

endothelial cell inflammation compared to pre-exercise MPs. In contrast, our overweight group (BMI of 28 kg/m^2) displayed the opposite response following the HIIT workout: Endothelial cell oxidative stress was not reduced and was significantly higher than the sitting condition.

Our study presents an interesting relationship between body composition and MP function. Acute exercise is thought to change MP cargo such as miRNA, cell adhesion molecules, and proteins.[59] This may have played a role in our study, as the stimulus for MP release is known to determine cargo and subsequent function.[6,59] For example, MPs stimulated by endothelial nitric oxide synthase (eNOS), a vasodilatory enzyme, appear to transfer mRNA to target cells that promote angiogenesis.[104] In contrast, MPs stimulated by vascular disruption are known to carry inflammatory cell adhesion molecules such as e-selectin, vascular cell adhesion molecule 1 (VCAM-1), and intracellular adhesion molecule 1 (ICAM-1).[3] As such, our data suggest that after a HIIT workout, recreationally active overweight individuals may experience greater oxidative stress following the high-intensity workout due to the transfer of MP contents compared to their lean counterparts. In contrast, lean individuals may experience less exercise-induced ROS from high-intensity aerobic exercise.

Total MP and platelet MP counts:

We observed a 128% increase in total MP numbers at 60 minutes after the HIIT workout among all participants compared to baseline. After participants were split into overweight and lean groups based on BMI, the increase appeared to be experienced primarily by the lean group. Only one other study has reported on total MP counts in response to acute exercise. Serviente and colleagues observed a decrease in circulating total MPs 30 minutes after an acute bout of moderate-intensity exercise in healthy active women (BMI of 23 kg/m^2).[59] Our lean participants (BMI of 22 kg/m^2) showed a different response to HIIT, with significantly elevated total MP

numbers at 60 minutes post-HIIT workout. There are three potentially important distinctions between the former and present studies: exercise intensity, age, and sex. Serviente and colleagues utilized a moderate intensity bout of treadmill exercise with an all-female group of middle aged women approximately 20 years older than our cohort.[59] Our participants did not exhibit changes in MP counts with MICT, which could represent an interactive effect of sex and/or age and exercise. No other studies have focused on sex differences with total MP counts and acute exercise. However, Durrer and colleagues reported that endothelial MP counts in males responded differently than females following high intensity and continuous exercise.[29] Females underwent no change with HIIT whereas endothelial MP numbers decreased after continuous exercise.[29] This suggests that MP number may respond to different intensities of acute exercise based on sex. Sex differences in endothelial MPs were not the focus of the present study, as our hypotheses involved body composition-based effects of acute aerobic exercise on MP number and function. However, we cannot discount the role this may have played in our data on total MPs. Although we hypothesized that participants would experience a decrease in MP counts following exercise, this was not the case. It appears that changes in total MP counts after exercise may be affected by body composition and exercise intensity, but also age and sex.

It is important to note that there are key methodological differences between the two studies which may have affected the difference in results. Although we were aware of these differences, it is unknown what role they play in determining how MP number responds to acute exercise. Serviente and colleagues did not include a specific antibody stain for total MPs, instead relying on size gating for quantification and analysis.[59] In contrast, our study utilized an annexin-V+ stain to quantify total MPs. This is considered a pan-MP marker because of its binding affinity for phosphatidylserine, which is commonly externalized on MPs.[86] Serviente and

colleagues also reported total MPs as total events instead of μL of plasma run through the flow cytometer.[29,62] Differences in reporting of extracellular vesicle data have long been common in the field and do not always allow for direct assessments between studies.[105] As such, we cannot directly compare results between the two studies and can only report that the total MPs of various populations may respond differently to acute exercise based on intensity, sex, and age.

Changes in Platelet MPs with acute exercise have also received very little attention in the literature. We observed that platelet MP counts were elevated by 75% following the HIIT workout among all participants, and this was primarily driven by the lean group. Wahl and colleagues reported that platelet MP numbers decreased at 60 and 180 minutes following a HIIT workout in triathletes.[62] An important distinction between the two studies is that our participants were recreationally active, while the triathletes were highly trained. In contrast, two other studies have reported increased platelet MP numbers up to two hours after a single bout of high-intensity exercise in subjects more similar to ours in terms of fitness status.[106,107] One potential theory is that there can be changes in platelet MP numbers without any discernable effect on function. Platelet MPs are thought to be associated with procoagulant pathways and the formation of thrombin.[107] This can have implications on the development of cardiovascular disease as well as the immune response. Maruyama and colleagues tested the effect of exercise on platelet MP numbers as well as plasma levels of soluble fibrin, a factor associated with procoagulant activity and platelet MP function.[107] Although an increase in platelet MP numbers was observed following exercise, there were no meaningful changes in soluble fibrin. The authors concluded that soluble fibrin would have likely been affected in a less healthy population, but it did not change in healthy active individuals.[107] Although we observed elevated platelet MP counts following HIIT, the increase may not be related to a meaningful rise in procoagulant activity

brought on by the platelet MPs. More research on platelet MP function and related mechanisms is necessary to further develop these theories.

Endothelial MP counts:

Following an acute bout of HIIT exercise, we observed that CD62e+ (activated) and CD31+/CD42b- (apoptotic) endothelial MP counts were elevated by ~100% 60 minutes after the HIIT workout compared to the MICT workout. Upon further analysis, this appeared to be driven by the lean group. The effects of acute high intensity exercise on endothelial MP counts have been previously reported with somewhat mixed results. Durrer and colleagues showed a 36% decrease in CD62e+ endothelial MPs among overweight males following a HIIT workout,[29] while our overweight group exhibited no significant changes in CD62e+ endothelial MPs among any conditions. It is important to note that the aforementioned study did not utilize an annexin-V stain, which may have affected their MP count data.[29] While our study used an almost identical HIIT workout stimulus to Durrer and colleagues, their participants were all previously sedentary with mean BMI of 30 kg/m^2, classifying them as obese. Our participants were recreationally active with mean BMI of 28 kg/m^2, classifying them as overweight.[29] The differences in fitness status and BMI classification may partially explain distinctions in endothelial MP count data between the two studies. In addition, female participants in the study did not experience any changes in endothelial MPs following the HIIT workout, which suggests a potential sex difference in the response to high intensity exercise among overweight individuals. This may partially explain why we saw no change in our overweight group, as our male and female participants may have responded differently to the HIIT workout. Although the exercise stimuli were similar between the two studies, sex differences and fitness status may have played a role.

Our lean group exhibited significantly higher CD62e+ endothelial MP counts 60 minutes post-HIIT compared to the MICT workout. Serviente and colleagues reported a similar rise in this MP subset, but that was following a moderate intensity workout in middle-aged women.[59] The higher age of their participants may have played a role in their MP response to a lower-intensity workout. Our finding is also supported by Lansford and colleagues who reported a significant 107% increase in CD62e+ endothelial MP numbers in healthy male participants after an acute bout of moderate-high intensity continuous exercise.[108] CD106+ endothelial MPs have also been shown to increase significantly following a HIIT workout in healthy active males, which may also support our finding.[109] Although CD106+ endothelial MPs are not the same as the CD62e+ subset, they share similar functions as both markers are associated with activation of endothelial cell adhesion molecules involved in the immune response.[6,110] Our data seem to support what others have shown, suggesting that higher intensity exercise may induce elevated CD62e+ endothelial MPs compared to lower intensity exercise. This may be indicative of increased post-exercise endothelial cell activation. However, it is important to note that this rise in circulating MP numbers came along with a decrease in endothelial cell oxidative stress in the lean group, indicating that a change in MP counts may not be correlated with a rise in vascular dysfunction. As such, further research may be needed into the mechanisms behind CD62e+ endothelial MPs and cellular activation.

CD31+/CD42b- endothelial MP counts among lean participants also tended to increase in response to the HIIT workout. Our finding is not supported in the literature, as multiple studies have reported no significant change in the apoptotic endothelial MP subset following exercise in both lean and overweight individuals.[29,108,111] Our lean participants exhibited decreased endothelial cell oxidative stress following the HIIT workout, hinting that the cargo of these MPs

may have been altered by the HIIT workout to be less inflammatory within endothelial cells even with an increase in number. Interestingly, Sapp et al. discussed a possible reason for this, as they reported HIIT-related increases in multiple markers of endothelial disruption such as von Willebrand factor and miR-126 among young healthy participants.[111] The upregulation of these factors may have been a result of the increased exercise intensity and not necessarily direct damage to the endothelium.[111] This possible explanation, along with our functional data, could serve to explain the apparent rise in MP counts post-exercise without detrimental changes in MP function.

Measurements of Augmentation Index and Blood Pressure:

Our overweight group showed elevated carotid SBP at 60 minutes following the HIIT workout compared to the lean group. Overweight participants also had elevated carotid SBP at 15 and 60 minutes post-MICT, respectively. Our data suggest that body composition affects how carotid SBP responds acutely to exercise. Carotid SBP is considered to be more indicative of cardiovascular disease risk than peripheral blood pressure measurements.[103] Carotid SBP, considered a measurement of central blood pressure, may be differentially affected by acute exercise based on body composition.[112,113] However, it is important to note that carotid SBP did not differ in the overweight group before and after exercise. Instead, we only observed differences between the overweight and lean groups.

Healthy individuals regularly experience a decrease in systolic blood pressure following exercise, which is known as post-exercise hypotension.[114] However, individuals with higher BMIs are known to have diminished post-exercise hypotension due to chronic inflammation and diminished vascular health.[114–116] Instead, overweight and obese individuals appear to experience post-exercise hypotension much later after the cessation of exercise compared to lean

individuals.[116] This may partially explain why our overweight group's carotid SBP was not reduced following the HIIT and MICT workouts. Interestingly, this group showed significantly elevated endothelial cell ROS production post-HIIT workout. While other inflammatory variables should be considered in future studies, our ROS data in overweight individuals may play a role in the lack of an exercise-related response in carotid SBP. Interestingly, ROS are a known to contribute to the development of chronic inflammation and arterial stiffness.[96] The relationship between SBP and endothelial cell ROS production following incubation with MPs has not been studied in this context. However, endothelial MPs are known to produce ROS, which diminishes NO production and may increase arterial stiffness.[56,97] Our data bring up interesting questions about potential connections between acute exercise, vascular health, and MP function.

Overweight participants on average showed significantly higher baseline carotid, brachial, and radial SBPs compared to the lean group. This difference in baseline measurements may also help to explain why carotid SBP did not respond to exercise in overweight individuals, as the measurement was already elevated prior to exercise. Differences in baseline blood pressure measurements have been previously reported, as overweight individuals tend to show higher resting central SBP due to chronic inflammation and greater vascular dysfunction than their lean counterparts.[114,117]

We observed that radial AIx in the overweight group was significantly lower at 15 minutes post-HIIT workout compared to MICT. This contrasts with our other vascular data, as arterial wave reflection indicated by radial AIx appeared to briefly improve after high-intensity exercise compared to moderate intensity in the overweight group. A post-exercise decrease in AIx has been previously reported in the literature in middle-aged overweight diabetic individuals

following both HIIT and moderate-intensity workouts.[118] Although our participants were not middle-aged or diabetic, our radial AIx data suggest that overweight individuals undergo some immediate benefit to arterial wave reflection from high-intensity exercise which may quickly resolve after the 15-minute post-exercise time point. Participants in the study by Way and colleagues experienced decreased AIx in response to both intensities, but participants' responses to the moderate-intensity workout may have been influenced by age.[118] However, it appears that a HIIT workout may acutely influence radial AIx in younger otherwise healthy overweight individuals. While a decreased cardiac afterload within the first 60 minutes post-exercise has been theorized as having played a role, we observed no change in carotid SBP to support this.[118] Other studies have shown that post-exercise vasodilation may be enhanced in overweight individuals.[119,120] Vasodilation may have played a role in the present study, as carotid SBP in the overweight group did not increase during the HIIT workout. The lack of change in carotid SBP indicates that there may have been some level of compensatory vasodilation preventing a rise in SBP. Elevated post-exercise vasodilation would lead to delayed wave reflection and therefore a lowered AIx.[121] While carotid SBP remained elevated in the overweight group compared to lean, there were no differences between time points within the HIIT workout. We cannot discount the possibility that vasodilation contributed to the AIx response we observed in the overweight group. However, if the post-HIIT effect on radial AIx was driven by increased vasodilation, we would have expected to see a decrease in SBP as well.

Increased resting sympathetic nerve activity has been connected to increased AIx in men, while the inverse has been shown in women.[122] This suggests that there are potential sex differences between AIx responses that may stem from sympathetic nerve activity. However, it is unclear if sympathetic nerve activity contributes to AIx-based sex differences after acute

exercise. Instead this may depend on whether AIx is adjusted for heart rate.[123] In addition, sex differences in this regard are unlikely to have played a role in our study, as we had a relatively even split between males and females in the overweight group. Therefore, the AIx responses we observed were unlikely to have stemmed from sympathetic nerve activity. More research is needed to further define these topics and show whether our findings in radial AIx are related to MP function.

Strengths & Limitations:

This study was the first to observe changes in MP function following an acute bout of HIIT exercise. While others have studied the effects of different aerobic exercise modes on circulating MP counts, our study was the first to examine exercise-related changes in function via endothelial cell oxidative stress. The findings from this study may inform future research on MP function and may serve as a jumping off point for studying mechanisms related to MPs and body composition. Much of the previous work on MP counts and exercise lacked a stain for annexin-V, which is a MP-specific marker.[29,59,61,108] As such, our study offers an updated look at how various MP subsets respond to different modes of aerobic exercise with more specific MP count data free from cellular debris.

The present study was not without limitations. Previous studies have reported exercise-related changes in markers of vascular health and angiogenesis such as von Willebrand factor, vascular endothelial growth factor, and hepatocyte growth factor.[62,111] Our study did not include analysis of these markers, which may have strengthened our MP counts and functional data. However, it is important to note that the purpose of the present study was to examine body composition-related changes in MP counts and function following acute aerobic exercise. As such, the analysis of those factors may be outside the scope of this study. In addition, our data on

MP counts and oxidative stress were non-normally distributed. This may stem from high variation between participants' MP counts quantified via flow cytometry. Although trends within the cohort were relatively consistent, the variance did cause us to alter our statistical approach. We utilized the Wilcoxon signed-rank test, which assigns rankings to change scores within each variable. This alternative approach could make our data less comparable to that of others studying MP counts and function.

Finally, we were unable to use only endothelial MPs when incubating MPs with HUVECs and instead utilized total MPs for those experiments. As such, we cannot discount the possibility that other MP subsets may have influenced our results. Our approach may not specifically address the question of how *endothelial* MPs affect HUVEC ROS production following acute aerobic exercise. However, the utilization of isolated endothelial MPs for this experiment would pose logistical issues as so few are available in each 10 mL blood sample. By using total MPs for this experiment, we created an environment that was perhaps more indicative of what occurs in circulation. Indeed, our data may provide a broader picture of how MP function is affected by acute aerobic exercise.

Conclusions:

We have demonstrated for the first time that there is a post-exercise rise in endothelial cell ROS following incubation with MPs from overweight recreationally active individuals. Overweight individuals also exhibited significantly elevated carotid SBP but lowered radial AIx after high intensity exercise, which may relate to the changes we saw in MP function. In contrast, incubation with MPs from lean recreationally active individuals led to decreased endothelial cell ROS production following a HIIT workout. More research is needed to uncover how other factors related to vascular health and ROS play a role in these mechanisms and whether MP

cargo is altered with exercise. In addition, MP counts among all subsets in lean individuals appear to be elevated following a HIIT workout, but this was not accompanied by detrimental changes in endothelial cell ROS. These data help broaden our understanding of the relationships between body composition, acute aerobic exercise, vascular health, and MPs. Our findings indicate that high-intensity acute exercise may have differential effects on MPs in people with different BMI classifications. As such, this could potentially inform exercise prescription for overweight and lean individuals and highlights novel differences in how each group responds to acute exercise.

Chapter 4: Effects of aerobic exercise training on circulating microparticle counts and function in previously sedentary older adults

Introduction

The development of cardiovascular disease and other disorders linked to its development is of great concern among aging adults. As such, it is continually relevant to explore mechanisms related to these diseases. A decline in vascular function is a strong indicator of cardiovascular health among older adults, which can deteriorate even faster when combined with a sedentary lifestyle.[124,125] Vascular aging is especially relevant, as it signifies a decrease in vascular function independent of biological age. As such, older adults who are also sedentary may experience a steeper decline in vascular function, putting them at great risk of developing cardiovascular disease.[124,125] Although there is a large amount of previous research on vascular health and aging, pathways of vascular inflammation need further development. Novel biomarkers connected with vascular dysfunction such as circulating microparticles (MPs) require further exploration among these individuals.

One important mechanism related to vascular dysfunction is the buildup of oxidative stress within endothelial cells. This comes in the form of reactive oxygen species (ROS) that scavenge nitric oxide (NO), leading to diminished vasodilation as well as chronic inflammation within the endothelial layer of the arteries.[56,72,126] A variety of mechanisms contribute to endothelial cell dysfunction, but the functions of circulating MPs may be significantly involved. Circulating MPs are a subset of extracellular vesicles that are shed from parent cells in response to cellular apoptosis or activation of the immune response.[6,56,126] Their main function is cell-to-cell communication, through which they deliver their cargo to target cells in the form of proteins, cell adhesion molecules, and miRNAs. It is thought that their stimulus of release determines the

make-up of this cargo, ultimately affecting MP function.[6] Increased amounts of circulating endothelial MPs have been associated with disrupted vascular function among individuals at risk for developing cardiovascular disease.[126] The continuous release and uptake of MPs may also influence the spread of inflammation and subsequent disease.[6,126] These novel biomarkers of inflammation warrant further investigation regarding exercise training.

Aerobic exercise is a well-known therapeutic stimulus that can ameliorate the effects of vascular aging. The positive effects of aerobic exercise training on cardiovascular disease risk have been previously reported, and sedentary older adults can significantly benefit from the adoption of regular exercise.[64,124,125] Measurements of vascular health and function appear to improve in sedentary older adults following the completion of an aerobic exercise training program.[64,124] While several studies have reported on the effects of a single bout of exercise on circulating MP number, fewer have examined the effects of aerobic exercise training. In one such study, just two weeks of aerobic exercise training at varying intensities elicited a 7% decrease in CD62e+ (activation) endothelial MPs in previously sedentary overweight middle-aged adults, but there were no changes in CD31+42b- (apoptotic) endothelial MPs suggesting a longer training program may have been needed.[63] Babbitt and colleagues utilized a much longer 6-month aerobic exercise training program in previously sedentary older adults and reported a 48% reduction in CD62e+ endothelial MPs counts as a result of the training.[64] Participants also exhibited a significant reduction in flow-mediated dilation, suggesting an improvement in vascular function.[64] Both studies show promising findings in the relationship between circulating MP counts and aerobic exercise training; however, gaps remain regarding how exercise training may affect other MP subsets such as total MPs and platelet MPs, as well as MP function.

The purpose of this study was to determine the effect of aerobic exercise training on circulating MP counts in previously sedentary older adults as well as their ability to stimulate endothelial cell oxidative stress *in vitro*. We hypothesized that (1) circulating total MPs, endothelial MPs, and platelet MPs would decrease in older adults following 6 months of aerobic exercise training, and (2) endothelial cells incubated with MPs collected following exercise training would exhibit decreased ROS production compared to pre-training MPs, compared with the flexibility control group.

Methods

Ethical Approval:

All human subjects research performed in this study received prior approval from the University of Maryland Institutional Review Board (IRB). Written informed consent was obtained from each participant and the study conformed to the standards set by the latest revision of the Declaration of Helsinki.

Participants:

A total of 40 participants were recruited for the present study. Participants were healthy older adults aged 60-80 years with no symptoms of severe cardiovascular disease, asthmatic conditions, or history of transient ischemic attacks who could complete activities of daily living (ADLs). Participants were also required to obtain physician consent to perform a VO_{2peak} test as well as moderate intensity exercise for the 26-week training period. Participants were sedentary at the time of their first visit, meaning less than 30 minutes/day of activity on 3 days/week over the past 6 months. Potential participants were excluded if they had history of neurological conditions and medical conditions that could affect brain health.

VO_{2peak} Testing:

$VO2_{peak}$ was assessed at the baseline and 6-month time points using indirect calorimetry. The test was conducted on a treadmill using a modified Balke-Ware protocol (2.0 mph, grade increase 1% per minute) according to the American College of Sports Medicine Guidelines and lasted approximately 8-12 minutes.[78] Measurements of ventilation, rate of oxygen (O_2) consumption, rate of carbon dioxide (CO_2) production, and the respiratory exchange ratio (RER; CO_2 production/O_2 consumption) were obtained every 30 seconds throughout the exercise test with a calibrated metabolic measurement system. The Borg Ratings of Perceived Exertion (RPE) scale was used to monitor subjective effort every minute during exercise. The test was terminated upon reaching 85% of participants' predicted maximal heart rates based on age, or if participants indicated a desire to stop. VO_{2peak} was extrapolated from heart rate and submaximal oxygen consumption measurements collected throughout the test.

Exercise Training and Flexibility Control:

Participants completed either 6 months of exercise training or flexibility training to serve as a control starting at 2-3 days/week for the first 8 weeks and progressing to 4 days/week for weeks 9-26. All participants completed a 10-minute warm-up of light total body stretching, 5 minutes of balance/toning exercises, 2 minutes of in-place marching, and a 6-8-minute obstacle course. The balance and toning period consisted of multi-directional steps (forward, side, and back) with each leg; multi-directional leg raises (forward, side and back) with each leg; arm circles, and trunk rotations. Handrail or armchair support was minimized, leg raise height was increased, the range of motion for trunk bending and rotations was increased, and marching cadence and foot height was increased to encourage participants to continually challenge

themselves. The obstacle course consisted of functional and core movements including seated bicycles, leg extension holds, and sit-to-stand movements among others.

The exercise training group also performed functional and core movement interval workouts where each exercise was performed for 20 seconds with 10 seconds of active rest between each. Groups of exercises were performed multiple times within each workout. Exercises included one-sided jacks, alternating leg kicks, mini squats, and various marching workouts. Duration began at 10 minutes for weeks 1-4 then progressed to 20 minutes for weeks 5-8. Workout durations increased to 30 minutes and remained that way for weeks 9-26. Percent of heart rate reserve and rate of perceived exertion began at 40-50% and 9-11, respectively, for weeks 1 and 2, then increased to 45-50% and 9-11 for weeks 3-4. Intensity was again increased to 50-55% heart rate reserve and rate of perceived exertion 10-12 for weeks 5-8, then 60-70% and 11-15 for weeks 9-26. Participants also completed a cool-down consisting of light stretching.

The control group's training consisted of functional and core movements (described above) as well as an additional time-matched bout of core and flexibility exercises including total body static stretching (standing and seated) of all major muscle groups (neck, shoulders, arms, trunk, hips, legs, and ankles). Intensity progressed from 20-30% of heart rate reserve and rate of perceived exertion 8-10 for weeks 1-4 to 30-40% and 9-11 for weeks 9-26.

Microparticle Isolation:

All participants underwent blood draws at baseline, as well as >72 hours following the final training session. Blood samples were collected in ACD vacutainer tubes (BD Bioscience, SSS, YY), centrifuged, and plasma was stored at -80°C for future analysis. Plasma samples from each time point were used to isolate EMPs via sequential centrifugation. Briefly, 500µL aliquots from each time point were centrifuged at 1500 x g for 20 minutes at 20°C to obtain platelet poor

plasma. The top two-thirds of the supernatant were transferred to a new microcentrifuge tube and centrifuged again at 1500 x g for 20 minutes at 20°C to obtain platelet free plasma. 100µL of each sample was incubated with fluorescent monoclonal antibodies for quantification of each MP population, including annexin-V+ anti-annexin-V-violet 450 (v450) (BD, Franklin Lakes, NJ, USA, Cat#560508, RRID: Ab_2869356) which binds to phosphatidylserine, CD31+ anti-CD31-allophycocyanin (APC) (ThermoFisher Scientific, Waltham, MA, USA, Cat#47031942, RRID: Ab_10730582) for PECAM-1, CD42b- anti-CD42b-peridinin-chlorophyll-protein (PerCP) (ThermoFisher Scientific, Waltham, MA, USA, Cat#46042942, RRID: Ab_2762458) to remove platelets and CD62E+ anti-CD62E-phycoerythrin (PE) (BD, Franklin Lakes, NJ, USA, Cat#551145, RRID: Ab_394072) for e-selectin. MP sub-populations including Annexin-V+/CD31+/CD42b- and Annexin-V+/CD62E+ as well as Annexin-V+ for total MPs and Annexin-V+CD42b+ for PMPs were quantified via flow cytometry. Standardized 900 nm and 1000 nm nanobead NIST traceable particle size standards (Polysciences, Warrington, PA, USA) and CountBright absolute counting beads (ThermoFisher Scientific, Waltham, MA, USA) were used for proper quantification of MPs. Data analysis was performed using FlowJo V10.1rs (FlowJo, LLC, Ashland, OR, USA).

Endothelial Cell Culture:

Passage 3-6 male and female human umbilical vein endothelial cells (HUVECs) (Lonza, Basel, Switzerland) were seeded in T75 flasks with 15 mL of endothelial growth medium 2 (EGM2), (Lonza, Basel, Switzerland) then passed into separate 96-well plates with 200 µL of EGM2 with 2% fetal bovine serum (FBS) per well once they had reached ~80% confluence. Once they had once again reached ~80% confluence HUVECs were ready for subsequent experiments.

Microparticle Incubation and Intracellular ROS Quantification:

Five thousand MPs were isolated from each time point of each sample using the previously mentioned MP isolation technique, then added to each well. This amount is similar to amounts previously utilized to quantify ROS production in endothelial cells.[56] HUVECs were incubated with MPs for 1 hour.

Following incubation with MPs, Cell ROX Green Reagent (Life Technologies, Carlsbad, CA, USA) was added to the cells at a concentration of 5 μM and incubated for 30 minutes at 37ºC. Following incubation, cell medium was removed, and each well was washed two times with 1x phosphate-buffered saline (PBS). Endothelial cell ROS production was quantified using plate fluorescence (Synergy H1 Hybrid Reader; Biotek, Winooski, VT, USA). Microscope images of each well were captured following CellROX incubation using a Nikon Eclipse Ti-U microscope with DS-Ri2 camera (Nikon Instruments, Melville, NY, USA). Cell counts within each well were obtained using NIS-Elements AR 5.21.03 imaging software (Nikon Instruments, Melville, NY, USA) to compare ROS fluorescence between wells.

Statistical Analysis:

Following completion of data collection, a Shapiro-Wilk test of normality showed a non-normal distribution of microparticle count and functional data ($P < 0.05$). This was not resolved with log transformation; therefore, Wilcoxon signed-rank tests were used to analyze any differences between paired MP counts among time points. The Mann-Whitney U test was used to test between independent conditions. Unpaired t-tests were used to analyze any differences in participant characteristics between exercise training and functional control groups. Statistical significance was accepted at $P < 0.05$.

Results

Participant Characteristics:

Participant characteristics are summarized below in Table 3. There were no significant differences in demographic variables within or between groups.

Table 3. Participant characteristics

	ET BL	ET 6m	FC BL	FC 6m
Age	69 ± 1.3	69 ± 1.3	68 ± 1.2	69 ± 1.2
Males	4		4	
Females	16		16	
Weight (kg)	81.80 ± 4.42	82.86 ± 4.53	79.27 ± 2.56	79.39 ± 2.53
Height (cm)	163.23 ± 1.89		163.55 ± 1.65	
BMI (kg/m^2)	30.53 ± 1.36	30.53 ± 1.48	29.71 ± 1.02	29.64 ± 1.03
VO_{2peak} (mL/kg/min)	16.58 ± 0.92	16.53 ± 1.05	15.60 ± 0.95	16.24 ± 0.61

Data are means ± SEM. ET, Exercise Training; FC, Functional Control; BL, Baseline; 6m, 6-month; BMI, Body mass index.

Circulating total microparticle counts:

There were no significant differences in circulating total MP numbers between baseline (BL) or 6-month (6m) time points within the exercise training (ET) or flexibility control (FC) groups and values were not different between groups at either time point (Figure 27). The same was true for platelet MP numbers, which are shown in Figure 28.

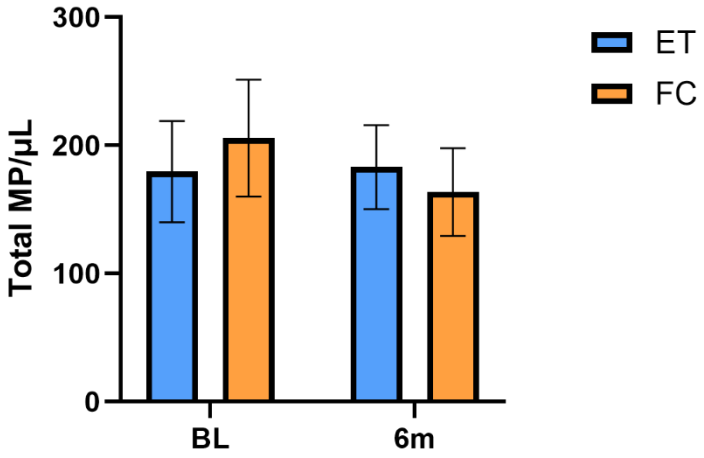

Figure 27. Total MP counts in response to 6 months of exercise training (ET) or functional control (FC). Mean ± SEM, n=20 for each group.

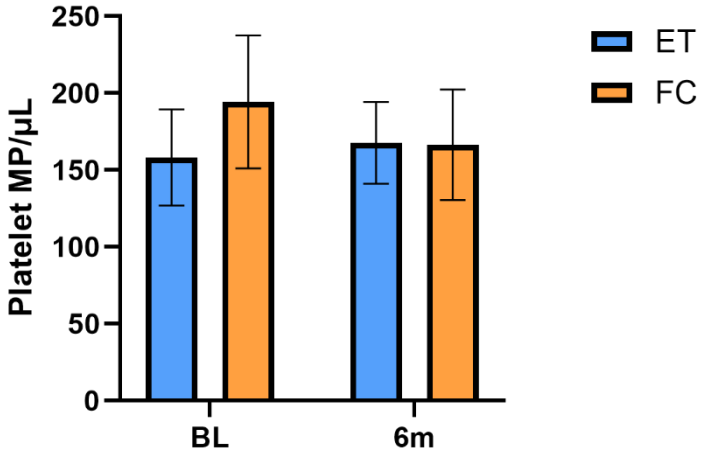

Figure 28. Platelet MP counts in response to 6 months of exercise training (ET) or functional control (FC). Mean ± SEM, n=20 for each group.

Endothelial MPs:

We saw no significant changes in circulating CD62e+ (activated) or circulating CD31+/42b- (apoptotic) endothelial MP numbers between BL or 6m time points within the ET

or FC groups (Figures 29 and 30). There were also no significant between-group differences in CD62e+ or CD31+/CD42b- endothelial MP counts.

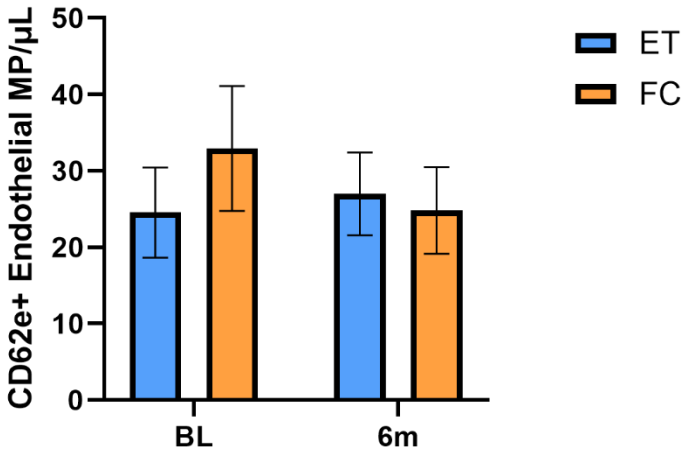

Figure 29. CD62e+ endothelial MP counts in response to 6 months of exercise training (ET) or functional control (FC). Mean ± SEM, n=20 for each group.

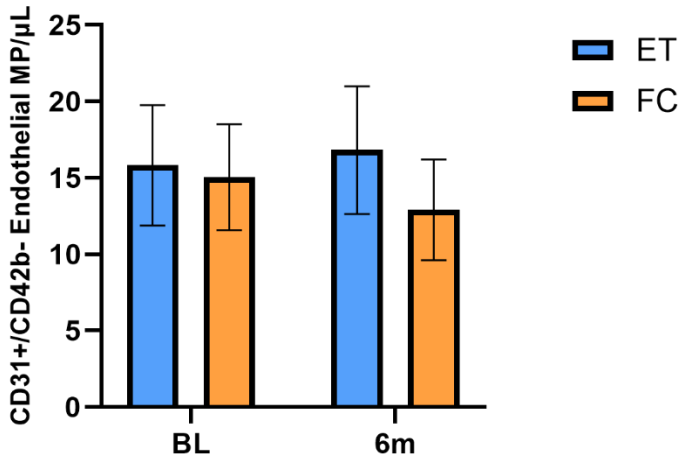

Figure 30. CD31+/CD42b- endothelial MP counts in response to 6 months of exercise training (ET) or functional control (FC). Mean ± SEM, n=20 for each group.

ROS production after MP incubation:

There were no significant differences in HUVEC ROS production between BL or 6m time points within the ET or FC groups (Figure 31). There were no significant between-group differences in HUVEC ROS production.

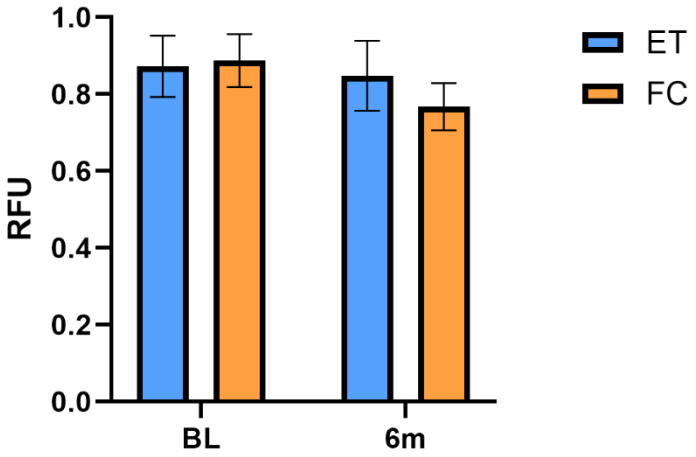

Figure 31. HUVEC ROS production in response to 6 months of exercise training (ET) or functional control (FC). Mean ± SEM, n=20 for each group.

ROS-producing capacity of total MPs:

We found no significant differences in ROS-producing capacity of total MPs between BL or 6m time points within the ET or FC groups (Figure 32). There were also no significant differences between the ET or FC groups.

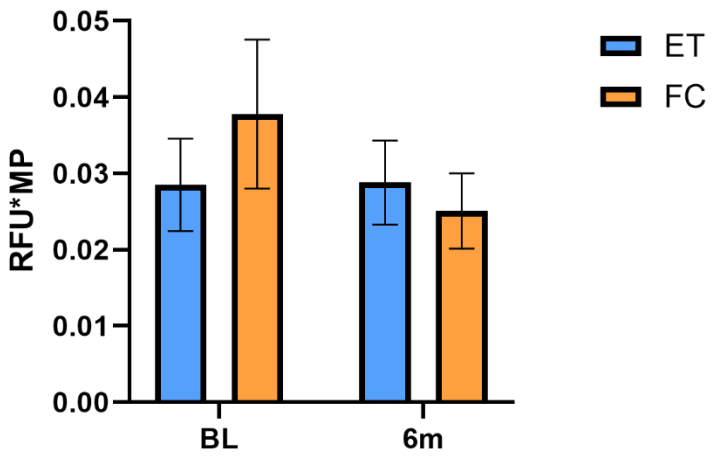

Figure 32. ROS-producing capacity of total MPs in response to 6 months of exercise training (ET) or functional control (FC). Mean ± SEM, n=20 for each group.

Discussion

Contrary to our hypothesis, we observed no significant changes in circulating MP numbers or MP function among previously sedentary older adults following a 6-month aerobic exercise training program. We initially aimed to investigate how aerobic exercise training may affect the function of MPs through their ability to elicit ROS production in endothelial cells. To our knowledge, this is the first study to analyze the effects of exercise training on MP function in sedentary older adults. While we also measured total MP, platelet MP, and endothelial MP counts in circulation, neither of these subpopulations changed as a result of the exercise training stimulus. We cannot accept our initial hypotheses. However, the data from this study does offer insight that may inform future studies on MPs and exercise training, especially among older adults. Our findings suggest that, while our participants did increase their physical activity levels for 6 months, the aerobic exercise training protocol did not elicit any changes to circulating MP counts or function.

Total MPs:

Total MP counts did not change in either group following the 6-month exercise training stimulus. A previous study from Rodriguez-Chiaradia and colleagues measured the ratio of endothelial MPs to total MPs following an 8-week exercise training program in hypertensive patients.[127] While the authors observed a decrease in endothelial MPs within this ratio, they reported no changes in total MPs isolated solely based on particle size.[127] Our data may align with this, albeit with some methodological differences. The previously sedentary participants in the study all had pulmonary arterial hypertension and were part of a pulmonary rehabilitation program focused on safely building exercise endurance. Our participants were also previously sedentary, and many had hypertension controlled with medication. A different study using 3 weeks of aerobic exercise training in mice reported an increase in the amount of exosomes in circulation, which are a subset of extracellular vesicles similar to MPs.[127,128] Post-training exosomes turned out to have a protective effect on cardiac health by carrying miR-342-5p to target cells, which is thought to inhibit cardiomyocyte apoptosis.[127,128] This idea of changes in extracellular vesicle cargo may partially explain why we saw no change in MP counts in our study even though we expected to see a decrease in total MP counts. Microparticle cargo is known to be largely determined by the stimulus of their release from parent cells.[3,6] Therefore, we cannot discount the possibility that exercise training brought about a change in MP cargo without a discernable change in the amount of MPs in circulation. While we measured MP function via endothelial cell ROS production following incubation with MPs, there may certainly be other mechanisms involved with changes in MP cargo. Future studies involving MPs and exercise training should consider a broader analysis of MP cargo.

Platelet MPs:

Our data showed no changes in platelet MP numbers as a result of the 6-month exercise training stimulus within or between both groups. There are no previous studies directly involving platelet MPs and exercise training. Guiraud and colleagues measured platelet MP responses to an acute bout of high-intensity exercise in older active coronary heart disease patients and observed no changes in response to exercise.[129] The absence of an acute effect of exercise was somewhat surprising, as platelet MPs are known to be chronically elevated in the progression of atherosclerosis as well as angiogenic regulation.[130] More research is needed to determine how platelet MPs respond to both acute and chronic forms of exercise, as they did not seem to be affected by our exercise training stimulus. In younger recreationally active adults, we have observed an acute increase in platelet MP counts following a HIIT workout. This suggests that exercise intensity or even age may play a role. Our participants were older sedentary adults and did not exhibit signs of chronic disease associated with elevated platelet MP numbers. Because there were no acute changes with exercise in our stable but diseased population, perhaps a higher intensity exercise training stimulus is needed to bring about discernable changes in platelet MPs.

Endothelial MPs:

We observed no significant changes in CD62e+ (activated) or CD31+/CD42b- (apoptotic) endothelial MPs in response to exercise training. Previous results on this topic vary, with studies showing both increases and reductions in endothelial MP numbers as well as unchanged counts.[63,64,131] There are very few studies involving exercise training and CD31+/CD42b- endothelial MPs. Rafiei and colleagues reported no change in this MP subset following 8 weeks of moderate or high intensity exercise training in overweight middle-aged adults.[63] Another study found no change following 12 weeks of moderate intensity aerobic and

functional exercise training in older adults with intermittent claudication.[132] However, the authors did show a significant increase in CD31+/CD42b- endothelial MPs acutely 2 hours after exercise.[132] While neither study had participants identical to ours, their findings support our data on this subset of endothelial MPs.

Both Rafiei and Babbitt showed reductions in CD62e+ endothelial MPs following exercise training. Rafiei reported a 7% reduction in this marker of endothelial cell activation following just 8 weeks of high-intensity interval training in overweight middle-aged adults.[63] Babbitt and colleagues utilized an older cohort with a longer 6-month aerobic exercise training program, showing a 48% reduction in CD62e+ endothelial MPs.[64] Our participants were older overweight adults with similar BMIs to those in the Babbitt study. However, they did not exhibit the same post-exercise decrease in endothelial MP counts. One potential reason for this could be differences in the modes of aerobic exercise included in the study, leading to varied responses in MP counts.

While participants in the Babbitt study completed an exercise training program with a similar progression to ours, they included a second VO_{2peak} treadmill test to account for changes in fitness following completion of the first half of the program.[64] This allowed the authors to adjust training intensity based on participant fitness gains as needed. Our training program did not include this, which may have affected our participants' level of cardiovascular fitness attained at the end of the 6-month program. Intensity in the training protocol was based on VO_{2peak} whereas our intensities were based on heart rate reserve. Interestingly, participants in the Babbitt study showed increased VO_{2peak} as a result of the training program, as well as significant reductions in BMI and triglycerides.[64] Even though our training program included a similar progression with duration and intensity, our participants did not increase their VO_{2peak} after the

training program. This key difference may support the lack of changes in MP counts in our study. The Babbitt study also included a variety of aerobic exercise training modes, while our program consisted of more functional movements and floor exercises in addition to the functional/core program completed by both groups. The addition of multiple training modes may have continually challenged their participants to work harder, increasing fitness gains. The Rafiei study utilized high-intensity interval training in their middle-aged participants. Their participants were younger than ours, which may have contributed to their ability to complete high-intensity exercise. However, a training program with varied exercise intensities may have been a more appropriate stimulus to elicit changes in endothelial MP counts.

In contrast, Kirk et al. reported no changes in CD106+ endothelial MPs among adults (24 ± 6 years), with polycystic ovary syndrome (PCOS) and healthy controls following 8 weeks of moderate intensity aerobic exercise.[131] While we did not specifically measure this MP subset, CD106 (vascular cell adhesion molecule-1) is a marker of endothelial cell activation similar to CD62e (e-selectin). The participants in this study were much younger than ours, making direct comparisons difficult. The authors also measured CD105+ endothelial MPs, which are considered endothelial-specific markers of inflammation. This subset was reduced in PCOS patients following exercise training to a level similar to baseline values of healthy controls.[131] While we did not observe any changes in our endothelial MP counts, disease state may have played a role, as the PCOS participants from Kirk et al. showed significantly higher baseline counts compared to the healthy controls. In addition, older individuals are known to have increased CD62e+ endothelial MP numbers.[133] This may have played a role in our study, as any reductions from the exercise training may have been blunted by already elevated endothelial MP numbers in our older population. There does not appear to be a consensus in the literature

regarding the effects of exercise training on endothelial MP counts. Instead, responses appear to be dependent on age and disease state of participants. Responses also may differ between endothelial MP subsets. While we saw no changes in CD62e+ and CD31+/CD42b- endothelial MPs, other subsets may be more likely affected by exercise training in an older population. Future studies should focus on exercise intensity-based differences in MP counts as well as how endothelial MP subsets may be affected by differently based on age and disease state.

Microparticle-induced endothelial cell ROS & ROS-producing capacity:

We did not observe differences in endothelial cell ROS production following incubation with MPs isolated before and after exercise training. Endothelial cell ROS production is known to contribute to vascular dysfunction by scavenging NO. Microparticles are thought to contribute to endothelial cell ROS production because of their involvement in cell-to-cell communication.[56] Burger and colleagues reported that endothelial cells incubated with MPs showed significantly compromised NO production.[56] As such, we attempted to quantify the ability of MPs to elicit endothelial cell ROS production before and after a 6-month exercise training intervention. There are no previous studies on this aspect of MP function with exercise training, so we cannot compare our findings with previous studies, but we found no differences in MP function between time points or groups. Similarly, we found no differences in the ROS-producing capacity of total MPs because of exercise training.

Exercise intensity may play an important role in endothelial cell ROS production that factored into our study. Wang and colleagues reported that moderate intensity exercise led to an acute increase in endothelial cell NO production compared to high intensity exercise.[134] Although we did not measure endothelial cell NO production, the study may still provide insights into our data. The authors reported that endothelial cell ROS produced following

moderate intensity exercise promoted an increase in NO formation, while ROS generated by high intensity exercise coincided with a decrease in NO.[134] This may have factored into our results, as any ROS produced through our moderate intensity exercise may have incidentally enhanced NO bioavailability in endothelial cells and suppressed spikes in inflammatory ROS production.[135] While we failed to show changes in endothelial cell ROS production before and after exercise training, the ability of ROS to elicit beneficial changes in NO cannot be discounted. These mechanisms require deeper exploration to delineate whether exercise intensity does indeed modify the ability of ROS to aid in NO production, and whether this relationship even extends to MP-derived ROS.

Strengths and Limitations:

Our study is the first to look at the effects of aerobic exercise training on both circulating MP numbers and function in previously sedentary older adults. Although we cannot report any significant changes in our measurements, the study does have strengths that contribute to future work in this field. We successfully administered 6-months of moderate intensity exercise training to previously sedentary older adults and thereby increased their physical activity levels. Our groups were equal in size and sex distribution. In addition, our FC group completed core and flexibility exercises, providing an attention-matched control while still allowing there to be a key distinction between the two groups, i.e. aerobic exercise training. While this may be seen as a limitation because both of our groups performed some type of exercise training, we were able to provide the flexibility control group with a way to increase their physical activity levels and potentially receive benefits from that, which would not have occurred if they had been asked to remain sedentary. Only a few previous studies have focused on MP counts with exercise training, and none have measured MP function. As such, our study adds to the current body of literature,

which may inform the analysis and discussions of future work. Finally, we introduced a novel method for quantifying MP function in the form of endothelial ROS production following MP incubation. This method may be used in future studies looking at changes in MP function with other stimuli.

The present study was not without limitations. Although we could not show any relevant changes in MP counts or MP-induced endothelial cell ROS production, there may have been changes in MP cargo in response to the exercise training. Unfortunately, we were unable to assess MP cargo within the scope of this study. In addition, our study would have been strengthened with a direct measurement of MP uptake versus release, which could have explained why we saw no changes in circulating MP counts. Finally, we only utilized functional movements and floor exercises as an aerobic exercise stimulus and our participants did not see any benefits to their cardiorespiratory fitness measured via VO_{2peak}. Increased cardiorespiratory fitness is not necessarily guaranteed to enhance vascular function in older adults, as it appears to be dependent on the effect of the training program on specific populations.[136] However, there are certainly strong associations between the two.[137–139] While our participants completed consistent physical activity, a training program with more varied intensities and modes of exercise may have elicited a greater effect on cardiorespiratory fitness. This in turn may have allowed us to see meaningful changes in MP numbers and function as a result of exercise training.

Conclusions:

We found no significant changes in circulating MP numbers across multiple subsets, nor any changes in MP function following 6-months of aerobic exercise training in previously sedentary older adults. However, our study still provides relevant insights when viewed through the context of previous work on this topic. We may conclude that exercise intensity could play a

significant role in the MP response to exercise training in older adults. A more varied approach to the mode and intensity of aerobic exercise could lead to a training program that benefits cardiorespiratory fitness, which in turn may reduce MP counts and change function. As such, our work contributes to the current body of literature and provides insight for future studies involving MPs and exercise training in older adults.

Chapter 5: Summary of findings

The purpose of this dissertation was to ascertain how MPs isolated from different populations are affected by a variety of stimuli. We explored their role in dietary inflammation among both high-fit and lower-fit adults by analyzing MP counts and function before and after our participants consumed a high-fat meal. We were able to assess post prandial MP function by measuring endothelial cell reactive oxygen species (ROS) production after incubation with MPs isolated from before and after the meal, allowing us to see how acute, diet-induced inflammation affected participants based on their fitness status. In the second study, we looked at how MPs are affected by moderate- and high-intensity acute exercise in recreationally active overweight and lean individuals by measuring MP counts and function before and after a high-intensity interval training (HIIT) workout, a moderate intensity continuous training (MICT) workout, and a sitting visit. We were also able to assess how these data may relate to vascular health as a function of body composition. In the third study, we measured how MPs are affected by 6 months of aerobic exercise training in previously sedentary older adults. This was done to study the chronic effect of exercise on MP number and function.

Each of the three studies utilized different populations based on fitness status, body composition, activity level, and age. After looking at how each affects MP number and function following a variety of stimuli, we may better understand the role that MPs play in the body. However, the traditional idea of MPs as biomarkers based solely on number requires further exploration. While previous studies have related increased MP number to cardiovascular disease and its contributing factors, our findings indicate that their function must be assessed as well.[6,22] For example, our results suggest that MPs from lower-fit individuals may induce increased endothelial cell ROS following a high-fat meal compared to high-fit individuals. This occurred

regardless of seeing no change in MP number in the lower-fit group. Our data suggest that changes in MP function may not always coincide with a change in MP number. Therefore, this relationship requires further exploration. We found a similar response with acute high-intensity exercise, as MP numbers in the overweight group did not change but endothelial cell ROS production following MP incubation was elevated in post-HIIT samples. This idea has not been discussed in the literature, but MP function is known to be determined by their stimulus of release.[3,6] Wahl and colleagues observed a significant decrease in endothelial MP number following high-intensity acute exercise, which corresponded with diminished endothelial cell apoptotic activity following incubation with post-exercise MPs.[62] If only MP number was of relevance, these findings would suggest that a decrease in MP number should coincide with a decrease in MP inflammatory function. However, we observed increased MP counts in our high-fit and lean groups after the high-fat meal and high-intensity exercise, respectively. This came with no change or a decrease in endothelial cell ROS following MP incubation from each perturbation in these groups. Our results indicate that MP number may not be indicative of their response to acute dietary inflammation and exercise. Instead, it is important to include measurements of their function as well to obtain a more complete picture of how they are affected by lifestyle factors that tie into cardiovascular disease risk such as diet and exercise.

While we had distinct groups within each study, our comprehensive data provide meaningful insight into the relevance of MPs as biomarkers of inflammation. Our results suggest that acute inflammation caused by both high-fat meal and high-intensity exercise may differentially affect individuals based on their fitness status and body composition. Furthermore, we have shown that MPs are affected differently by acute exercise versus exercise training in number and function. Our results have implications on how MPs can be connected with vascular

health and the development of cardiovascular disease. In addition, our data may inform exercise prescription among overweight and older individuals.

We utilized clinically relevant populations in each of our studies, looking at adults who were previously sedentary, recreationally active, and lower-fit, as well as overweight. For the first time, we measured MP function via endothelial cell ROS production following incubation with MPs isolated from timepoints before, during, and after each condition. Our results provide a novel look at how MPs are affected by stimuli outside of the number in circulation. While MPs are known to produce and carry ROS to target cells, previous studies have not looked at endothelial cell ROS production following incubation with MPs.[56] Furthermore, none have assessed how this aspect of MP function may be augmented by exercise or dietary inflammation.

We uncovered novel insights regarding how MP function may be differentially affected by both body composition and fitness status. In the first study, high-fit individuals experienced a numeric increase in MP counts, but this quickly diminished postprandially at 4 hours and was not accompanied by any change in MP function. However, lower-fit individuals experienced elevated endothelial cell ROS production following incubation with MPs isolated postprandially at 4 hours. We can make important conclusions from these findings, specifically regarding how fitness status affects one's response to acute dietary inflammation. Individuals of a higher fitness status appear to be somewhat protected from the inflammatory effects of a high-fat meal based on their MP counts and endothelial ROS production. In contrast, lower-fit individuals experienced a deleterious change in MP function. We can therefore conclude that fitness status plays a part in determining how one will respond to the acute inflammation caused by a high-fat meal. This is especially important for lower-fit individuals who regularly consume high-fat meals, as they may be continually exposed to inflammatory loads from which they may not

recover as quickly as their high-fit counterparts. As such, we can theorize that a high-fat diet may contribute to the rise of chronic inflammation, which has strong implications on cardiovascular disease development. Furthermore, MPs may play a significant role in the buildup of dietary inflammation through endothelial cell ROS production. While this is only one inflammatory mechanism involved with the development of cardiovascular disease, the relationship between fitness status and MP function within the context of dietary inflammation should be further explored.

While fitness status may contribute to inflammatory changes in MP function, we found that body composition plays a significant role as well. Following acute bouts of HIIT and MICT, our overweight participants experienced elevated endothelial cell ROS production with MP incubation compared to lean individuals. While the lean group showed increased amounts of circulating MP counts following the HIIT workout, this did not translate to any change in their MP function. Instead, the lean group experienced an acute decrease in endothelial cell ROS production with MPs isolated following the HIIT workout. From these data, we can conclude that overweight individuals may experience a greater inflammatory load following high-intensity workouts compared to their lean counterparts. This was mostly supported by our vascular data, as overweight individuals showed elevated carotid systolic blood pressure (SBP) throughout both exercise sessions compared to the lean group, such that they did not undergo an exercise-related response. However, radial AIx was lowered following high-intensity exercise in the overweight group, suggesting that this group may have experienced a compensatory increase in post-exercise vasodilation. While we were unable to show any correlations between our ROS data and blood pressure measurements, both point to young overweight individuals having some amount of low-grade chronic inflammation impacting their response to high-intensity exercise. Our findings in

overweight individuals may be influential in determining proper exercise protocols that allow this population to attain exercise benefits. In addition, we have shown that MPs can be considered novel biomarkers of acute inflammation via their function. While we could not directly associate MP outcomes to our vascular measurements through our statistical analyses, some connections have been shown previously.[6,64] Interestingly, circulating endothelial MP counts have been inversely correlated with flow-mediate dilation and correlated with endothelial dysfunction in coronary artery patients and obese women.[22,140,141] Elevated amounts of circulating endothelial MPs have also been associated with risk factors for cardiovascular disease in older adults.[142] It is therefore important to consider MP function when determining the impact of exercise on overweight individuals.

Our second and third studies provide novel insights into how MP number and function respond to both acute and chronic forms of exercise. While we showed significant changes in MP function after various forms of acute aerobic exercise, we could not report any changes following exercise training in previously sedentary older adults. Age may have played a role, as our participants in the second study were much younger. However, exercise intensity is a more likely contributor. Others have shown significant changes in MP counts following exercise training in both young and older adults.[63,64,127] Among older adults this was accompanied by a significant enhancement of VO_{2peak}. However, our participants did not undergo changes in aerobic exercise capacity following exercise training. Considering our findings with acute exercise in the second paper, a higher exercise training intensity may have been needed to see measurable changes in MP counts or function. More research is likely needed to determine how higher intensity exercise training may affect MP function in older adults, and whether there may

be any effect of age causing elevated ROS production similar to what we saw in overweight individuals.

Conclusions and Future Directions:

Our results introduce novel findings on how MP number and function contribute to inflammation caused by both diet and exercise. This dissertation achieves this by: 1) determining that high fitness level may have a protective effect against the inflammatory load posed by a high-fat meal on MPs, 2) identifying that MPs and vascular health in overweight individuals are differentially impacted by acute high-intensity exercise, and 3) providing novel insight into how exercise intensity may play an important role in determining the effect of aerobic exercise training on MP number and function in previously sedentary older adults. Our findings demonstrate that MPs are significantly involved in both dietary and exercise-related inflammation, serving as novel biomarkers with strong connections to cardiovascular disease. Furthermore, we have shown that MPs from overweight and lower-fit individuals respond differently to inflammatory stimuli compared to their lean and high-fit counterparts, respectively, indicating that they are more likely to experience forms of chronic low-grade inflammation. While MP number was elevated in the lean and high-fit groups after their respective stimuli, this may have been offset by no change or even a decrease in endothelial cell ROS production. This suggests that MP number and function should be studied in tandem to obtain a better understanding of how individuals respond to each stimulus.

Future research may significantly build on our findings from the high-fat meal study by performing a longer time course of postprandial blood sampling. This would provide deeper insight into how long MP function is affected by the high-fat meal in lower-fit individuals. In addition, a study looking at the effects of repeated high-fat meals or a high-fat diet on MP

number and function would further develop our work and give deeper significance to our findings. Combined, a longer time course and a subsequent study using consecutive high-fat meals may show how long MP function is affected and whether the rise in inflammation is chronically influenced by diet.

Subsequent studies looking at MP number and function following acute exercise may build on our work by looking at its effects on obese individuals versus overweight, and whether the inflammatory effect of high-intensity exercise is altered to a greater degree in obese individuals. This could establish further clinical relevance for our initial findings and may contribute to the development of more informed exercise recommendations for overweight and obese adults. In addition, an exercise training program using higher intensities and different modes of exercise would build upon our findings from the acute and chronic exercise studies. While we showed significant findings with acute exercise, the effect of exercise training on MP number and function in both overweight and older populations require further exploration.

Although we utilized endothelial cell ROS production following MP incubation as a measurement of MP function, there are a variety of mechanisms that could be used to further investigate MP function. MPs are tightly linked to procoagulant pathways, which can contribute heavily to the development of vascular dysfunction and atherosclerosis. As such, their contributions to the formation of thrombin and other procoagulant factors in this pathway would be highly beneficial to our understanding of MP function. In addition, we observed augmentations in MP function independent of changes in circulating MP counts. While circulating MP counts are still thought to be relevant biomarkers of inflammation, their cargo appears to be increasingly relevant when exploring their function. Future studies should put a stronger focus on how MP cargo such as miRNAs and cell adhesion molecules are affected by

both exercise and dietary inflammation as a function of fitness status and body composition. While MPs of various origins have been heavily studied previously, the field of extracellular vesicle research continues to develop. Our understanding of their function in clinical populations may be a key contributor to how MPs may be utilized as therapeutic targets in vascular dysfunction and other factors leading to the development of cardiovascular disease.

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