THESIS

THE INFLUENCE OF AEROBIC EXERCISE ON EXTRACELLULAR VESICLES IN OBESITY

Submitted by

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ABSTRACT

THE INFLUENCE OF AEROBIC EXERCISE ON EXTRACELLULAR VESICLES IN OBESITY

Purpose: Cardiometabolic disease is the leading cause of death in the United States. Metabolic syndrome describes several common metabolic parameters that increase risk of developing cardiometabolic disease(s). However, current risk factors of metabolic syndrome laid out by the American Heart Association have a poor correlation to cardiometabolic disease development. Prevention of metabolic syndrome and by extension cardiometabolic disease is perhaps the best method to combat morbidity and mortality. Exercise is one intervention that has proved not only to decrease the chance of developing chronic disease, but also reverse symptoms of those already experiencing metabolic dysfunction. Therefore, exercise could be the most potent treatment of metabolic syndrome and by extension cardiometabolic diseases. However, since exercise is a multisystemic and highly integrative stimulus, the mechanisms responsible for the beneficial adaptations of exercise are not yet fully understood.

Extracellular vesicles are a heterogeneous subclass of excreted biologically active molecules that function to facilitate cell-to-cell communication. Extracellular vesicles are released during cardiometabolic disease and in response to exercise, but their relationship to metabolic health is poorly understood. Therefore, our objective was to examine if aerobic exercise alters the plasma concentration and/or size of circulating extracellular vesicles during both an acute bout of exercise and from exercise training. We then examined the relationship between extracellular vesicle plasma concentration and our subject's characteristics such as age, sex, body mass index, percent fat mass, peak oxygen consumption, among other physiological characteristics.

Methods and Results: This study utilized plasma samples from subjects recruited from a recent clinical trial. Sedentary, overweight, but otherwise healthy men and women were invited to

participate in a SGLT2 inhibitor exercise study. Thirty adults were recruited, fifteen adults were given an SGLT2 inhibitor and fifteen were on a placebo. Here, we examined only placebo treated subjects (N=14; 6/8 M/F; 23 ± 8 y; 30.6 ± 3.8 kg/m2; mean ± SD). Subjects underwent several baseline tests including maximal and submaximal exercise tests and body composition analysis. The subjects then participated in twelve weeks of a supervised aerobic exercise intervention. The baseline tests were repeated immediately after the intervention. Blood samples were taken during the submaximal standardized exercise test that was conducted before and after the exercise intervention. Extracellular vesicles were isolated and analyzed for their concentration and size distribution using nanoparticle tracking analysis. After excluding severely hemolyzed samples, six subjects were included in the extracellular vesicle analysis (3/3 M/F; 28 ± 11 y; 30.7 ± 3.4 kg/m2; mean ± SD). Our results indicate that the acute standardized exercise bout did not elicit changes in the concentration nor the size of extracellular vesicles. Additionally, when comparing samples pre- and post-exercise training, there was no change in extracellular vesicle concentration nor size. Collectively, these data insinuate an acute bout of submaximal exercise and/or exercise training do not increase circulating extracellular vesicle concentration in sedentary obese individuals.

Linear regressions were performed, and Pearson correlation coefficients were reported in order to examine relationships between EV concentration and physiological factors. BMI, fat mass, percent body fat, lean mass, and oxygen consumption correlated with EV concentration in samples that were low to moderately hemolyzed (absorption <0.3 at 414 nm). However, the sample size was small and further investigations are needed.

Conclusion: This study did not find any changes in extracellular vesicle concentration/size in untrained or trained subjects. However, several correlations between extracellular vesicle concentration and subject characteristics were found in sedentary and trained overweight but otherwise healthy adults. This study had several limitations that could have restricted our results,

and therefore additional research is warranted to understand the connection between exercise and circulating extracellular vesicle characteristics.

ACKNOWLEDGEMENTS

The pursuit of anything worth having brings arduous challenges. These past two years presented me with a series of tasks that— at times—seemed too difficult to overcome. It was not without help from those around me that I was able to make it to this milestone, writing and completing my thesis. As I reflect back on my time in this master's degree, I have several people I owe my appreciation to.

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CHAPTER I: INTRODUCTION

I. Metabolic Syndrome Criteria and Limitations

Metabolic syndrome, also referred to as syndrome X, encompasses a cluster of compounding risk factors that increase the probability of developing cardiometabolic disease(s) such as: type two diabetes (T2D), cardiovascular disease (CVD), and stroke. According to the American Heart Association (AHA), an individual is diagnosed with metabolic syndrome if they concurrently display three or more of the following: 1) abdominal obesity (waist circumference > 102 cm in men, > 89 cm in women), 2) high circulating triglycerides (TG, ≥150 mg/dL), 3) impaired fasting glucose (≥100 mg/dL) or undergoing treatment for elevated glucose, 4) high blood pressure (systolic ≥130 and/or diastolic ≥85 mmHg) or taking medication for hypertension, 5) low high density lipoprotein (<40 mg/dL for males; <50 mg/dL for females) or on cholesterol treatment (Alberti et al. 2009). In 2012, The National Health and Nutrition Examination Survey reported metabolic syndrome prevalence to be between 27-36% in the United States. Furthermore, a longitudinal study conducted by Ridker et al. assessed typical risk factors for cardiometabolic disease in almost 25,000 women. After comparing predicted versus actual cardiovascular events, they reported that up to fifty percent of women were incorrectly classified for their risk of coronary heart disease (Ridker et al. 2007). Meaning, that even with a great effort to unify metabolic syndrome criteria, substantial disconnect between metabolic syndrome and risk of disease remains.

II. Exercise as an Intervention for Metabolic Dysfunction

Metabolic syndrome and cardiometabolic diseases are thought to be the result of excessive caloric intake via an increase in the consumption of high caloric food supplemented by increased sedentary behavior. Several studies have found that physical activity and/or exercise is effective in favorably modulating circulating lipids, plasma glucose, blood pressure, and body composition.

Furthermore, it is well known that aerobic fitness is a strong predictor of morbidity and mortality (Myers et al. 2002, Korpelainen et al. 2016). Therefore, caloric expenditure through physical activity is thought to not only have the capacity to decrease the chance of developing chronic disease, but also reverse symptoms of those already experiencing metabolic dysfunction (Vechetti et al. 2020). However, exercise induces highly complex systemic alterations, and thus mechanisms behind the beneficial adaptations from exercise on metabolic function have not yet been fully elucidated.

III. Interorgan communication

Both metabolic syndrome and exercise are complex, integrative processes and are dependent on interorgan communication or "crosstalk" between metabolically active tissues (Javeed 2019). Interorgan communication is integral in multicellular organisms and can be mediated through direct cell-to-cell contact or the transfer of secreted molecules. Crosstalk is facilitated via several extracellular molecules such as neurotransmitters, hormones, cytokines, chemokines, and extracellular vesicles (EVs).

EVs are implicated as important mediators of many diseases, including cardiometabolic diseases and metabolic syndrome (Simeone et al. 2020, Dickhout and Koenen 2018, Vajen, Mause and Koenen 2015). They are semi-protected from rapid degradation in the extracellular space and share several similarities with their originating cell. EVs are also secreted from every cell type and can mediate communication locally within a tissue and/or systemically. They are packaged with biologically active molecules such as proteins, lipids, and/or nucleic acids. After exocytosis, their contents are horizontally transferred to subsequently modify their target cell (Raposo and Stoorvogel 2013). Currently, EVs are better understood in the context of tumorigenesis, however they have promising potential as a tool for diagnoses, prognosis, and therapies for cardiometabolic diseases.

Both metabolic syndrome and exercise rely on EVs to modulate systemic adaptations, yet neither have yet to be fully understood. Therefore, the purpose of this study is to characterize how exercise alters EVs in circulation in both an acute and chronic manner in sedentary and overweight, but otherwise healthy adults, in order to understand the relationship between exercise and EVs. The long-term goal of this work is to determine whether EVs can be used as accurate and simplistic biomarkers and therapeutic tools to aid in metabolic syndrome diagnoses, prognoses and treatment in the future.

Statement of the problem

Current literature surrounding the effect of exercise on EVs in plasma is widely divergent and underdeveloped. Furthermore, the effect of exercise training on EVs in plasma is unknown. Understanding the effect of both an acute bout of exercise and exercise training on EV concentration and size may offer insight on how exercise mediates systemic beneficial adaptations. The purpose of this present study is to gain insight into how plasma EV concentration and/or size are affected by submaximal steady state exercise and if exercise training elicits effects on EV plasma concentration and/or size.

Hypotheses

We hypothesize that an acute bout of exercise will induce a systemic metabolic stress response that will in turn increase EV concentration in the plasma. Additionally, we hypothesize that exercise training will increase baseline EV concentration in the plasma, and that EV concentration will increase to a lesser extent from the same exercise stimulus after exercise training. We also hypothesize EV mean and modal size will be unaffected by exercise and exercise training.

CHAPTER II: LITERATURE REVIEW

I. Metabolic Dysfunction in Key Tissues and Metabolic Syndrome Pathogenesis

While mechanisms driving metabolic disease processes remain incompletely defined, three key tissues: the liver, skeletal muscle, and adipose tissue (AT) seem to experience the bulk of metabolic dysregulation. Hepatic steatosis, extensive adipose tissue hypertrophy and dyslipidemia are some of the first tissue alterations (McCracken, Monaghan and Sreeenivasan 2018). Insulin resistance (IR) with fatty acid (FA) flux and chronic low-grade inflammation are potential mechanisms that underly the pathophysiology of metabolic syndrome development and progression.

Insulin Resistance with fatty acid flux

Perhaps the most supported mechanism driving metabolic syndrome development is IR with FA flux (McCracken et al. 2018). Excessive caloric intake often leads to elevated circulating FAs and unhealthy storage of FAs in tissues other than AT, such as skeletal muscle and the liver. This change in proper FA regulation and storage stimulates several cellular maladaptations such as the activation of stress pathways and stress kinases. Phosphorylation of stress kinases such as mitogen-activated protein kinases (MAPKs), c-Jun N-terminal kinase (JNK), extracellular signal regulated kinase, inhibitor of nuclear factor kappa B (NFκB)-kinase (IKK), and protein kinases C phosphorylate insulin receptor substrate (IRS) is upregulated. Increased activation of IRS has been implicated in decreased insulin signaling, resulting in decreased insulin dependent glucose uptake. NFκB also interacts with peroxisome proliferator activated receptor (PPAR)γ. PPARγ is involved in adipocyte differentiation and reduction of this transcription factor limits adiponectin production. Adiponectin is not only vital in increasing FA uptake, but it also decreases glucose production in hepatocytes and increases FA oxidation in the liver and skeletal muscle. Adiponectin functions to increase systemic insulin sensitivity. Additionally, hepatic glucose production is

downregulated less with liver IR, resulting in increased glucose production by the liver. IR also increases adipocyte lipolysis resulting in high circulating FA, propelling hepatic steatosis and IR even further. Therefore, IR with FA flux is the leading hypothesis behind metabolic syndrome pathogenesis.

Chronic low-grade inflammation

Novel research implicates chronic low-grade inflammation as an additional source in metabolic dysfunction (Esser et al. 2014). AT, skeletal muscle, and the liver are sites of increased inflammation in the presence of excessive caloric intake. Macrophages and other immune cells within these tissues shift from an anti-inflammatory to a pro-inflammatory state and in turn interfere with insulin signaling in the peripheral tissue and elicit β-cell dysfunction (Esser et al. 2014). Additionally, inflammation is thought to further propel chronic disease by decreasing exercise capacity. Chronic low-grade inflammation is thought to deter movement both physically and psychologically from inflammatory-induced fatigue movement reduction and depressive symptoms, thus further propelling disease states (Golbidi, Mesdaghinia and Laher 2012).

II. Extracellular Vesicle Classifications, Biogenesis, and Uptake

Prokaryotes and eukaryotes secrete EVs to facilitate cell-to-cell communication. Furthermore, EVs have been found in a wide range of biofluids including blood, saliva, urine, breast milk, ascites fluid, and cerebral spinal fluid (Raposo and Stoorvogel 2013) and have been implicated in autocrine, paracrine, and endocrine signaling. EVs have also been divided into three main categories, apoptotic bodies, microvesicles, and exosomes (Fig. 1, Raposo and Stoorvogel 2013).

EV Classifications and Biogenesis

Apoptotic bodies are fragments of cells undergoing apoptosis. They originate from the plasma membrane, typically range in size of 500 – 2,000 nm, and facilitate phagocytosis. They have

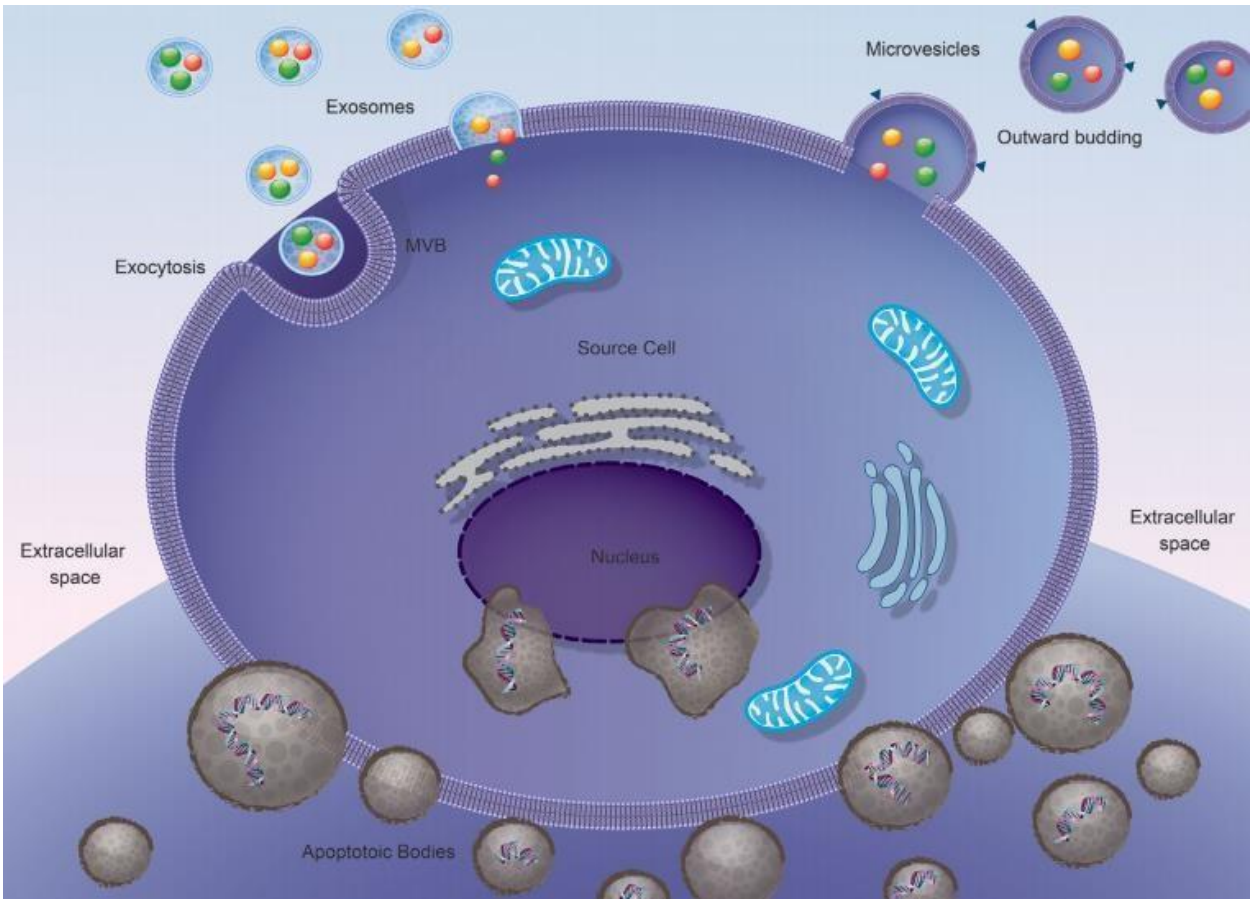

Figure 1. Graphical representation of extracellular vesicle subtypes: Apoptotic bodies, microvesicles, and exosomes (Vechetti et al. 2020).

been implicated in DNA transfer and may aid in the repair of damaged cells by signaling progenitor cell activation (Hristov et al. 2004).

Microvesicles (MV) facilitate cell-to-cell communication and also originate from the plasma membrane. These vesicles are slightly smaller than apoptotic bodies, with a typical size range of 50-1,000 nm. MVs are formed after biological material is clustered at the edge of a cell in the cytoplasm. The contents will start to push the lipid membrane of the cell outwards until the membrane separates from the newly formed vesicle. Proteins such as aminophospholipid translocases, scramblases, calpain and ESCRT-III aid in this process (Tricarico, Clancy and D'Souza-Schorey 2017).

Exosomes are the smallest of these general classifications and range in size from 40-120 nm and also function to enable cell-to-cell communication. However, unlike MVs, exosomes are derived from an endocytic pathway. Meaning, that instead of budding away from the surface of a cell, exosomes are generated from late endosomes that bud inward into a cell. Once inside a cell,

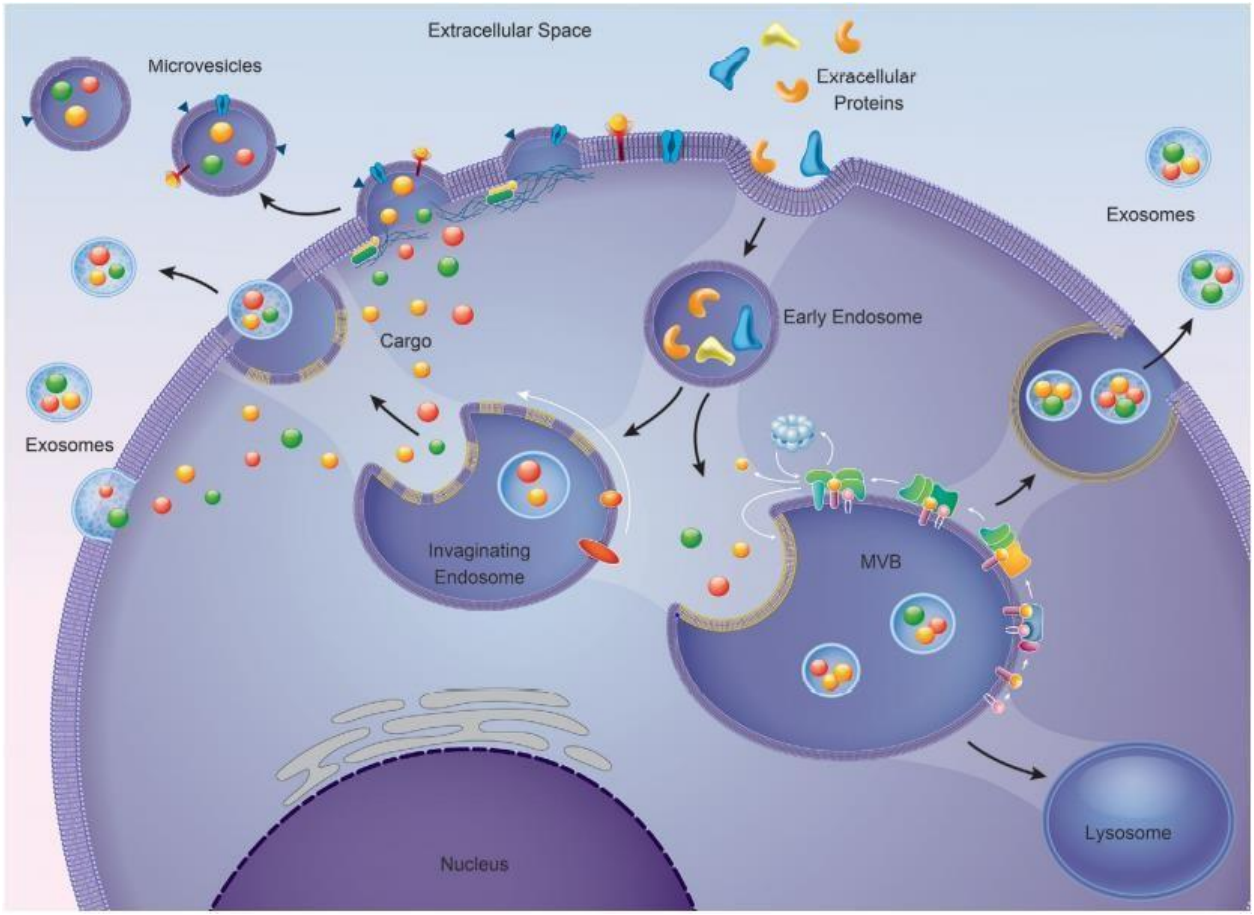

Figure 2. Graphical representation of microvesicle and exosome biogenesis (Vechetti et al. 2020).

these late endosomes are termed multivesicular bodies and are often abbreviated as MVB. MVBs are packaged with exosomes. From there these vesicles have two fates, either they will fuse with the plasma membrane to release the exosomes into the extracellular space or fuse with the lysosome for potential degradation (Fig. 2). The ESCRT proteins (ESCRT-0, -I, -II, -II, and -IV) are central to the packing and exocytosis of exosomes (Colombo et al. 2013).

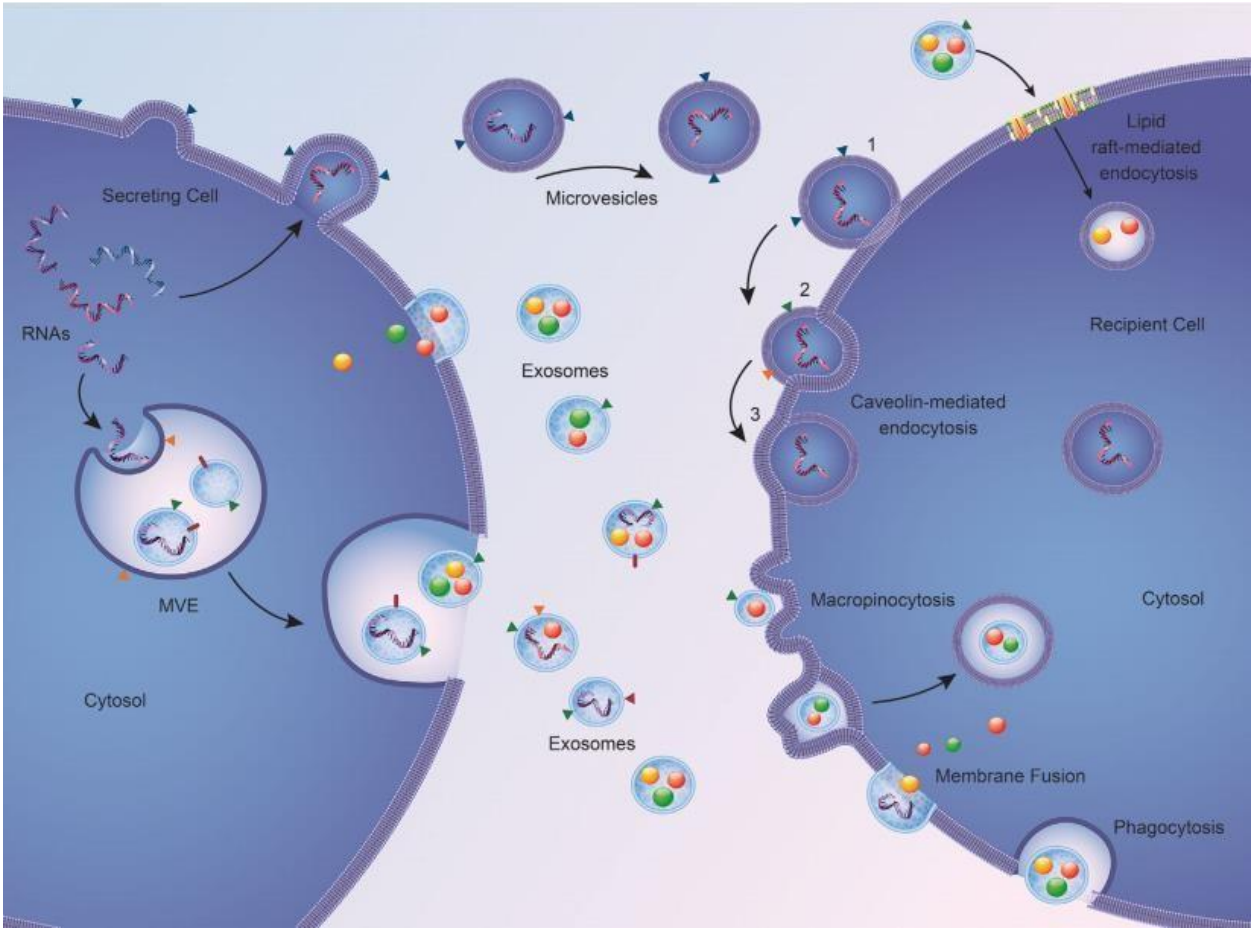

Figure 3. Graphical representation of microvesicle and exosome uptake by target cell (Vechetti et al. 2020).

Importantly, MVs and exosomes are synthesized and secreted in a regulated manner. Typically, EVs are enriched in protein markers such as CD63, CD81, CD9, ALG-2-interacting protein X (ALIX), tumor susceptibility gene 101 (TSG101), heat shock protein 70 (HSP70) and annexin V (Akbar et al. 2019). Secretion is thought to be controlled by calcium signaling and signals such as ATP, neurotransmitters, depolarization, thrombin receptor activation, and lipopolysaccharides in monocytes, oligodendrocytes, neurons, platelets, and dendritic cells, respectively can stimulate EV biogenesis (Raposo and Stoorvogel 2013). However, exact mechanisms that regulate synthesis and secretion of EV have yet to be discovered.

EV Uptake

Uptake of EVs is complex and has just started to somewhat be understood. Several molecular pathways have been implicated in endocytosis of EVs such as clathrin- or caveolin-dependent endocytosis, phagocytosis and micropinocytosis. (Fig.3, (Vechetti et al. 2020, Svensson et al. 2013). Clathrin-mediated uptake is dependent on cellular endocytosis of molecules which in turn generate clathrin-coated vesicles. These vesicles act by altering the membrane to activate inward budding of the plasma membrane (Kaksonen and Roux 2018). Whereas caveolin-dependent endocytosis functions independently of clathrin and utilizes caveolae that are internalized by the cell (Kiss and Botos 2009). Macropinocytosis is a nonselective form of endocytosis that is actin dependent. This process is initiated from surface membrane ruffles and generates large endocytic vacuoles referred to as macropinosomes, which are subsequently engulfed by the cell (Lim and Gleeson 2011). During phagocytosis, cells rely on the plasma membrane to uptake large vesicles ultimately generating a phagosome (Richards and Endres 2014).

EV uptake and degradation is further complicated by cells beyond the originating and recipient cells (Mulcahy, Pink and Carter 2014). Not only have EVs been implicated in modulating the immune response, but immune cells such as leukocytes and lymphocytes have been implicated in their uptake (Veziroglu and Mias 2020, Mulcahy et al. 2014, Freeman et al. 2018). EVs may also degrade from exposure to matrix metalloproteinase at the cell surface. Additionally, it is thought that the contents of EVs potentially mediate cellular signaling via receptor binding (Akbar et al. 2019).

III. Extracellular Vesicles and Metabolic Syndrome

Metabolic dysfunction is associated with changes in plasma concentrations and content of EVs. Research indicates that individuals with T2D have higher circulating EVs compared to their non-diabetic controls. More specifically, erythrocyte derived EVs are elevated in T2D subjects. Furthermore, IR and b-cell dysfunction have been implicated in increased secretion of EVs. Interestingly, leukocytes preferentially degraded EVs, sequentially altering leukocyte function, in

diabetics (Freeman et al. 2018). Furthermore, higher levels of EV inflammatory proteins, such as VEGF, are associated with T2D (Wu et al. 2020). Therefore, EVs are integral in T2D pathogenesis. Increasing our understanding of the role EVs play in metabolic syndrome will increase the therapeutic capacity of EVs.

IV. Extracellular vesicles and Exercise

It has been proposed that an acute bout of exercise induces secretion of EVs that stimulate beneficial systemic modifications. However, the literature is still underdeveloped and divergent. Several studies have reported contradicting reports of how exercise alters EVs. A study by Fruhbeis et al. reported that exercise induced a rapid release of small EVs into circulation. They recruited healthy subjects to perform both a cycle ergometer and treadmill maximal exercise test. After both exercise protocols, they found an increase in circulating EVs (Frühbeis et al. 2015). Brahmer et al. studied male athletes during an incremental cycling test until exhaustion. They found that exercise increased EV concentration, with the highest concentration at peak exercise. Another study conducted by Just et al. again with healthy human subjects, did not report EV concentration at all, but instead reported an increase in EV surface markers and EV miRNA content (Just et al. 2020). Rigamonti et al. compared EV profiles in obese and normal weight subjects. They found that acute exercise actually decreased EV concentration in both groups, and that EV secretion after exercise was high in their normal weight control group. Furthermore, females had lower exosomes and higher microvesicles compare to males. They also reported a tissue specific alteration within their isolated EVs. Rigamonti et al. thus concluded that EV response not only decreased to exercise, but is also tissue, sex, and BMI specific (Rigamonti et al. 2020). Guescini et al. wanted to know if skeletal muscle contributed more to the EV pool after exercise. They found an increased contribution of miRNA 206 (a miRNA found to be in high concentrations in skeletal muscle). Furthermore, they also observed a correlation between aerobic fitness and muscle-specific miRNAs. They declared that EVs are a novel means of muscle

communication (Guescini et al. 2015). Additionally, a study by Oliveia Jr. et al. found that acute aerobic exercise in rats increased concentration with increased exercise intensity but did not affect EV size (Oliveira et al. 2018).

CHAPTER III: METHODS

I. Human Subjects

This study utilized plasma samples from a previous randomized, double-blind repeated measures parallel study in which thirty sedentary, overweight, but otherwise healthy men and women were invited to participate in a twelve-week supervised aerobic exercise intervention. Half of the subjects were randomly selected to consume a daily dose of a SGLT2 inhibitor while the other half were selected to consume a placebo. Inclusion criteria included the following 1) between 18 to 50 years old, 2) between 25 to 45 kg/m^2 body mass index (BMI), 3) maintained a sedentary lifestyle (no more than two weekly exercise bouts that were longer than twenty minutes in the past two years) and 4) willing and able to participate in endurance exercise. Subjects were excluded if there was evidence of cardiovascular, respiratory, renal, hepatic, pulmonary, gastrointestinal, hematological, neurologic, psychiatric, or other diseases that may alter the subject's physiological adaptations, thereby jeopardizing the safety of the subjects and interfering with metabolic/performance outcomes. Furthermore, subjects were excluded if they were currently using prescription drugs, unless the prescription fell under the following categories: birth control or gastroesophageal reflux disease, seasonal allergy, and/or depression medication. Additionally, subjects were also prohibited if they used tobacco within the previous 2 years or were pregnant and/or breastfeeding (Fig. 4).

For the purposes of this study, we utilized the plasma samples from those who were on the placebo, to avoid any potential effects of the SGLT inhibitor. Plasma samples from fifteen subjects were originally available for analysis; however, one subjects samples were unable to be located after storage in the freezer. Therefore, fourteen subjects were originally included in this study (Table 1).

Figure Four- Participant Screening

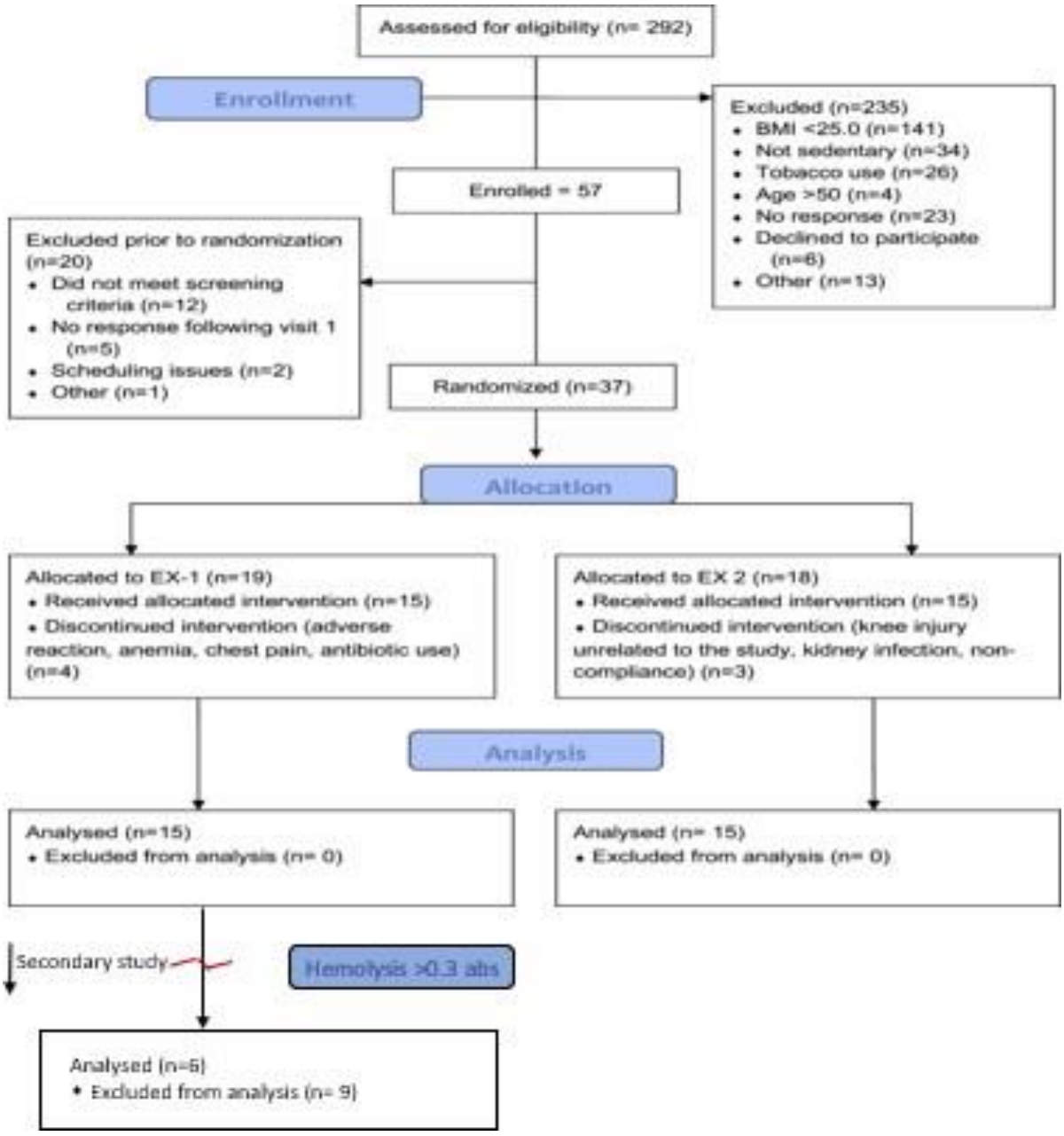

Figure 4. Participant screening process from original study. 292 participants originally contacted the research lab. 57 fit the parameters of the study. Several were excluded for various reasons and 37 were randomized into either the intervention or control group. 7 subjects failed to conclude the study after starting the exercise program.

Table One: Subject Characteristics

Male/Female	6/8
Age (years)	24 ± 8
Height (m)	1.72 ± 0.09
Body Mass (kg)	88.6 ± 12.0
BMI (kg/m^2)	30.6 ± 3.8
Blood Pressure (mmHg)	125/79 ± 8/7
VO_{2peak} (L/min)	2.7 ± 0.8
VO_{2peak} ($mL/Kg_{body\ weight}$/min)	30.1 ± 6.3
VO_{2peak} ($mL/Kg_{fat\ free\ mass}$/min)	47.5 ± 8.5
Fasting glucose (mg/dL)	75.8 ± 5.1
Fasting insulin (mU/L)	8.9 ± 6.7

II. Experimental Design for Exercise Intervention

This trial was registered at ClinicalTrials.gov (identifier: NCT02371187) and conducted in accordance with the Good Clinical Practice guidelines. All procedures were reviewed and approved by the institutional review board at Colorado State University in accordance with the principles set out in the Declaration of Helsinki. All participants were provided a written informed consent and were verbally instructed of risks before signing or any involvement in the study.

Exercise Protocol Overview

This study was a repeated measures clinical trial in which sedentary and overweight, but otherwise healthy, men and women participated in a 12-week supervised endurance exercise training program. A third party, employed by the Health and Exercise Department at Colorado State University, blinded the researchers and subjects to all data.

Baseline testing

The screening visit encompassed questions about the subject's medical history and habitual physical activity, a physical examination, and an exercise stress test. During the exercise stress test, the subjects' blood pressure and heart were monitored to ensure a healthy response from rest to maximal exercise. An adult blood pressure cuff, a 12-lead electrocardiogram, and a stationary electronically braked cycle ergometer were used. The same cycle ergometer was used for all exercise testing (Dynafit Velotron; Racermate Inc., Seattle, WA).

During a follow up visit, a dual-energy x-ray absorptiometry (Discovery W, QDR Series; Hologic, Bedford, MA) was used to measure body composition. Additionally, height and body weight were measured using a physician's stadiometer and balance. These data were used for BMI calculations. Waist circumference measurements were taken midway between the lower border of the costal margin and the uppermost border of the iliac crest with a tape measure.

Then, subjects underwent a continuous incremental exercise test from rest to volitional fatigue. This was used to determine oxygen consumption (VO_2) at ventilatory threshold (V_T) and maximal oxygen uptake (VO_{2max}). Resistance was selected in accordance with the screening visit so that the duration of the test lasted between 10-12 min. VO_2 at V_T was determined by visual inspection of breath-by-breath data in agreement with established criteria. The criteria being: an increase in the slope of the line showing the relation between CO_2 produced and VO_2 in which arise in the ventilatory equivalent for the volume of O_2 and in end-tidal O_2 pressure with no simultaneous rise in the ventilatory equivalent for CO_2 production, or a decrease in end-tidal CO_2 pressure. Maximal oxygen uptake was determined with the greatest mean breath-by-breath volume of O_2 consumed for one continuous minute. Maximal oxygen uptake was confirmed if subjects achieved at least three of the following criteria: 1) a plateau in VO_2 despite increasing work rate, 2) an RER ≥ 1.15, 3) a final HR within 10 beats/min of the age-predicted maximal HR (208 − (0.7 × age)), and/or 4)

subjective fatigue. Breath-by-breath indirect calorimetry (Parvo Medics, Sandy, UT) and heart rate (HR) were measured for the duration of the test. HR was collected via short-range telemetry (Polar Electro, Inc, Lake Success, NY).

On a subsequent day, subjects completed a standardized graded exercise test. After a self-paced five minute warm up, three 10-minute stages of were completed. Resistance for stages one, two and three were set at 25, 50, and 100 watts, respectively. VO_2, respiratory exchange ratio (RER), HR, ratings of perceived exertion (RPE), and circulating concentrations of lactate (2900 STAT Plus Glucose Lactate Analyzer; YSI Inc., Yellow Springs, OH) were recorded within the final two minutes of the three stages.

Additionally, an oral glucose tolerance test (OGTT) was conducted. Subjects were asked to abstain from consuming anything other than water 12 hours prior along with abstaining from vigorous exercise 24 hours prior to the start of the visit. For the OGTT, ten milliliters (mL) of venous blood were collected immediately before ingestion of 75 grams of glucose diluted in water. Subjects were instructed to consume the entire glucose drink within five minutes. Ten milliliters of blood were repeatedly and methodically sampled over two hours at 5, 10, 20, 30, 45, 60, and 120 minutes post glucose consumption. Blood samples were immediately analyzed for glucose and lactate concentrations (2300 STAT Plus Glucose Lactate Analyzer, YSI Inc.) and insulin (enzyme-linked immunosorbent assay; Crystal Chem, Inc., Elk Grove Village, IL). Insulin sensitivity was estimated via the Matsuda index.

Endurance Exercise Training Protocol

The aerobic training program began with subjects completing three bouts of a self-selected duration of 20 to 40 minutes of work. All exercise visits began with a five-minute warm up and ended with a five-minute cool down, in which subjects did not have to remain within a set intensity.

Steadily, subjects increased the duration of exercise (self-selected) from week one until week four. At week four, exercise visits increased to four per week with a minimum of 40 minutes per session. By week eleven or before, subjects exercised for 60 minutes. Subjects reported their HR and RPE four times throughout each supervised exercise visit and were instructed to stay within 70-80% of their heart rate reserve. Subjects rotated through treadmill walking/running, stationary cycle ergometer, and elliptical ergometer for exercise visits. The exercise modalities were varied between every visit, but not during the same visit. Subjects were to complete 44 exercise sessions, those who failed to complete 40 sessions or two sessions within 7 days were eliminated from the study.

III. Experimental Design for EV Isolation and Storage

Blood Sample Collection and Storage

Plasma samples collected during the submaximal exercise test were stored in EDTA tubes and placed on ice. The samples were then spun at 3,600 RPM for 10 minutes at 4°C to remove red blood cells and the buffy layer. From there, the plasma was allocated into several 1.5 mL Eppendorf tubes and frozen at -80 °C.

The plasma samples were then thawed and spun again at 18,000 x g for 30 minutes at 4°C to remove platelets. The supernatant was collected into a new tube, the platelets were discarded to generate platelet free plasma (PFP). The samples were once again frozen at -80°C.

Hemolysis Analysis

The samples were analyzed for their hemolysis with the Nanodrop 2000 (ThermoFisher Scientific, USA). The absorbance was set at 414 nm and 2 uL of the PFP was placed on the pedestal. The pedestal was cleaned with 0.22 um filtered water and ethanol between readings. Samples that

were severely hemolyzed (absorbance >0.3) were excluded. If one PFP sample for a subject was excluded, all of the subject's data was excluded from subsequent analysis.

EV Isolation

250 uL of the platelet free plasma was incubated with 2.5 uL of thrombin at room temperature for five minutes with constant mixing. Thrombin functions to precipitate fibrinogen to fibrin. Fibrin is a protein involved in the formation of blood clots and is therefore highly abundant in plasma, but not in EVs. Therefore, the thrombin incubation was included in the EV isolation protocol to aide in removal of plasma specific proteins in order to better enrich for EVs. After thrombin incubation, samples were spun at 9,600 x g for five minutes.

A visible white fibrin pellet was formed post thrombin incubation. The supernatant was aspirated off and the plasma was moved to a new 1.5 ml Eppendorf tube. ExoQuick (EQ, EV Isolation System for Serum and Plasma; System Biosciences, LLC Palo Alto, CA) was then added to the sample at 1:4 ratio (63 uL to the 250 uL plasma sample). Samples were inverted and quickly vortexed, then incubated at 4°C for one hour. Immediately post EQ incubation, samples were spun at 1.5 x g for 30 minutes at 4°C. A visible off-white EV pellet formed. The supernatant was aspirated off and discarded. The sample was spun again at 1,500 x g for 5 minutes at 4°C. The remaining liquid was aspirated and discarded.

250 uL of PBS was added to the EV pellet formed. The sample was pipetted and vortexed to resuspend the EV pellet as quickly and effectively as possible. Once the pellet was completely suspended (about ten minutes on the plate shaker), the sample was aliquoted into smaller volumes for subsequent analysis, then placed in -80 °C for later use.

EQ is activated when introduced to a certain salt condition. The polymer then generates ‘‘polymer nets" that capture EVs ranging in size between 60–150 nm. The polymer bags of EVs make it possible to recover EVs at lower speeds negating the need for ultracentrifugation. EQ isolates all

types of EVs independent of their composition, increasing total EV yield. The PBS resuspension works to dilute and dissolve the polymer nets to liberate intact EVs. This rapid and effective isolation protocol results in higher EV yields than ultracentrifugation, chromatography, and antibody-coupled magnetic bead methods (Peterson et al. 2015).

IV. EV Analysis

The 250 uL sample of isolated EVs were then analyzed for EV isolation quality and for EV concentration and size. A Bicinchoninic Acid (BCA) assay was performed to analyze an overall reduction in sample protein. Polyacrylamide Gel Electrophoresis was performed to ensure a reduction in plasma specific proteins such as albumin in EV samples compared to PFP. An Exo-Check immunoassay (System Biosciences, LLC; Palo Alto, CA) was performed to ensure an enrichment of EV specific markers. Then, nanoparticle tracking analysis (NTA, Manta/Horiba ViewSizer 3000; Irvine, CA) was performed to measure EV concentration and size.

BCA Assay

Both the EVs and PFP were analyzed for protein concentration. The BCA was conducted per protocol with the limited protein protocol followed. In short, 10 uL of standards or diluted sample were added to each well. EV and plasma samples were diluted to 1:50 and 1:100 respectively. The working reagent was made per protocol and 200 uL were added to each well. The standards and samples were run in triplicate. The 96 well plate was then incubated at 37°C for thirty minutes. Absorbance was measured at 562 nm on a Spectramax M2 spectrophotometric plate reader (Molecular Devices). The data was exported to Excel where the protein concentration was determined based on the slope and y-intercept of the protein standards. Samples were re-analyzed if the SD was greater than 10%. All standard curves had r^2 values greater than 0.97.

Polyacrylamide Gel Electrophoresis

After conducting the BCA assay, EVs samples were compared to their PFP samples to show a reduction in albumin, the most abundant plasma protein. For this samples were prepared according to the NuPAGE sample preparation and sample buffer preparation protocol. In short, samples were prepared by combining the sample, NuPAGE LSD sample buffer, NuPAGE reducing agent, and PBS. Samples were prepared at a final protein concentration of 1 ug/uL. Samples were then incubated at 70°C for ten minutes, placed on ice, and then loaded on to a 4-12% Bis-Tris Gel (NuPAGE). 7 ug (7 uL) of protein was loaded in each well. The gels were then washed and stained according to the SimplyBlue stain protocol.

Exo-Check assay

To ensure enrichment of EVs, an Exo-check antibody array (System Biosciences, LLC Palo Alto, CA) was performed on select samples. This assay was conducted per the manufacturers protocol. In short, a PVDF membrane embedded with antibodies for GM130, FLOT-1, ICAM, ALIX, CD81, and TSG 101. GM130, a protein present in the Golgi and therefore acted as a cellular contamination marker. All other markers are reported to be highly enriched in EVs. The membrane was imaged using the FluorChem E by ProteinSimple.

Quantification of EV markers was performed using ImageJ. EV markers were normalized to the negative control and then analyzed for their relative increase compared to GM130.

Nanoparticle Tracking Analysis

Nanoparticle tracking analysis was performed to analyze EV concentration and size distribution. For this, we used the Manta/Horiba Viewsizer 3000. The Viewsizer utilizes the properties of both light scattering and Brownian motion to report the concentration and size distribution of particles in liquid suspension. Several EVs are tracked individually and simultaneously. The Stokes-Einstein equation is used to determine the diameter of particles.

This instrument is unique and novel because it utilizes three spectrally distinct lasers to simultaneously measure a wide range of particle sizes. This is an improvement over single laser instruments that have limited detection range due to the exponential relationship between size and light scattering.

All NTA measurements were performed by a trained technician. Each sample analysis began with making sure the equipment was as clean as possible. This was ensured by adding 0.22 μm filtered water to the cuvette and visually inspecting the contents of the cuvette with the ViewSizer 3000 computer program. Once the technician ensured minimal particle presence, she added the sample directly into the cuvette. Two videos were captured and analyzed. Once a concentration of 100-150 particles per video was reached (to avoid over or underestimation of the particle concentration), 25 videos were captured and analyzed. Each video was 10 seconds with a 3 second mix in between each video. The following recording settings were used: Frame Rate = 30 fps, Exposure = 15 ms, Gain = 30 dB, Stir time between videos = 5 seconds, Wait time between videos = 3 seconds, Blue Laser Power = 210 mW, Green Laser Power = 12 mW, Red Laser Power = 8 mW, Frames per video = 300, Videos Recorded = 25, Temperature = 22C. Videos were processed using ViewSizer software using the following settings as recommended by the manufacturer: Detection Threshold Type = Manual, Detection Threshold = 0.8, Auto Threshold = Disabled, Feature Radius = 40 pixels, Drift Correction = 0%. EV concentration is reported as particles/mL/mg tissue.

Image Quantification

Both the SDS gels and Exo-check antibody PVDF membrane were quantified with ImageJ (Version 1.52a).

Statistical Analysis

Anthropomorphic data collected before and after exercise training was compared using parametric, paired Student t tests. EV data were compared by either a two-way analysis of variance (ANOVA) or linear regression analysis. The level of statistical significance was set at $p < 0.05$. Data are reported as mean and standard deviation, unless otherwise indicated. The statistical calculations were done with statistical software (GraphPad Prism 8.0, Graphpad Software Inc., CA, USA).

CHAPTER IV: RESULTS

I. Plasma Hemolysis

There was no correlation between hemolysis and EV concentration and size when all subjects were included (Fig. 5A). However, when only considering low to moderately hemolyzed samples, there was a modest correlation of $r = 0.56$ (Fig. 5B). With this cut off value, only six subjects were included in the subsequent analysis (Table 2).

Table Two: Subject Characteristics with an Absorbance <0.3

Male/Female	3/3
Age (years)	28 ± 11
Height (m)	1.73 ± 0.1
Body Mass (kg)	92.0 ± 12.5
BMI (kg/m^2)	30.7 ± 3.4
Blood Pressure (mmHg)	123/78 ± 3/7
VO_{2peak} (L/min)	2.7 ± 0.6
VO_{2peak} ($mL/Kg_{body\ weight}$/min)	29.1 ± 5.0
VO_{2peak} ($mL/Kg_{fat\ free\ mass}$/min)	45.4 ± 4.4
Fasting glucose (mg/dL)	75.0 ± 5.4
Fasting insulin (mU/L)	8.5 ± 9.0

Figure Five- Hemolysis in Plasma

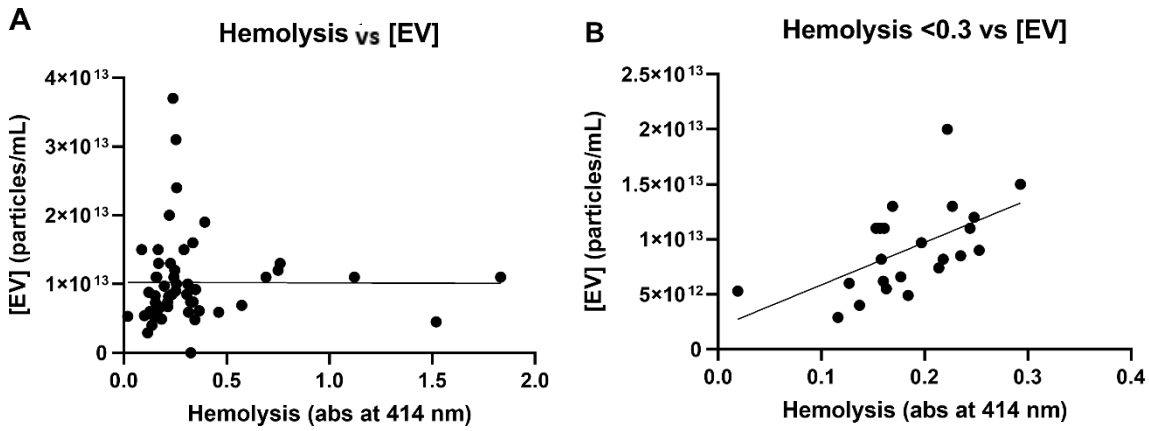

Figure 5. There was no correlation between hemolysis and EV concentration (A, n = 14) however when only analyzing samples with a <0.3 absorbance at 414 nm, a correlation of 0.56 was observed (B, n=6).

II. Exercise Training Response

Endurance exercise training favorably modifies metabolic parameters such as maximal work rate and HR, at a given workload. In this study, maximal work rate during the maximal exercise test increased after training (Fig. 6A), with an average increase of 43.8 ± 23.1 Watts. Baseline HR and HR at the end of the 100-Watt bout decreased (Fig. 6B/C), with an average decline of 6 ± 10 bpm and 17 ± 11 bpm respectively. These data taken together suggest that the exercise intervention was effective.

III. Submaximal Exercise Response

An acute bout of exercise should increase metabolic demand via an increase in RER, oxygen consumption (VO_2), and HR. In this study, RER (Fig. 7A/B), HR (Fig. 7C/D), and VO_2 (Fig. 7E/F) all increased during both the baseline and follow up standardized exercise tests, with an average increase of 68 ± 22 bpm, 0.71 ± 0.25 L/min, and 0.07 ± 0.04, respectively $p < 0.001$). These data suggest that the submaximal exercise induced metabolic stress.

Figure Six- Aerobic Training Effects

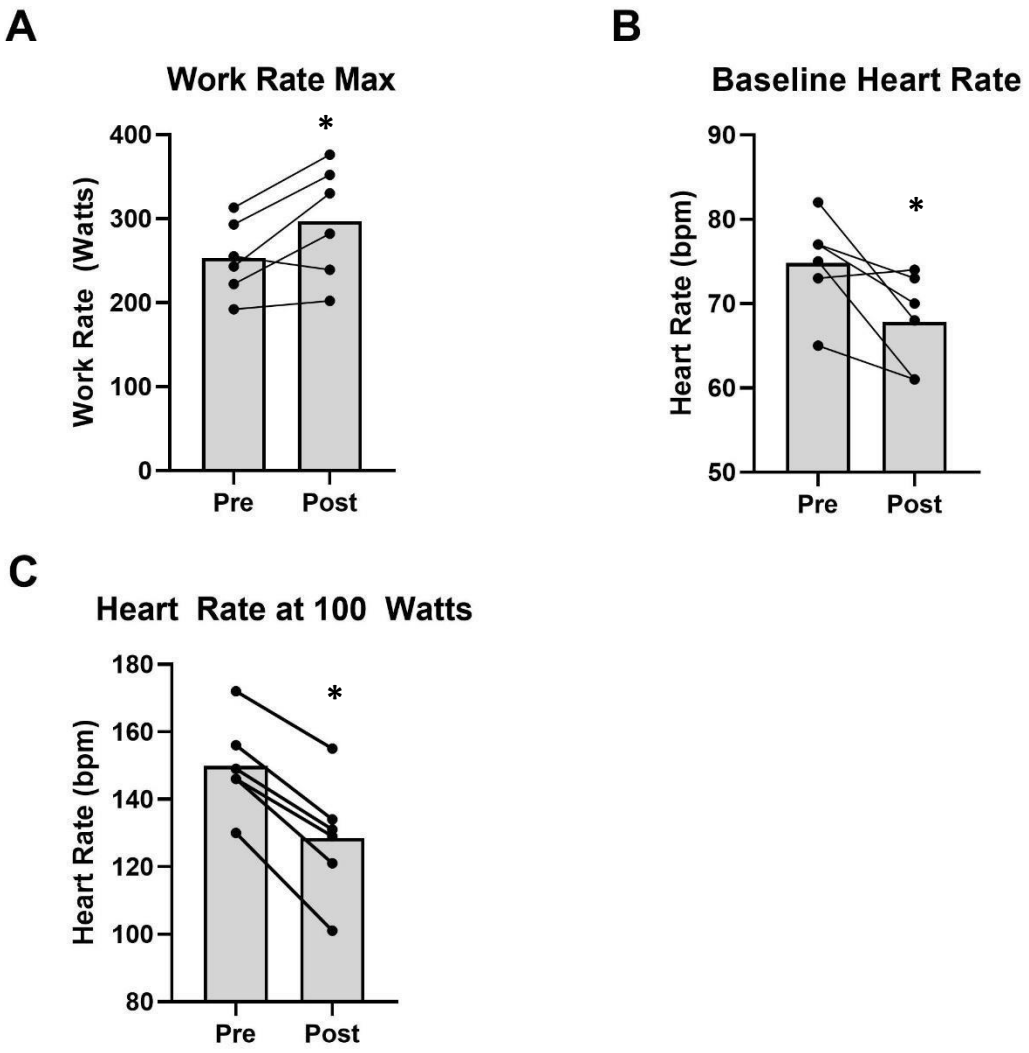

Figure 6. Exercise training increased maximal work rate (A), decreased baseline HR (B) and decreased heart rate at 100 Watts of resistance (C) (n = 6, p <0.05). * indicates significance.

IV. Extracellular Vesicle Isolation Verification

The blood samples were isolated for EVs via Exoquick precipitation. We verified EV isolation with several assays.

Figure Seven- Acute Submaximal Bout of Exercise Effects

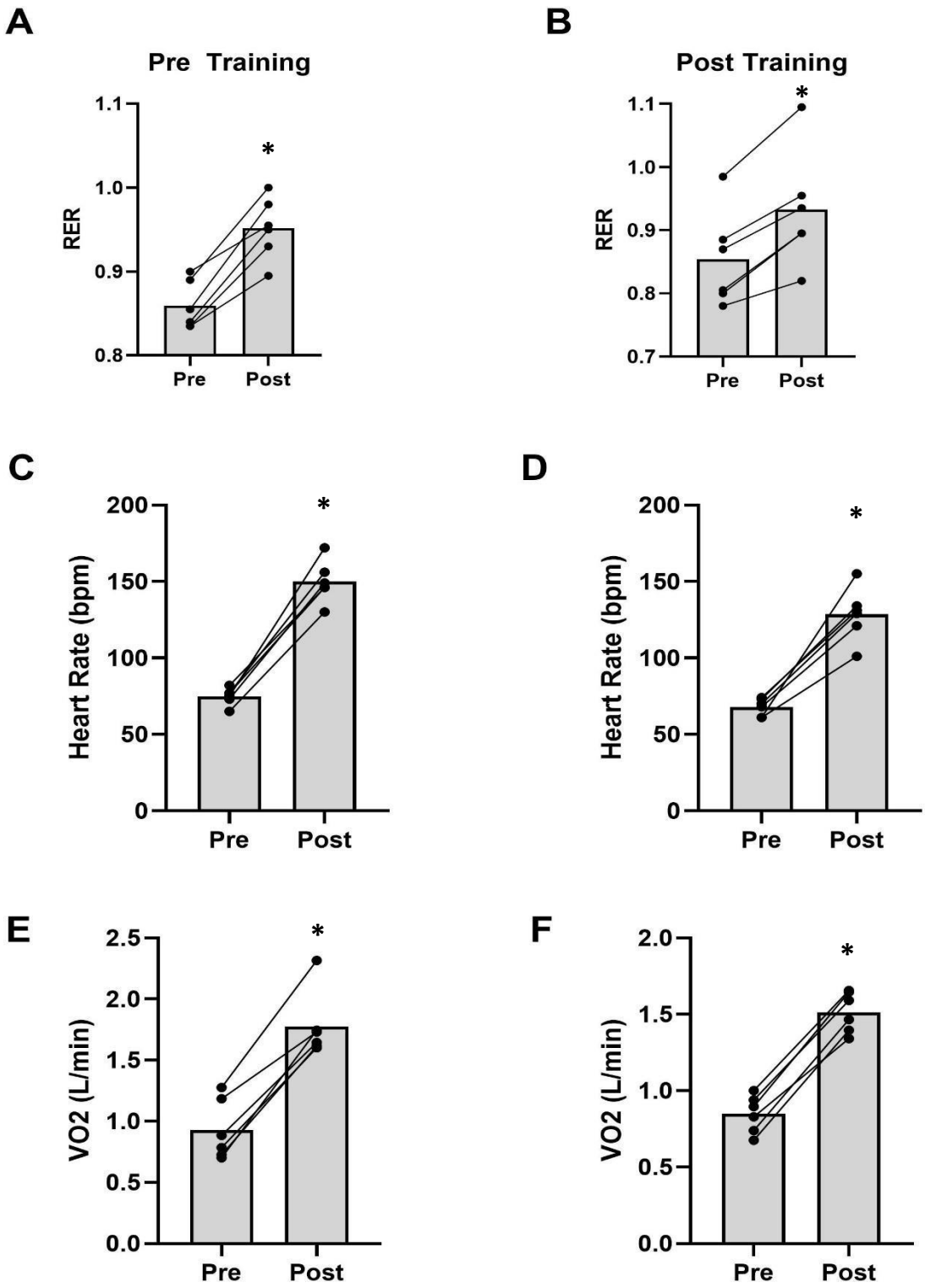

Figure 7. The standardized exercise test induced metabolic stress reflected by the increase in RER (A/B), HR (C/D) and oxygen consumption (E/F) from baseline (pre) to the end of the exercise bout (post, n=6, $p < 0.001$). * indicates significance.

The BCA assay gave us insight as to whether the protein concentration was different between the PFP and isolated EVs and allowed us to standardize the protein in the Polyacrylamide Gel.

Electrophoresis assay and the Exocheck assay. Protein concentration in the PFP was on average 61.7 ± 28.4 ug/uL. Average protein concentration in the isolated EV samples was 16.5 ± 4 ug/uL. Therefore, EV isolation reduced the protein concentration 45.2 ± 26.7 ug/uL or about 300% on average. The BCA assay confirmed that the EV isolation decreased the protein concentration (Fig. 8A, $p < 0.001$).

The Polyacrylamide Gel Electrophoresis assay segregated the protein in the PFP and EV samples by size. This assay gave insight into the crude change in albumin presence between the PFP and EV samples. Albumin is the most abundant plasma protein but has not been reported in EVs. Our isolation reduced the contribution of albumin to total protein by about 250% on average (Fig. 8B/C, $p < 0.001$).

The Exocheck assay probed for several EV specific surface markers and GM130 (a protein in the cis-Golgi stack). GM130 presence was used as an indicator of cellular contamination. The Exocheck assay showed a substantial enrichment of EV markers FLOT-1, ICAM, ALIX, CD81, CD63, EpCAM, ANXA5, and TSG101 relative to GM130 (Fig. 8D/E).

These data suggest our EV isolation protocol was sufficient in decreasing plasma proteins and cellular contamination, as well as enriching our sample with EVs.

V. Extracellular Vesicle Concentration and Size Distribution

After confirmation of EV isolation, NTA was utilized to measure EV concentration and size distribution. The average baseline EV concentration before training was $9.18 \times 10^{12} \pm 4.62 \times 10^{12}$ particles/mL (mean = 102 ± 13 nm, mode 67 ± 7 nm) and 9.52×10^{12} particles/mL (mean = 95 ±

8 nm, mode = 67 ± 6 nm) after training. At the end of the acute bout of exercise before training the EV concentration was 8.04 x 10^{12} particles/mL (mean = 99 ± 12 nm, mode = 68 ± 7 nm) and 1.17 x 10^{13} particles/mL (mean = 98 ± 8 nm, mode = 66 ± 5 nm) after training. There was no change in EV concentration nor size at baseline or at the end of the acute bout from exercise training (Fig. 9).

Interestingly, several subject characteristics correlated with EV concentration. BMI, fat mass, percent body fat highly correlated to baseline EV concentration (Fig. 10A-C) and after the submaximal exercise bout (Fig. 11A-C) conducted before exercise training, with Pearson correlation coefficient values of r = 0.69, 0.76, 0.89 at baseline and r = 0.78, 0.81, and 0.58 post the acute bout respectively. The submaximal exercise bout conducted after exercise training exhibited a similar correlation between BMI, fat mass, and percent body fat (Fig. 10D-F and 11D-F). However, the correlations all decreased slightly, with the correlation between baseline EV concentration and percent body fat diminishing (Fig. 10F). BMI post training had a correlation value of r = 0.59 at baseline and r = 0.47 post the submaximal exercise, fat mass had a value of r = 0.47 at baseline and r = 0.59 post exercise, and percent body fat had a r = 0.20 correlation at baseline and r = 0.51 post exercise.

There was also a correlation between lean mass before the acute bout of r = -0.62 and EV concentration and between lean mass and the change in EV concentration of r = 0.55 at baseline (Fig. 12A/B). Only the change in EV concentration correlated with lean mass with a value of r = -0.63 (Fig. 12C/D). Relative and absolute oxygen consumption strongly correlated with baseline EV concentration pre training as well, with correlations of r = -0.71 and r = -0.81 respectively (Fig. 13A/B). Post training absolute and relative oxygen consumption correlated with the change in EV concentration during the acute submaximal bout with values of r = -0.62 and r = -0.60 respectively (Fig. 13C/D). Further, the percent of the working oxygen consumption relative to maximal oxygen consumption (VO_2working/VO_2maximal) at the end of both pre- and post-training submaximal

Figure Eight- EV Isolation Verification

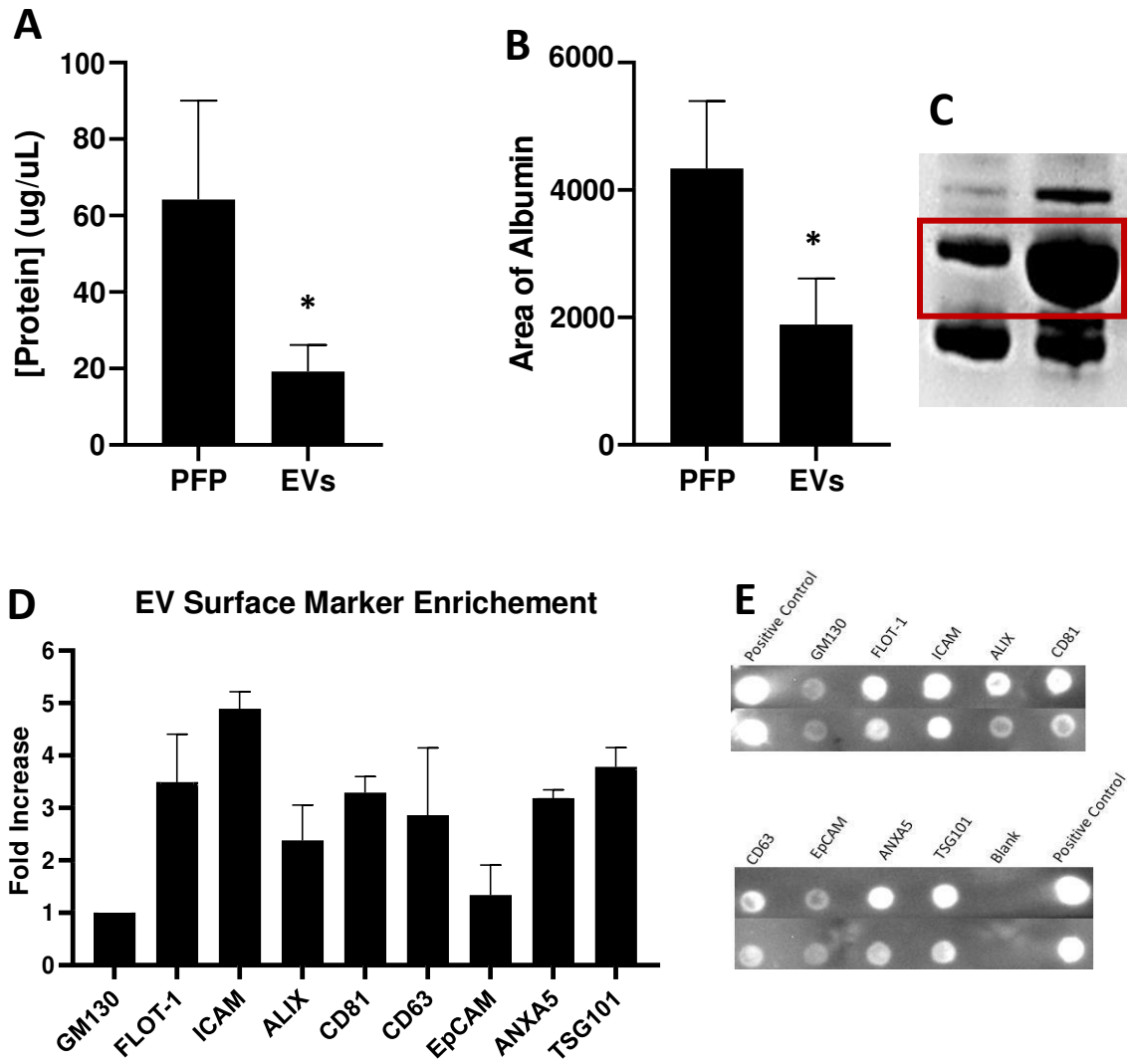

Figure 8. Extracellular Vesicle Isolation Verification: Protein concentration was reduced (A, n=5, $p < 0.001$). Albumin was reduced (B/C, n=5, $p < 0.001$). EV markers were enriched with minimal cellular contamination (D/E, n=2, $p = 0.001$).

exercise bouts had large, negative correlations with the change in EV concentration, $r = -0.90$ and $r = -0.83$, respectively (Fig. 13E/F).

Figure Nine- Exercise Effects on EV Concentration and Size

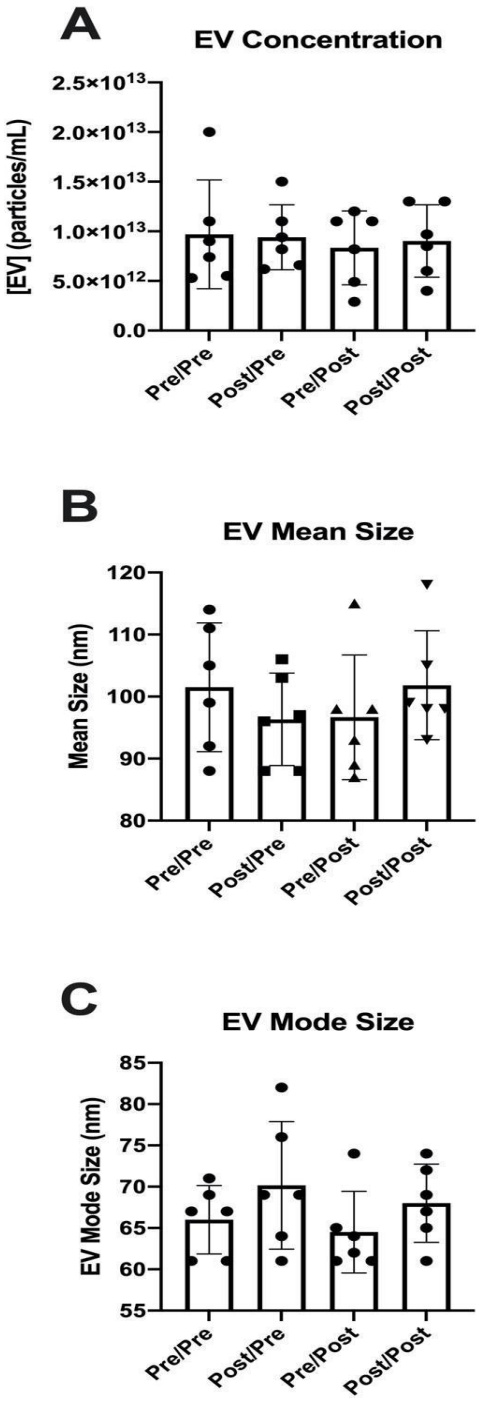

Figure 9. There were no changes in EV concentration (A), EV mean size (B), nor EV mode size (C) were observed during the acute bout of submaximal exercise (n = 6) Pre/Pre indicates before the acute submaximal exercise bout and before training. Post/Pre indicates after the acute bout and before training. Pre/Post is before the acute bout and after training. Post/Post is after the acute bout and after training.

Figure Ten- EV Concentration Correlates with BMI, Fat Mass, and Fat Percent Pre-Aerobic Training

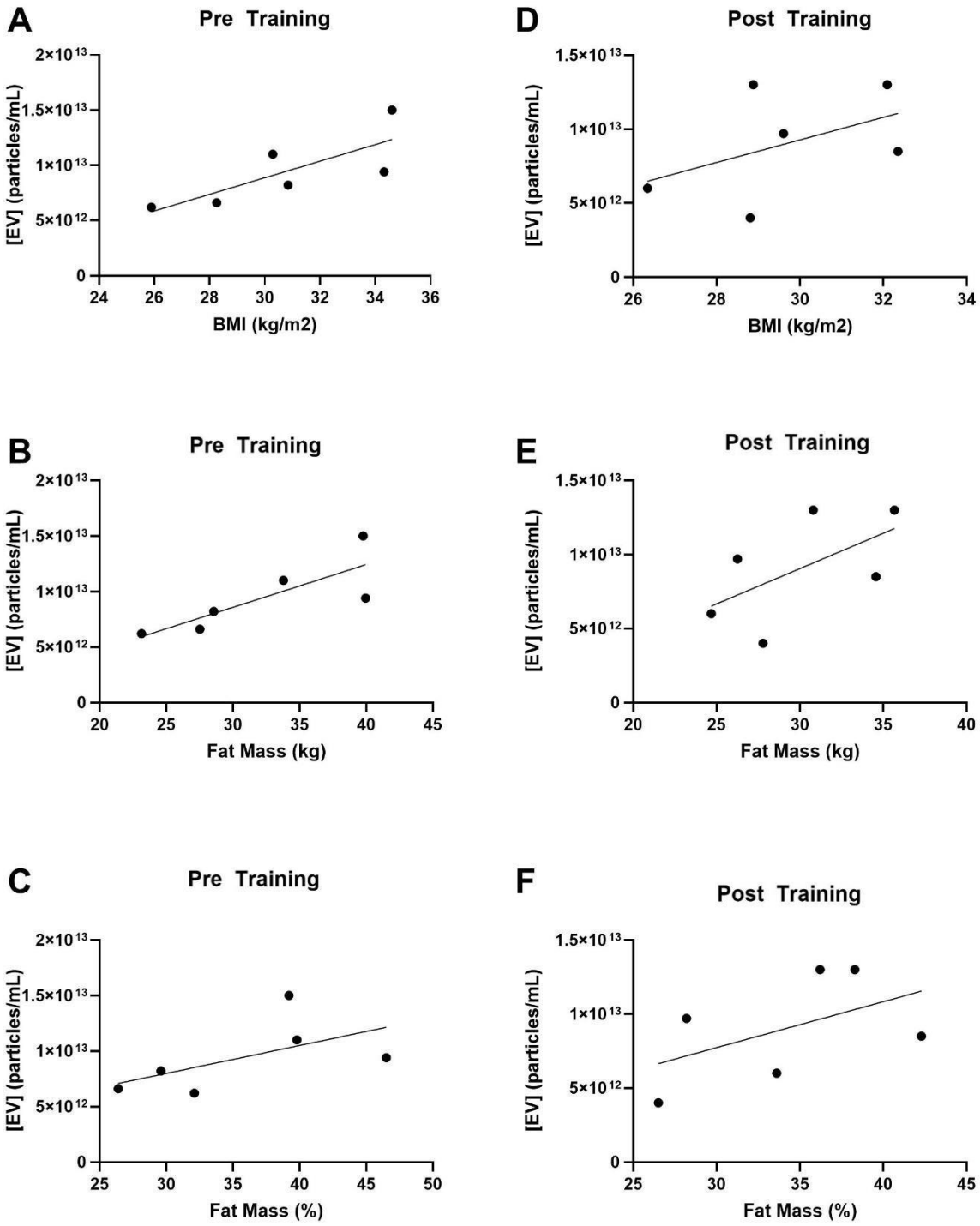

Figure 10. There was a moderate to strong correlation between EV concentration and BMI (A/D), Fat mass (B/E), and percent fat (C/F) before and after exercise training at baseline, with the exception of percent body fat with no longer correlated after training (F, n = 6).

Figure Eleven- EV Concentration Correlates with BMI, Fat Mass, and Fat Percent Post Aerobic Exercise Training

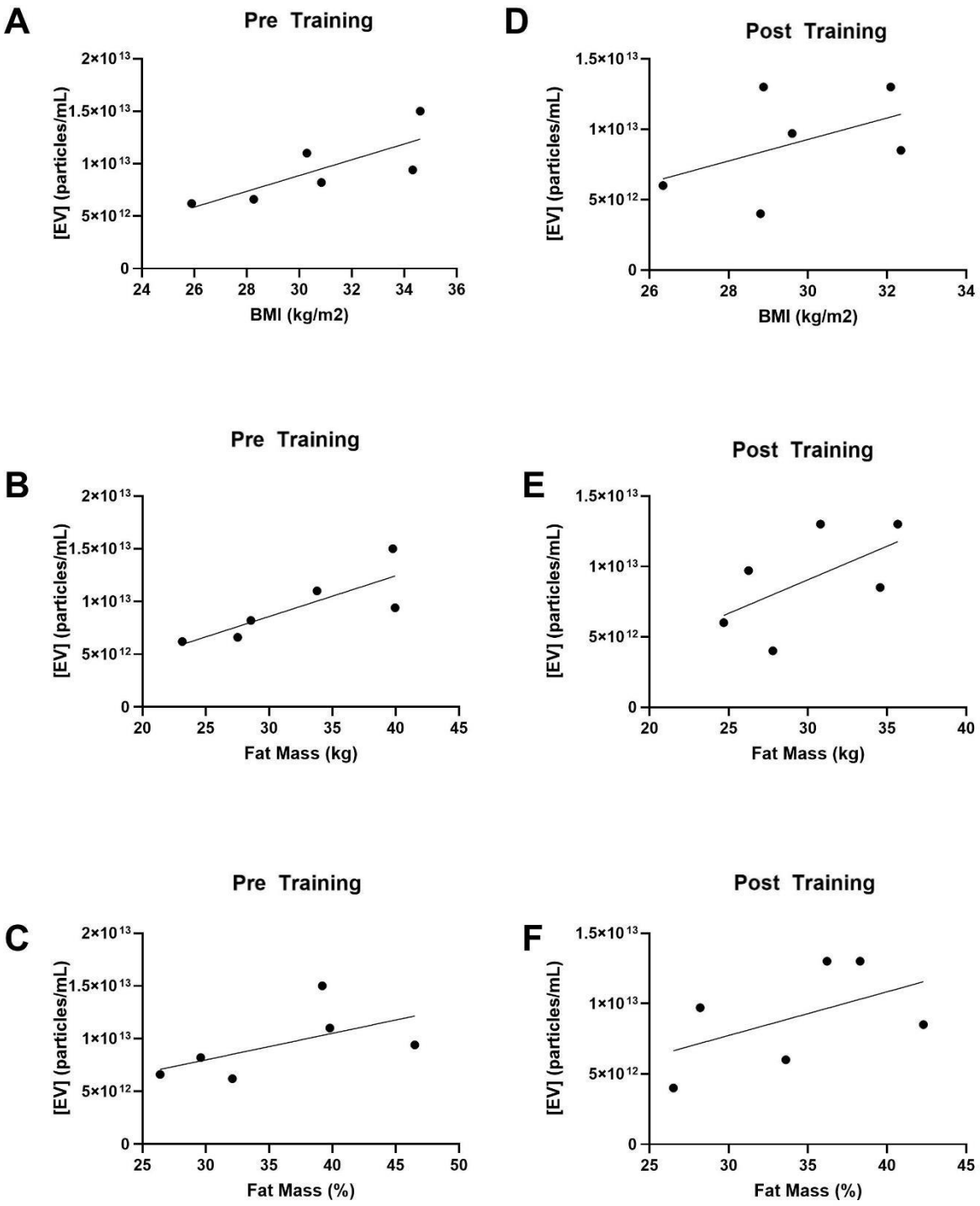

Figure 11. There was a correlation between EV concentration and BMI (A/D), Fat mass (B/E), and percent fat (C/F) before and after exercise training at baseline, with the exception of percent body fat with no longer correlated after training (F, n = 6).

Figure Twelve- EV Concentration Correlates with Lean Mass

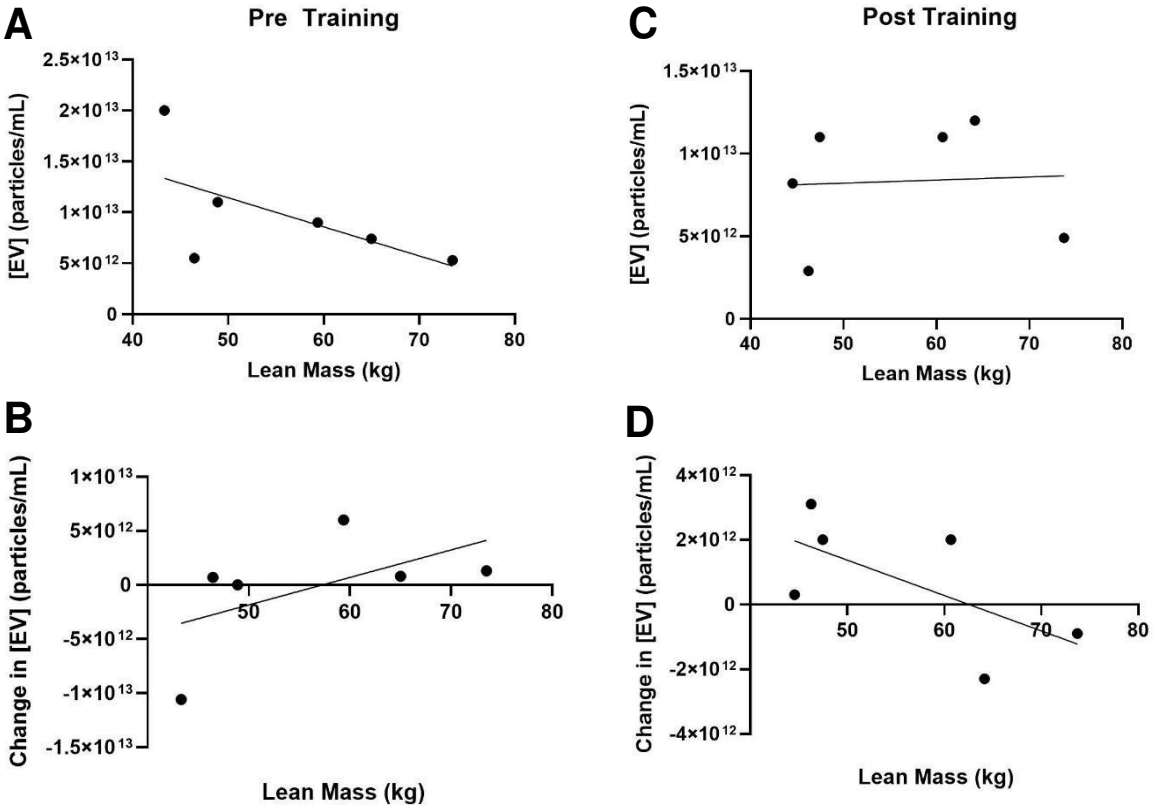

Figure 12. There was a correlation between EV concentration and the change in EV concentration and lean mass at baseline before and after exercise training (n = 6).

Figure Thirteen- EV Concentration Correlates with Maximal Oxygen Uptake

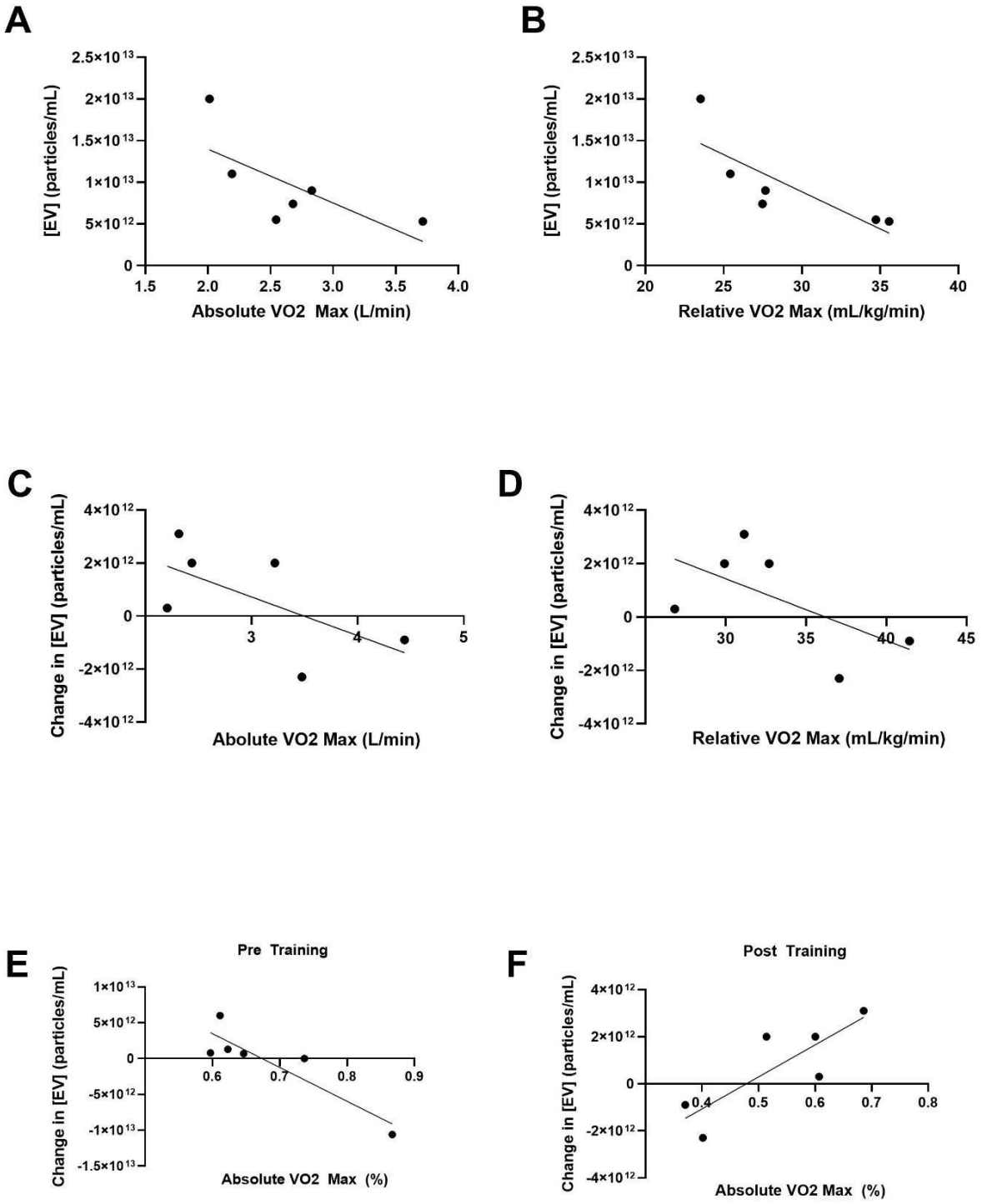

Figure 13. There was a correlation between EV concentration and absolute and relative maximal oxygen consumption before training (A/B). There was also a correlation between the change in EV concentration during the acute bout of exercise and the absolute and relative maximal oxygen uptake after training (C/D). Finally, there was a correlation between the change in EV concentration and the percent of absolute oxygen consumption subjects were working at both before and after training (E/F, n = 6).

CHAPTER V: DISCUSSION

Exercise is associated with improved prognoses and potential reversal of obesity, T2D, CVD, and stroke symptoms (Sattelmair et al. 2011). Furthermore, EVs are rapidly growing in popularity as potential novel biomarkers and therapies of almost every chronic disease including aforementioned diseases (Simeone et al. 2020, Dickhout and Koenen 2018, Vajen et al. 2015). Understanding how plasma EVs are altered with exercise could provide valuable insight into how exercise elicits its beneficial effects as well as increase our understanding of disease pathogenesis and aide in prevention (Eichner, Erdbrügger and Malin 2018). Therefore, the current study was conducted to examine the influence of aerobic exercise on plasma EV concentration and size. We hypothesized that an acute bout of exercise would induce a systemic metabolic stress response that would in turn increase EV concentration in the plasma. Additionally, we hypothesized that exercise training would increase baseline EV concentration in the plasma, and that EV concentration would increase to a lesser extent from the same exercise stimulus after exercise training. We also hypothesized EV mean and modal size would be unaffected by exercise and exercise training. The primary findings from this protocol are that an acute exercise bout and exercise training may not robustly influence EV concentration nor size. However, after considering hemolysis, preliminary prospective correlations between body mass, BMI, fat mass, fat percentage, lean mass, peak and working oxygen consumption, and EV concentration showed large, positive correlations in low to moderately hemolyzed samples. Importantly, because several characteristics were related to EV concentration, multiple regression analyses could be used to better determine the most significant correlates of circulating EV level. However, due to the of the small number of subjects we chose to utilize single linear regressions. Further investigations between subject characteristics and EVs is needed to confirm our initial findings.

Interestingly, several studies analyzing plasma EVs during exercise do not report nor discuss hemolysis in their plasma samples (Frühbeis et al. 2015, Oliveira et al. 2018, Rigamonti et al. 2020, Brahmer et al. 2019, Whitham et al. 2018). Perhaps they, like us, found no correlation between hemolysis and EV concentration nor size (Fig. 7). However, one study by Just et al. excluded samples that were affected by hemolysis. They approximated hemolysis using a Nanodrop 2000 and set the absorbance to 414 nm to estimate free hemoglobin. Any samples with an absorbance of >0.2 were excluded. There was no explanation as to why this was the cut off value they selected, and upon further investigation a cut off value of 0.3 absorbance has been found to sufficiently segregate samples at risk of moderate to severe hemolysis (Shah, Soon and Marsh 2016). Intriguingly, after only considering low to moderately hemolyzed samples, EV concentration moderately correlated with hemolysis (Fig. 7B). Based on this cut off value, six subjects were included in our analysis.

We did not observe differences in plasma EV concentration or size in response to a submaximal exercise bout either before training or after exercise training. This bout was standardized between all individuals and ended with a ten-minute cycling segment at 100 Watts. The average working oxygen consumption at this resistance was 1.73 ± 0.23 L/min, which equated to an average of 68 ± 16% (36%-94%) of their peak oxygen consumption. Other studies that observed changes in EV characteristics used exercise protocols that stressed participants more, such as working until exhaustion (Frühbeis et al. 2015, Just et al. 2020, Rigamonti et al. 2020, Brahmer et al. 2019) or running at 60% VO_{2max} or until exhaustion (Rigamonti et al. 2020). Those that ran at 60% of their VO_{2max} had an average max HR of 170 and 166 bpm in the obese and normal-weight groups whereas our subjects only reached a max HR of 149 and 131 bpm pre- and post-training. Additionally, a study in mice by Oliveira Jr. et al. showed that exercising mice had a higher EV concentration compared to non-exercised mice (Oliveira et al. 2018). Interestingly, low and high intensity exercisers had the highest concentration, while medium intensity exercisers EV

concentration was higher than non-exercisers and lower than the low and high exercisers. Therefore, our submaximal exercise protocol may not have been a sufficient stressor to induce an increase in EV concentration.

Interestingly, several different research groups have reported contrasting changes in EV concentration after an acute bout of exercise. This may be due to the fact that changes in EVs concentration in the plasma may be dependent on the fitness level of the participants and/or the modality of exercise. A study conducted by Rigamonti et al. evaluated plasma EVs in obese (BMI > 40 kg/m^2) and normal weight subjects before and after they ran at 60% of their VO_{2max} for 30 minutes or until exhaustion. Subjects were reported to be moderately active, participating in 60 minutes of physical activity twice a week. They found that their subjects, both obese and normal weight decreased their plasma EV concentration. Their findings are inconsistent with the general consensus in the field of extracellular vesicles, which is that stress such as exercise should increase EV concentration. Additionally, in line with the field, studies on trained male athletes reported an increase in EV concentration after running and/or cycling until exhaustion (Frühbeis et al. 2015, Brahmer et al. 2019). Just et al. also reported an increase in EV concentration five minutes post blow flow-restricted resistance exercise in recreationally active males (Just et al. 2020).

We expected exercise to cause an increase in EV concentration. However, the acute bout of exercise caused some subjects to increase their EV concentration, while others decreased. If metabolic stress does increase EV concentration in the blood, it could be that the rate of disappearance varied between subjects. Most if not all studies that have measured EV characteristics *in vivo*—including this one—fail to measure or consider the uptake or degradation of EVs. Recent studies have provided insight as to how EVs disappear from circulation. There is of course the uptake from the target cell via several different pathways and may depend on the proteins and glycoproteins on the surface of the vesicle and target cell (Mulcahy et al. 2014).

There has also been published data to suggest that EV concentration in circulation is dependent on immune cell activity (Eitan et al. 2017, Murphy et al. 2019, Freeman et al. 2018). That is, the higher the b cell and macrophage activity, the more rapid uptake and clearance from circulation. This could explain why we saw a slight decrease of EV concentration during the first submaximal exercise bout. Since our subjects were overweight and sedentary individuals, it is possible that they were already in a pro-inflammatory state and by exercising, immune cells were even more upregulated, thus increasing EV clearance during exercise (Nieman and Wentz 2019). During the post submaximal exercise bout, EV concentration slightly increased. This difference in EV response during the two bouts could be due to exercise training shifting the subjects from a pro-inflammatory state to a more anti-inflammatory state. This is also in-line with other studies findings. The study by Rigamonti et al., which recruited obese and non-aesthetically trained individuals, observed a decrease in EV concentration post exercise whereas studies that recruited trained individuals observed that exercise increased EV concentration in plasma (Frühbeis et al. 2015, Whitham et al. 2018, Rigamonti et al. 2020). However, without testing immune responses we cannot confirm this without further investigation.

In conclusion, our study found no change of EV concentration nor size from an acute bout of submaximal exercise or exercise training. However, this study found a potential correlation between baseline EV concentration and select subject characteristics. Much is still needing to be elucidated about EV kinetics and biodistribution in response to exercise.

I. Limitations

Study Design and Blood Samples

The sample size of this study was small ($N = 6$) and limited the overall power to detect changes in this study. Next, there was no control group, reducing our ability to detect the impact of being overweight on our results. All subjects were similar in fitness and physical activity prior to the

study. A more diverse subject recruitment would add additional insights and increase our understanding of the exercise effects on EVs. Diet was not controlled prior to the submaximal exercise nor during the exercise training however it is suspected to alter EV secretion. For example, palmitic acid (a common saturated fatty acid) and an oral glucose load suppressed EV secretion (Maly and Hofmann 2020, Eichner et al. 2019). Furthermore, fasting increases EV secretion (Flaherty et al. 2019). Therefore, diet may be a confounding factor that impacts our conclusions.

Additionally, the original collection of blood only removed the red blood cells and buffy coat layer, but not the platelets. Therefore, the platelets could have contributed to the EV count artificially. It has been well described that platelets contribute the greatest majority of EVs into the blood and secrete EVs in response to shear stress (Antwi-Baffour et al. 2015). The longer the platelets were in the plasma, the greater chance of a non-physiological contribution of EVs from the platelets. Next, the samples underwent several freeze thaw cycles. They were frozen after the original blood collection, thawed and refrozen after the platelet spin, for the EV isolation and for the NTA analysis. There were also stored for a substantial period of time (> 1 year). The study was designed to minimize freeze thaw cycles; however, long-term storage and repeated freezing and thawing could cause an unknown degree of EV degradation.

EV Research Limitations

EV research is a relatively new field of study. The first reports of EVs can be traced back to the early 1980's when two lab groups, Harding, Heuser and Stahl, along with Pan and Johnstone, almost simultaneously reported reticulocytes dumping their contents in 50 nm vesicles extracellularly (Harding, Heuser and Stahl 1984, Pan and Johnstone 1983). The lack of uniformity between scientists in the terminology, description, and classifications of EVs research has and still continues to hinder progression of EV research, leading to conflicting reports of EVs (Théry

et al. 2018). Originally, EVs were thought to only function to excrete cellular components that were no longer needed by the cell (Johnstone et al. 1987). While this is certainly a function of EVs, the majority of the field now understands that they have much bigger implications in maintaining systemic homeostasis through intercellular signaling, cellular stress response, maturation, and development (Grillo 1970, Allan et al. 1976, Pan and Johnstone 1983, Anderson 1969, Ali, Sajdera and Anderson 1970). However, even with great attempts to characterize EVs, there are still substantial gaps in the literature, frequent misinterpretations of EV data, and lack of uniformity in the description between research groups. This is further complicated by the small size of EVs making characterization even more difficult.

The size of EVs present massive challenges in research. The size of these vesicles is difficult to research for multiple reasons. First, the technology for studying single EVs is not widely accessible nor reliable yet. Many have relied on equipment like flow cytometers for EV analysis, however a standard cell flow cytometer is not ideal for studying sub-micron sized particles like EVs. This machine uses hydrodynamic focusing to allow one single droplet of solution to cross the laser interrogation point at a time. This single droplet has ideally one single cell encapsulated in it. When using this same machine for something as small as an EV, the chance of coincident events is greatly increased. This limitation can be slightly mitigated with highly trained lab personnel coupled with very deliberate gating strategies, but single EV analysis is needed to confirm our current understanding.

Next, both the nanoparticle tracker and flow cytometer, along with other EV analysis technology, use lasers to describe EVs. However, light is not a continuous straight beam but moves as a wave. Therefore, the small size of EVs increase the chance that the particles will elude the interrogation point, and thus go unanalyzed. Even with some of the most powerful lasers, EVs can still be missed. This results in the under estimation of EV concentration and overestimation of EV size.

EV Isolation

Currently the most widely used isolation methods include ultracentrifugation, ultrafiltration, size exclusion chromatography (SEC), antibody-based affinity capture, and polymer-based precipitation (Konoshenko et al. 2018). Each isolation method presents unique advantages and disadvantages. Ultracentrifugation is the current gold standard in this field. This is done by pelleting small particles at high speeds for a long duration, such as 100,000 x g for an hour or overnight. Unfortunately, high g-forces elicit effects on the purity and yield of EVs. Furthermore, different cell lines have been found to pellet very differently (Jeppesen et al. 2014). Contamination is also high in these samples since this centrifugation pulls all particles in the size range of EVs into the pellet.

Ultrafiltration is similar to ultracentrifugation by the fact that ultrafiltration does not target a specific attribute within the EV sample. Instead, this is a protocol in which a series of filtration steps of varying pore sizes is used. The downside to this isolation method is that this is both a timely isolation and EVs are known to bind to the filters, decreasing sample yield (Konoshenko et al. 2018).

SEC allows for separation of molecules by hydrodynamic radius. However, several plasma proteins such as albumin and apolipoproteins can co-precipitate with EVs with SEC alone. Therefore, ultrafiltration and/or ultracentrifugation is suggested along with SEC to remove free proteins and lipoprotein contaminations (Konoshenko et al. 2018, Diaz et al. 2018). Additionally, proteinase K, a serine proteinase, used with the above methods has been found to increase purity further (Diaz et al. 2018). Importantly, treatment with proteinase K can digest EV transmembrane proteins. Furthermore, this method can be more time consuming that other methods.

Antibody-based affinity capture targets lipids, proteins, and polysaccharides that are exposed on the surface of EVs. EV isolation with antibodies typically target tetraspanins, heat shock proteins, and MHC antigens that are hypothesized to be enriched in the EV population (Konoshenko et al. 2018). EVs are extremely heterogeneous so by targeting specific surface markers, many EVs will not be captured in the isolation, thereby underestimating EV particles along with enriching for specific populations of EVs (Willms et al. 2018).

Polymer-based precipitation is the second most used isolation method after ultracentrifugation (Konoshenko et al. 2018). This method requires sample incubation of polyethylene glycol (PEG) and undoubtably provides the advantage of a rapid and simple protocol. Additionally, a standard benchtop centrifuge can be used, reducing the specialized equipment required. Particle size isolated with PEG is similar to ultracentrifugation and ultrafiltration, however EV particle yield is substantially increased (Konoshenko et al. 2018). Unfortunately, co-precipitation of contaminates such as albumin, thrombin, and other plasma molecules elicit markedly decreased purity compared to other isolation methods (Konoshenko et al. 2018). To negate some of these contaminating substances the Exoquick protocol includes the preliminary step of a thrombin incubation. This thrombin incubation precipitates a large majority of the fibrinogen protein that would otherwise contaminate the EV pool. Arakelyan et al. advised the omittance of thrombin, stating that this precipitation step may entrap many EVs, leading to an underestimation of particle count (Arakelyan et al. 2016). However, since PEG precipitation displays a higher EV yield and contamination than other isolation methods, we still included this step.

CHAPTER VI: CONCLUSION AND FUTURE DIRECTIONS

In summary, this study aimed at characterizing the plasma concentration and size distribution of EVs in response to an acute bout of exercise and exercise training in sedentary overweight individuals. The stress induced by aerobic exercise appeared to not change the concentration nor size of EVs in circulation. Furthermore, there was also no effect of exercise training on the concentration nor size distribution. Both of these observations aligned with our hypothesis that the size would remain unaffected, however, no change in the concentration of EVs went against our original hypothesis. Additionally, there were several correlations between baseline EV concentration and the subject's characteristics. Having a similar group of subjects may have limited our results. Future investigations are necessary to understand if exercise has an acute and/or chronic effect on circulating EVs with larger and more diverse sample sizes. Research studies including a wider range of subjects as well as a more intense exercise protocol could give additional insight into this relationship. Additionally, this study was limited by only analyzing EV concentration in plasma, not the contribution of EVs from skeletal muscle nor the secretion and uptake rates at the level of the tissue. Therefore, further probing into how exercise alters EVs in plasma is needed. For this reason, our group is also utilizing a specialized Cre recombinase "dual fluorescent reporter" mouse model to distinguish between vesicle populations. This mouse expresses a membrane targeted tdTomato red fluorescent protein in all cells except skeletal muscle and an enhanced green fluorescent protein (GFP) only in skeletal muscle cells. Therefore, all skeletal muscle EVs will be the only EVs to display this GFP. With the help of fluorescence-assisted vesicle sorting, RNA-seq, and protein mass spectrometry, we will be able to characterize skeletal muscle specific EVs. With careful consideration of our current findings and the data collected with the mouse model, future investigators should focus on characterizing the effect of exercise on EVs and the promising capability of EVs as potential therapeutic tools and as biomarkers of metabolic dysfunction.

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