IMPACT OF EXERCISE TRAINING AND FEEDING ON CIRCULATING EXTRACELLULAR VESICLES IN ADULTS WITH OBESITY

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ABSTRACT

First described by Wolf in 1967 as only "cell dust", extracellular vesicles (EVs) have emerged as novel biomarkers and/or mediators of chronic diseases such as cardiovascular disease (CVD) and type 2 diabetes (T2D). As both exercise and diet interventions are known to reduce CVD and T2D risk, it is clinically relevant to determine the impact of such interventions in relation to EV count and subtype. Despite this relevance, most previous work in the field lack sensitivity to optimally enrich and phenotype EVs, as the current approach of frozen samples and conventional flow cytometry may impact the clarity and precision of results. Therefore, the combination of fresh blood samples and imaging flow cytometry to characterize EVs in relation to feeding and exercise interventions is crucial for understanding the roles EVs may play in cardiometabolic disease risk and progression. The focus of Aim 1 was *to characterize EVs utilizing fresh blood samples and imaging flow cytometry in relation to cardiorespiratory fitness*, a strong independent predictor of all-cause mortality and CVD. We found that in adults with obesity, those with lower levels of fitness (15.4±0.6 ml/kg/min) had higher levels of total, Annexin V- (AV) platelet ($CD31^+/CD41^+$) and endothelial-derived ($CD31^+/CD41^-$) EVs than those with higher levels of fitness (25.9±3.0 ml/kg/min), independent of age or body fat. Aim 2 was *to determine the impact of high-glucose feeding on EV count in adults with both normal glucose tolerance (NGT) and prediabetes (PD)*. We found that regardless of glucose status, total, AV+ endothelial ($CD31^+/CD41^+$) and AV+ $CD31^+$ platelet endothelial cell adhesion molecule 1(PECAM-1) EVs were significantly lowered following a 75 g oral glucose challenge, whereas postprandial elevations in total EVs were also related to decreased arterial stiffness and increased insulin resistance. In Aim 3, *the impact of short-term exercise intensity on circulating EVs in adults with prediabetes was determined*. We found that while 12 days of training had no effect on platelet or leukocyte EVs, interval exercise significantly decreased the endothelial EV AV- CD105 compared with continuous training. Those individuals who had the greatest improvements in cardiorespiratory fitness saw the greatest decreases in AV- CD105,

supporting our findings in Aim 1 that EV count may in part be modulated by differences in fitness. When accounting for changes in dietary sugar consumption, however, the intensity effect was no longer significant, supporting our results presented in Aim 2 that showed EVs might also be modulated by high glucose conditions. Taken together, our findings from this work suggests that both dietary and exercise interventions modulate EV count in conjunction with changes in clinical outcomes such as cardiorespiratory fitness, arterial stiffness and insulin resistance.

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APPROVAL OF THE DISSERTATION

This dissertation, "Impact of Exercise Training and Feeding on Circulating Extracellular Vesicles in Adults with Obesity," has been approved by the Graduate Faculty of the Curry School of Education in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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DEDICATION

This work is dedicated with love to my parents and family, especially John and Esther Eichner, Fausto and Marie Tomassoni, and finally, Christopher Lewis. I thank God everyday for your continuous support and encouragement.

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CHAPTER 1: INTRODUCTION

Nearly 40% of adults in the United States have obesity and this number is expected to increase in the upcoming decades. This is concerning since obesity is a major and independent risk factor for development of chronic diseases including cardiovascular disease (CVD) (10), the leading cause of death worldwide. As such, it is of paramount importance and clinical relevance to study CVD risk assessment in individuals with obesity. However, the American Heart Association estimates that the current biomarkers utilized to assess CVD risk, such as blood pressure and cholesterol, account for only 40-50% of known risk (9). A greater understanding of this remaining unknown risk will allow for improved CVD assessment and treatment in the future.

Extracellular vesicles (EVs) have emerged as novel biomarkers of CVD and are elevated in various diseased states, including obesity and type 2 diabetes. Derived from various types of cells, such as the endothelium, platelets and leukocytes, EVs are membrane-bound particles generated in response to cell activation, injury or apoptosis. Despite a great interest in the field, the lack of standardization of nomenclature or methodology in the pre- and post-analytical phases of analysis has led to conflicting results in the present literature thus making comparisons difficult. However, utilization of fresh blood samples and imaging flow cytometry, combined with rigorous characterization of EVs through cryo-electron microscopy, sizing, and Western blotting, would allow for improved sensitivity and accuracy in analysis of EVs in relation to diseases such as obesity, type 2 diabetes, and CVD.

As exercise and diet are key mediators of health and intimately related to chronic diseases such as obesity and CVD, it is relevant to study the response of EVs to these interventions. To date, previous literature has reported acute bouts of exercise to increase endothelial EV count, with little effect on platelet or leukocyte-derived EVs (2, 5, 6, 8, 11). It is important to note that these effects may be temporal in nature and depend upon when the EVs were characterized in relation to the exercise bout. Conversely, chronic exercise training appears to decrease endothelial, platelet, and

leukocyte EVs (1, 3, 7). However, these results may be confounded by other factors such as weight-loss. Therefore, it is important to study the effect of short-term exercise training on EV count and subtype, independent of weight loss, in relation to relevant clinical outcomes. Like acute bouts of exercise, current *in-vitro* work has suggested hyperglycemic conditions to increase endothelial-derived EVs (4). To our knowledge, no work has been done examining the effects of a high glucose load *in-vivo* in adults with obesity.

To fill these aforementioned gaps in the current literature, we proposed to examine the impact of exercise and feeding on circulating EVs in adults with obesity utilizing fresh blood samples combined with imaging flow cytometry. Consequently, three aims were developed. In Aim 1, we sought to first characterize Annexin V+ (AV+) and Annexin V- (AV-) total, platelet endothelial cell adhesion molecule (PECAM) ($CD31^+$), platelet ($CD31^+/CD41^+$), endothelial (CD105; $CD31^+/CD41^-$) and leukocyte ($CD45^+$; $CD45^+/CD41^-$) derived EVs, utilizing fresh blood samples and imaging flow cytometry, in relation to a well-known independent risk factor for CVD, cardiorespiratory fitness. This work would serve as pilot data for subsequent aims. Based on previous literature, we hypothesized that these EVs would be elevated in individuals with lower levels of fitness as assessed by VO_2peak. To further delineate the potential clinical relevance of these EVs, we also assessed them in relation to other outcomes such as blood pressure, arterial stiffness (assessed by augmentation index, utilizing aplanation tonometry), and glucose tolerance (assessed using a 75 g oral glucose tolerance test (OGTT)). We found that adults with obesity classified as having very poor fitness (n=13; 15.4±0.6 ml/kg/min) had significantly higher levels of AV- platelet ($CD31^+/CD41^+$) and endothelial ($CD31^+/CD41^-$) derived EVs when compared to obese adults with higher levels of fitness (n=13; 25.9±3.0 ml/kg/min), independent of age or body fat. Elevations in the endothelial EV $CD31^+$/CD41- were also related to increased pulse pressure, whereas the endothelial EV AV- CD105 was related to postprandial hyperglycemia, suggesting clinical relevance.

Postprandial elevations in blood glucose are a better predictor of CVD compared to assessment of fasting glucose alone. However, the manner in which postprandial hyperglycemia confers greater risk is unclear. Characterization of EVs in the postprandial state may help better understand this risk. Our pilot data from Aim 1 suggests that circulating endothelial-derived EVs are related to postprandial hyperglycemia in adults with obesity. Therefore, we hypothesized that postprandial hyperglycemia may be a key modulator of EV release in adults with obesity. To test this hypothesis, Aim 2 was developed in which we characterized the EV response to a 75 g OGTT in adults with both normal glucose tolerance (n=7; fasting plasma glucose: 95.2±1.3 mg/dl) and prediabetes (n=18; fasting plasma glucose: 104.9±2.1 mg/dl) utilizing fresh blood samples and imaging flow cytometry, combined with rigorous characterization of EVs through cryo-electron microscopy, sizing, and Western blotting. We found that regardless of glucose status, AV+ platelet ($CD31^+/CD41^-$) and PECAM ($CD31^+$) EVs were significantly lowered at the 2-hour time point of the OGTT. These reductions in EVs in response to the glucose load were also associated with insulin sensitivity and arterial stiffness. Interestingly, when accounting for group differences in fitness, the effect of the OGTT on postprandial EVs was no longer significant, suggesting that baseline levels of fitness may play a crucial role in relation to postprandial physiology and EV count.

Exercise is known to reduce CVD risk in adults with obesity through improvements in outcomes such as glucose control, blood pressure, and cardiorespiratory fitness. How exercise elicits these beneficial effects, however, remains unclear. Based on pilot data from Aim 1, as well as our results from Aim 2, we hypothesized that improvements in fitness as a result of an exercise intervention may also be a key modulator of EV count. We found that a short-term exercise intervention comparing the effects of a continuous training protocol (CONT) versus a high intensity interval-training program (INT) on total, platelet, leukocyte and endothelial EVs had a differential impact on the endothelial EV AV- CD105. Independent of clinically meaningful changes in weight loss, those individuals in the INT group saw significantly lower AV- CD105 following only 12 days

of training, whereas individuals in the CONT group saw increases in AV- CD105. Since those individuals in the INT group saw greater improvements in fitness and decreased AV-CD105 EVs, we speculate that INT may have mediated lowering of AV- CD105 through a fitness-mediated mechanism. However, it is important to note, that increased sugar consumption blunted the effects of exercise intensity on EV count, suggesting that the interaction between EV count, exercise and ad-libitum dietary sugar intake may be relevant.

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CHAPTER 2: LITERATURE REVIEW

ABSTRACT

Regular exercise is important for reducing type 2 diabetes (T2D) and/or cardiovascular disease (CVD) risk. However, only about 40-50% of this CVD risk reduction is accounted for by adiposity, hyperglycemia, hypertension, and dyslipidemia. Herein, we present the novel hypothesis that Extracellular Vesicles (EVs) are candidate biomarkers that may relate to impaired endothelial function and insulin resistance independent of obesity risk factors. EVs are small membrane-bound particles that are generated by cells following stimulation, stress or activation. They carry markers of their parent cell and are thought to be potent bioactivators and communicators. We discuss the underlying physiology of specific cell type EVs, as well as examine how acute and chronic exercise interventions impact EV count and phenotype. We also propose that current gaps in the field are in part related to use of different detection techniques and the lack of standardized measurements of EV affecting the pre- and post-analytical phase. Ultimately, improving the understanding of how EVs impact cardio-metabolic health and their function will lead to improved approaches for enhancing diagnostic options as well as designing exercise interventions that treat and/or prevent T2D and CVD.

1. INTRODUCTION

Nearly 33% of all deaths globally each year are attributed to cardiovascular disease (CVD) (1). In fact, CVD mortality has increased from 12.59 to 17.82 million deaths between 1990 and 2015 (2). Individuals with type 2 diabetes (T2D) are 2-3 times more likely to have CVD than their healthy counterparts, indicating that abnormalities in glucose metabolism share a CVD pathogenic root (3). However, glucose alone may not be a primary driver of CVD in people with T2D given recent interventions focused on lowering glucose alone have failed to significantly lower CVD risk and mortality (4). As such, it is not surprising that elevated blood pressure and dyslipidemia in people with hyperglycemia are considered critical drivers of CVD that are linked together by insulin

resistance (5). Insulin resistance can be defined as the reduced responsiveness of skeletal muscle, liver, adipose and vasculature tissue to insulin for the maintenance of nutrient delivery and utilization. Although the exact cause of insulin resistance is unclear, endothelial dysfunction is a leading candidate for promoting these nutrient disturbances (6). Endothelial function is the ability of the endothelium to respond to both metabolic mediators (e.g. insulin, nitric oxide, etc.) and/or shear stress that enhance blood flow. Recently the American Heart Association suggested that current biomarkers (e.g. blood pressure, lipids, etc.) do not account for the majority of adverse outcomes, and may account for only 40-50% of CVD risk (7). Thus, there is an urgent need to identify new treatment targets for T2D and CVD that mediate health and well-being.

Extracellular Vesicles (EVs) have emerged as novel biomarkers of T2D and CVD (8,9). EVs belong to a heterogeneous population of vesicles summarized with the generic term "Extracellular Vesicles" (EVs). Interestingly, most of the studies analyzing EVs in metabolic diseases have focused on larger EVs (>100-1000nm) (***Tables 2 and 3, respectively***), called microparticles/microvesicles, generated by the low centrifugation speed of up to 20,000 Gs, but our own data (10) and that of others (11-13) indicates that we also see smaller EVs (<100nm, called exosomes) in these EV preps. As these studies have likely analyzed a mix of larger and smaller EVs we will only use the term EV. EVs are unique biomarkers as they are also believed to carry and transfer proteins, lipids and nucleic acids, they facilitate communication between cells. How EVs regulate vascular health remains to be fully determined, but obesity related insulin resistance might be a potential reason through oxidative stress and inflammatory related mechanisms (14). Interestingly, physical inactivity also increases EV levels in association with worsening of insulin resistance and endothelial dysfunction, suggesting that muscle contraction alters disease risk in an EV mediated manner (15). However, there is limited research regarding the effects of physical activity and/or exercise on EVs in healthy and disease populations. In particular, we propose that EVs may be a novel mediator of T2D and CVD risk. First, we highlight the biogenesis of EVs and the purported mechanism relating EV to insulin resistance

and endothelial function. Next, we examine the gaps in knowledge regarding the effectiveness of acute and chronic exercise on EVs. We also discuss the mechanistic role of cell specific EVs related to leukocytes, platelets and the endothelium as mediators of cardiometabolic risk. Lastly, we analyze how current EV methodologies could play a role in discrepancies seen across exercise studies and discuss new methodology to advance understanding of EVs and exosomes that could improve diagnostic and treatment options for T2D and CVD.

2. EXTRACELLULAR VESICLE BIOGENESIS

First described as merely "cell dust" by Wolf in 1967, EVs are now recognized as cell bioactivators and communicators of cardiometabolic health (16). Smaller EVs (<100nm, also called exosomes) are thought to derive from multivesicular bodies inside the cells and then secreted into different body fluids. Whereas larger EVs (>100-100nm, also called microparticle/microvesicles) are believed to be shed from cells into body fluids/tissue upon stimulation or activation. These larger EVs are likely the product of outward membrane budding through cytoskeletal rearrangement and a loss of calcium-dependent membrane phospholipid asymmetry (17). These vesicles consist of membrane proteins and cytosolic material from the cell they originate from. Indeed, EVs are derived from cells in circulation (i.e. endothelial, platelet, leucocyte), erythrocytes (18), as well as progenitor cell populations (19) (***Table 1***). Additionally, EVs are found in many other body fluids besides blood, including urine (20), which increase potential for clinical collection sites. EVs are released during conditions of stress that initiate cell activation and/or apoptosis (21). In particular, proinflammatory stimuli (e.g. oxidative stress/cytokines), bioactive lipids (22), and hyperglycemia (23) are considered key stimuli that impact EV release, phenotype and function. In particular, hyperglycemia increases endothelial derived EV formation, size and reduced surface charge that collectively prompt greater pro-coagulant activity (24). Moreover, high glucose conditions increase NADPH oxidase activity in endothelial EVs that work to amplify the effects of oxidative stress-mediated inflammation on the endothelium (23) that decrease endothelial nitric oxide synthase (eNOS) (25), thereby potentially

impairing vascular function and raising CVD risk. There is also work suggesting that EVs may not only release inflammatory cytokines (26), but also act as deliverers of bioactive lipids (22), protein and genetic material (10) between cells. Taken together, EVs represent a potentially novel paradigm in cell-to-cell communications between various organs important for T2D and CVD. For a comprehensive discussion of biogenesis of EVs we will refer the reader to other review papers (27).

3. EXTRACELLULAR VESICLES IN THE PATHOGENSIS OF T2D AND CVD

EVs are composed of parental proteins, nucleic acids and cytoplasm based on the stimuli (10). This is physiologically important because carrying markers of the parent cell allows for specific subpopulations identification (e.g. endothelium or leukocyte-derived) (10) that can influence cross-talk between tissues and cells (28). Indeed, elevated endothelial EVs are thought to reflect vascular injury, whereas increased leukocyte and platelet EVs signify pro-inflammation and coagulation, respectively. This notion is consistent with literature reporting that different subtypes of EVs are elevated in people with prediabetes (29), T2D and CVD (8,9) as well as hypertension (30), chronic kidney disease (31), and heart failure (32). Even obesity, independent of co-morbidities, presents with elevated platelet EV levels (33) in relation to reduced fibrinolytic ability. Subsequently, these observations support that EVs likely play a key physiologic role above and beyond a biomarker.

Circulating EVs are believed to play an important physiologic role in vascular physiology (34) (***Figure 1a***). Werner et al. reported that elevated endothelial EVs (CD31+/annexin V+) are correlated with reduced endothelium-dependent vasorelaxation (35). This is consistent with others reporting that elevations in these same endothelial EVs are related to reduced flow-mediated dilation as well as increased pulse wave velocity and carotid intima-media thickness (36,37). Together, these findings suggest that higher levels of EVs relate to poor blood flow and arterial stiffness. There are several putative mechanisms that may explain how EVs promote dysregulation of blood flow, although most data exists from *in vitro* experiments and more human work is needed. EVs are

thought to directly produce reactive oxygen species (ROS). Endothelial EVs (CD144, Annexin V^{+ve}) increase production of superoxide anion and hydrogen peroxide in cultured endothelial cells through NADPH oxidase and mitochondria (38,39), although others suggest xanthine oxidase may contribute in endothelial (CD144-PE) (40), lymphocytic (CD4, CD3+, CD8, CD11a, Fas, and FasL) (41) and monocyte derived EVs (42). Additionally, EVs are hypothesized to promote *in vivo* inflammation through stimulation of pro-inflammatory cytokines and the recruitment of inflammatory cells (26). In *in vitro* experiments, leukocyte EVs (CD14+) promote the release of IL-6 and IL-8 in cultured endothelial cells (43). In addition, T-cell EVs promote TNF-α and IL-1b by monocytes (44), as well as promote the interaction and adhesion of leucocytes to endothelial cells (22). These later findings are consistent with work by Mastronardi (45) demonstrating that injection of EVs from blood of patients with sepsis into mice promotes increased expression of iNOS, COX-2, and NFk-B in the heart and lung, thereby supporting a direct role of EVs at producing inflammation. Lastly, circulating EVs express the functionally active eNOS protein (46). This is clinically germane as patients with endothelial dysfunction have EVs with reduced expression and release of nitric oxide (46).

Another possible mechanism by which EVs contribute to T2D and CVD relates to the interaction and transfer of EV contents to the cell (***Figure 1a***). EVs have been proposed to physically alter cell targets receptors that modify signal transmission. For example, blocking EGF receptors in endothelial cells inhibits EV-mediated ROS production and inflammation (38). Additionally, other work has suggested macrophage-derived EVs (M0 THP-1) interfere with GLUT-4 translocation in human adipocytes by decreasing p-Akt, thereby inducing insulin resistance (47). The exact cause of this insulin resistance is unclear, but activation of NfK-B was noted, suggesting that inflammation may play a role. Indeed, it is also possible that miRNA transcripts from EVs play an important role in communicating signals to local and systemic tissues for the alteration of cell activity (48). For instance, Rautou et al. (18) demonstrated that EVs (CD31+) derived from apoptotic plaques transferred ICAM-1 to endothelial cells, suggesting that EVs play an important inflammatory

response mechanism in atherosclerosis. In addition to ICAM-1, other studies have reported adipocyte-derived EVs (CD14+) to interfere with insulin signaling in both the liver (49) and skeletal muscle (50) via transfer of adipokine content, thereby inducing insulin resistance. However, not all studies support the observation that EVs fuse and transfer content to cells (51), as there are different ways in which EV promote cell to cell communication or even EV uptake (52). In either case, EVs appear to mediate angiogenesis and induce endothelial repair (34,53-55) by at least partially (56) vascular endothelial growth factor-A (57) or eNOS (46). In this way, EVs may promote increased angiogenesis and blood flow via cargo such as eNOS induced nitric oxide. In turn, this compensatory response of increased blood flow may allow nutrient delivery to tissue, thereby contributing to insulin-mediated GLUT-4 translocation. Given the literature linking oxidative stress and inflammation to the pathogenesis of insulin resistance and endothelial dysfunction (58), the identification of how EVs may be modified or targeted for metabolic health warrants attention.

4. EFFECTS OF ACUTE EXERCISE BOUTS ON EXTRACELLULAR VESICLES

A majority of the chronic exercise training induced effect on insulin resistance and endothelial function is considered to be the result of the last bout of exercise (59). Subsequently, understanding the acute exercise effect on EVs provides insight independent of cardiorespiratory fitness adaptation and weight/fat loss. However, to date there are limited studies examining the effects of acute aerobic (60) (61) or resistance exercise (62) on EVs (***Table 2***). For instance, Mobius-Winkler et al. tested the effect of a 4-hr cycling protocol at 70% of the anaerobic threshold in 18 young, lean, healthy males (63) and found no change in endothelial EVs (CD42b-, CD42b-/CD62E+) in the immediate post-exercise period, despite increases in the cytokine IL-6. It was speculated that the lack of exercise effect might have been due to the population studied (health vs. diseased) or the low to moderate intensity exercise prescribed. We add to this by speculating the lack of EV differences following exercise could also be related to technical differences of EV detection. Blood was collected and EV

pellet enriched from platelet poor plasma using conventional flow cytometry. Smaller EVs (e.g. <500nm) might not have also been captured with this approach. In addition, targeted phenotyping was limited to detection of surface marker for CD62E and CD42. CD62E is found on activated endothelium, but other endothelial markers might reflect better the endothelial changes during exercise. Nonetheless, these findings are consistent with Guiraud et al. who showed that there were no changes in endothelial EVs (CD31+, CD62E+, CD42b-) or platelet EVs, (CD42b+) in 19 male coronary heart disease patients when measured up to 72-hr following either high intensity interval or moderate intensity cycling exercise (60). In contrast, Chanda et al. reported that a maximal bout of exercise (defined as a VO_2max test) elicited an approximate 40% increase in platelet (CD41a) EVs in healthy adults (61). While these later findings suggest that exercise intensity raises EV, it should be noted that maximal exercise would be considered a stressful perturbation to the system and it is known that high intensity exercise raises oxidative stress and inflammation in the immediate post-exercise period, thereby conferring a stimulus for metabolic adaptation (64). Indeed, *in vitro* experiments by Wilhelm et al. demonstrate that EVs generated after intense exercise in healthy young men enhanced endothelial proliferation, migration and tubule formation compared with EV derived from rest (65). Interestingly, platelets EVs (CD41+) from the same patients were elevated during 1-hr of high (67% VO_2max), but not moderate (46% VO_2max), intensity exercise. These later findings are of potential significance as they suggest exercise intensity promotes angiogenesis for improved blood flow and nutrient delivery. Whether EVs from people with T2D or CVD respond to exercise comparably to lean healthy people remains to be seen. This is particularly of interest given recent work highlighting that single bouts of exercise increase EVs (ACTN4, ADAM10, ALIX, ANAX11, CD81) and miRNA to potentially coordinate communication of nutrient homeostasis between muscle, endothelium as well as liver (66,67).

Another possible reason explaining why acute exercise has yielded equivocal EV results may relate to sex differences. Toth et al. reported elevated total Annexin V EV, platelet EV (CD63, P-

selectin-exposing) and endothelial EV (CD62, E-selectin-exposing) in 27 young healthy women compared to men while at rest. It was reported that elevated EVs (Annexin V-binding EV, CD61, P-selectin-exposing EV and E-selectin-exposing EV) in women were related to the menstrual luteal cycle (68). However, no significant sex or menstrual cycle dependent differences were observed in the endothelial EV (CD144+). Lansford et al. (69) recently tested the effect of an acute bout of exercise (60-75% VO_2max) on endothelial EVs (CD62E+, CD31+/CD42b-, CD34+) in recreationally active men and women, and demonstrated that endothelial EV (CD62E+) increased by 107% in men but not in women. Conversely, women displayed a 253% elevation in mononuclear EVs (CD34+). Based on previous research (70,71), these results suggest that increased levels may prime CD34+ peripheral blood mononuclear cells for paracrine angiogenic effects in females. Interestingly, the endothelial EVs (CD31+/CD42b-) remained unchanged following exercise in either sex. These results suggest that only certain phenotypes of endothelial EVs (CD62E+) or other not yet tested EV phenotypes may be affected by sex and exercise. In another study, Durrer et al. examined the effects of high intensity continuous versus interval exercise on EVs in young, overweight inactive adults, and reported that both exercises lowered EVs in men (n=6), but endothelial EV counts (CD31+/CD42b-) were unaffected in females (n=7) (72). However, high intensity continuous exercise increased endothelial EVs (CD62E+) in females. Although this was a relatively small sample size, the data suggest that sex may be an important factor explaining differential EV responses to exercise. Further work is needed to elucidate the mechanism by which men and women differ in EV profiles in order to individualize exercise to treat and/or prevent disease.

It reasons that dietary intake may also influence EV responses post-exercise since circulating bioactive lipids are considered a stimulus for EVs biogenesis. In fact, high fat meals induce endothelial dysfunction in healthy and T2D individuals (73). Although Jenkins et al. reported that a high fat meal had no independent effect on endothelial EVs, acute exercise at 70% VO_2max lowered endothelial EVs (CD62E+ and CD31+/CD42b-) by 55% and 30%, respectively (both: $P<0.05$)

compared to a sedentary control in healthy, recreationally active men (74). Interestingly, the lowering of endothelial EVs (CD62E+ and CD31+/CD42b-) post-exercise was associated with blunted ROS production during postprandial lipemia. This finding supports the notion that EVs may induce vascular dysfunction through an oxidative stress mediated mechanism. The modulation of oxidative stress post-exercise may also be clinically relevant since it relates to fasting and postprandial endothelial dysfunction in obese individuals with prediabetes (75). In addition, a lowering of endothelial EVs (CD31+, CD31+/CD42b-), which may be indicative of endothelial activation and apoptosis, suggests that exercise confers cardiovascular protection through modulation of EV phenotype. However, Harrison et al. reported that high intensity exercise performed at ~70% VO_2max for 90 min had no effect on high fat fed induced elevations in endothelial EVs (CD31+/CD42b-) in recreationally active young men (76). This observation is in stark contrast to Jenkins et al. (74). Despite both studies prescribing exercise at 70% VO_2max, Harrison et al. included ten 1 min sprints. This subtle difference in exercise protocols may be of relevance since high intensity exercise could have promoted greater vascular injury and prohibited the lowering of EVs. Additionally, differences in EV preparation and analysis, such as centrifugation at 1500 g for 20 min at room temperature (74) as opposed to 1600 g for 15 min at 4°C (76) may account for differences between the two studies. In either case, additional work is required to determine if exercise restores diet-induced EV levels to optimize exercise prescription for disease prevention in men and women given postprandial metabolism is a strong predictor of CVD (77).

5. EFFECTS OF CHRONIC EXERCISE TRAINING ON EXTRACELLULAR VESICLES

Exercise training improves whole body insulin sensitivity (78,79) and glucose tolerance (80,81) in adults with prediabetes and T2D. Additionally, chronic exercise enhances endothelial function in healthy individuals (82) and those at risk for (83) or with CVD (84,85). Therefore, it would be expected that long-term exercise training would also have favorable effects on EV

phenotype and count. Bruyndocyx et al. recently demonstrated that 10 months of exercise training significantly decreased endothelial EVs (CD31+/CD42b-) as measured by conventional flow cytometry in 33 overweight children (86). In addition to decreasing endothelial EVs, exercise training significantly improved microvascular function (measured via pulse amplitude tonometry), increased circulating adiponectin, and reduced body fat and high sensitivity C-reactive protein. These findings are consistent with other work reporting that 12-24 weeks of aerobic exercise with weight loss significantly lowered endothelial EVs (CD31+/CD41a; CD62E+) in middle aged men with erectile dysfunction (87) or pre-hypertensive men and women (88), as well as in African American women (89,90) (***Table 3***). Interestingly, changes in endothelial EVs (CD62E+), IL-6 and IL-10 accounted for nearly 11% of the improvements in flow mediated dilation following the exercise training in the later studies (89,90).

Although exercise training appears to favorably lower endothelial EVs (CD31+/CD41a; CD62E+), not all individuals appear to respond the same (91). Kretzschmar et al. (89) demonstrated that endothelial EVs (CD31+/CD42b) (92) only decreased in pre-menopausal compared with post-menopausal women following exercise training. It is not clear why post-menopausal women did not respond to exercise, but it is consistent with work suggesting some individuals are "exercise resistant" (75). Another plausible reason may relate to the notion that estrogen provides protective heart effects and lower CVD risk in women (93). Notwithstanding these hormonal differences across the lifespan in women or compared with men, fitness may be an additional determinant of EV improvement post-training. Indeed, recent work by our group (94), utilizing EV-Track (www.evtrack.org) reporting guidelines, as well as advanced imaging flow cytometry (*see below for details*), showed that EVs correlate with aerobic fitness and other cardiometabolic health factors in obese adults with prediabetes, highlighting again the potential role fitness may play in modulating EVs. Furthermore, Van Craenonbroeck et al. reported that pre-intervention endothelial EVs (CD31+/CD42b) count significantly predicted improvements in VO_2max despite no effect of a 12-

week training program on these EVs in 200 individuals with coronary artery disease (95). Together, these later findings suggest that EV may modulate training responses through a yet to be defined mechanism (96).

As exercise training promotes weight-loss and decreases adipose derived inflammation (97), it is reasonable to expect that habitual exercise improve EVs originating from platelets and leukocytes. Murakami et al. reported that platelet EVs (CD41+) were significantly correlated with subcutaneous fat area in 49 obese, non-diabetic subjects following 12 weeks of a restricted caloric diet or a restricted caloric diet plus exercise (33). Although EVs did not correlate with visceral fat, which is considered a chief site for inflammatory production, this finding is reasonable since subcutaneous tissue is a primary supplier of free fatty acids, and elevated free fatty acids may act as a bioactive lipid that stimulates coagulation and platelet recruitment (98). Whether exercise or exercise plus diet alter free fatty acid mediated EV levels or function waits to be tested. In either case, in the only studies to investigate exercise on leukocyte EVs (CD16+, CD14+), it was shown that training decreases neutrophil and monocyte derived EVs (CD16+, CD14+). This observation highlights that exercise has multi-cell EV effects that may favor improved in cardio-metabolic health (99,100).

6. EXTRACELLULAR VESICLE ANALYSIS AND GAPS

To date most exercise studies lack sensitivity to optimally enrich and phenotype EVs. A leading challenge in doing so is the lack of consensus on the nomenclature of EVs as well as the precise detection method or sample preparation (i.e. the pre-analytical phase) (12,101). In fact, the pre-analytical phase includes several important steps that could impact the clarity and precision of results, including but not limited to: blood collection technique (e.g. needle size or blood draw rate that impacts shear stress), sample centrifugation, timing of sample processing, sample freezing, thawing and storage (102). Generally speaking, EVs collected from fresh blood is considered more accurate and reflective true *in vivo* EV levels when compared with frozen samples (101,103), but

plasma frozen for only 24 hr may yield comparable counts when compared to fresh sample (104). In either case, it is suggested that samples should be analyzed after the same "freezing period" (105) to enhance accuracy of sample analysis. Centrifugation speed crucially affects the type of EV population isolated. Most of the studies analyzing EVs in exercise interventions have utilized low centrifugation speeds (***Tables 2 and 3, respectively***). As different speeds are used they have likely isolated different EV populations. In addition they might have enriched for larger EVs, and therefore used the term microparticles. However, this topic is still in debate and our work (10, 94) and that of others (11-13) indicates that we also see smaller EVs (e.g. exosomes, <100nm) in these EV preparations, thereby making it difficult to distinguish between various types of EVs (11).

Conventional flow cytometry is the most commonly used technique for phenotyping and enumeration of EVs (106). However, many older flow cytometer models limit detection of smaller EVs, thereby contributing to potential gaps in our understanding of all subtypes of EVs (10). Indeed, recent evidence suggests that while >80% of EVs are < 500 nm, most conventional flow cytometers have a detection threshold greater than 500 nm, suggesting that a vast majority of EVs may not be quantified (107) with this technique. To address this discrepancy, an alternative approach has been developed, combining conventional flow cytometry with resolution imaging (called imaging flow cytometry). Erdbrügger et al. found that by adding imaging to flow cytometry, EVs can be clearly differentiated from the beads and cells, as well as debris. It also provides the advantage of confirming the presence of these vesicles based not only on fluorescence, but by scatter and morphology as well (108). The detection threshold is likely down to 100-200nm. To date though, no prospective exercise research exists utilizing this approach to assess EV phenotypes. As interest in the role of EVs as mediators and markers of disease continues to grow, implementation of standardized EV approaches will be needed to elucidate the exact role of EVs in chronic disease. One approach to close this methods gap is that future studies consider using established guidelines by the EV-TRACK Consortium to improve transparency in reporting EV research (92) and follow minimal

experimental requirements for definition of EVs and their functions, as published by the International Society for Extracellular Vesicles (12). Finally, implementation of these minimal experimental requirements described (12) is crucial in moving forward with functional studies combined with content analysis (genetic, proteomic, metabolomics) in order to better advance our understanding preventing/treating chronic disease in relation to EVs.

7. ANALYSIS OF SMALLER EXTRACELLULAR VESICLES

Most of the studies discussed so far have used low centrifugation speeds to enrich for EVs, but likely analyzed a mix of large and smaller vesicles in their preparations. A few studies have focused on use of high centrifugation speed of 100,000 G to enrich for smaller EVs called exosomes. It is important to study all subtypes of EVs given that they play roles in immune modulation (109,110), activating tissue repair (111) and angiogenesis as the following studies demonstrate. Interestingly, Fruhbeis et al. was one of them to report that cycling exercise increased smaller EVs (called exosomes, positive for Flot1, Hsp/Hsc70, and IntαIIb) to a greater extent when compared to treadmill exercise, but the rise in these smaller EVs (exosomes) remained elevated for a longer period of time into recovery with treadmill exercise (112). The reason for these differential responses between treadmill and cycling exercise is not clear, but it might relate to the higher heart rate and eccentric muscle contraction associated with running. This would be consistent with prior work (65), suggesting that EVs are important for vascular repair and adaptation. Moreover, recent work from Safdar et al. has suggested that smaller EVs (exosomes) may be essential following endurance-oriented exercise as a means to treat metabolic disease (113). This assertion is supported by evidence from Bei et al. who demonstrated that exercise-induced increases in circulating EVs enhanced the protective effects of endogenous EVs against cardiac ischemia/reperfusion injury (114). These later findings are consistent with new work highlighting that exosomes play critical roles in inter-organ crosstalk during exercise to regulate energy homeostasis (66). Taken together, these preliminary data

suggest more work is needed to characterize all subtypes of EVs, including smaller (exosomes) and larger (microparticle) EVs in people with T2D and CVD following different doses of exercise, with or without diet modification, to improve clinical practice for patient care.

8. CONCLUSION AND CLINICAL PERSPECTIVES

The precise mechanism by which exercise lowers CVD is unclear, as only 40-50% of the reduction in CVD risk in subjects reporting >1500 kcal/week of exercise is attributed to non-traditional CVD risk factors (115). EVs have emerged as novel markers of T2D and CVD that have potential functional and therapeutic benefit by transferring proteins, lipids and nucleic acids. In fact, EV physiology appears critical towards the production of oxidative stress (54), inflammation (23), and/or physical contact/release of signaling molecules (i.e. miRNA) that modulate endothelial function (116). Herein, we present evidence that suggests EVs represent a potentially novel mechanism by which exercise could fill a "cardio-protection risk gap". Exercise may impact EVs by not only reducing substrates thought to drive EV functional responses, but also alter the release of oxidative stress, inflammatory cytokines as well as miRNA (***Figure 1***). Indeed, the acute effects of exercise on EVs are limited to endothelial derived EVs (CD62E+, CD31+/CD42b-, CD144+) with little change or slight increases and few to no work on platelet or leukocyte derived EVs (***Table 2***). In contrast, exercise training appears to have more robust effects on decreasing endothelial, platelet and leukocyte derived EVs in men and women (***Table 3***). However, these studies are limited in that conventional flow cytometry has been used, thereby providing less sensitivity to detecting a variety of EV sizes (< 500nm) as well as distinguishing EVs from small cells/debris. Further work is needed using various tools including imaging or high-resolution flow cytometry, tunable resistive pulse sensing or nanoparticle tracking device and electron microscopy before and after exercise interventions in order to ascertain a comprehensive EV profile in adults at risk for and with T2D or CVD. Knowledge of EV content and function may ultimately lead to improved patient care by

enabling health care providers to provide bioengineered agents that mitigate "cargo" released from these EVs and/or deliver exercise derived EVs to as therapeutic options for optimization of T2D and CVD management.

LIST OF ABBREVIATIONS

Extracellular Vesicles (EVs)

Type 2 diabetes (T2D)

Cardiovascular disease (CVD)

Extracellular vesicles (EVs)

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TABLE LEGENDS

Table 1. Most commonly used Extracellular Vesicles**.**

Table 2. Summary table of the acute effects of exercise on circulating Extracellular Vesicles. Abbreviations: Individual anaerobic threshold (IAT); High Intensity Interval exercise (HIIE); Moderate intensity continuous exercise (MICE); Repetition Max (RM); High volume training (HVT); Peak power output (PPO); High Intensity Continuous Exercise (HICE); Ventilatory threshold (VT).

Table 3. Summary table of the chronic effects of exercise on circulating Extracellular Vesicles.

FIGURE LEGEND

Figure 1. Working hypothesis by which Extracellular Vesicles (EVs) interact with exercise to influence vascular function and insulin sensitivity. Reactive oxygen species (ROS) are generated by EVs in response to bioactive lipids, glucose and inflammatory cytokines and act as important cellular regulators cell health. In addition, EVs may bind to cells and interfere with receptor related mechanisms and/or release microRNA (miRNA) to influence cell activity. Lastly, EVs may release inflammatory cytokines and impact cell NFkB activity, which influences cell vascular function. Exercise (B) decreases circulatory lipids, glucose and cytokines, thereby improving EV levels and function. We hypothesize herein that EVs not only serve as a biomarker of type 2 diabetes and cardiovascular disease, but also regulate vascular function independent of traditional obesity related risk factors. Future work should consider studying the interaction of EV and exercise doses in order to identify optimal treatment plans for preventing type 2 diabetes and cardiovascular disease.

Table 1. Most commonly used Extracellular Vesicles**.**

EV Origin	**Surface Markers**
Endothelium	CD31+/CD41-(PECAM+/ITGA2B-) CD31+/CD42- (PECAM+/GPIb-) CD31 (PECAM, Platelet cell adhesion molecule) CD144 (VE Cadherin, vascular endothelial- Cadherin) CD146 (MCAM, melanoma cell adhesion molecule) CD105 (Endoglin) CD106 (VCAM, vascular cell adhesion molecule) CD62E (E-selectin, endothelial-selectin)
Platelet	CD41 (ITGA2B, Integrin alpha 2 b) CD42 (GPIb, Glycoprotein Ib) CD31 (PECAM, Platelet cell adhesion molecule)
Leukocyte	CD45 (PTPRC, Protein tyrosine phosphate receptor type C) CD11b (ITGAM, integrin alpha M) CD14 (co-receptor of lipopolysaccharide) CD16 (on surface of neutrophils, monocytes, macrophages) CD62L (L-selectin, LyEVhocyte-selectin)
Red blood cell	CD235 (Glucophorin A)

Table 2. Acute effects of exercise on circulating Extracellular Vesicles.

Author	***Exercise Dose***	***Subtype EV***	***Pre-Analytical Phase***	***Collection Time***	***Response***
Harrison et al. *(70)*	Cycling at for 90 min at 70% VO_2peak followed by ten 1 min sprints interspersed with 1 min of recovery in young, recreationally active men.	CD31+/CD42b-	1,600 g for 15 min at 4°C	- Morning following exercise bout	No change
Mobius-Winkler et al. *(57)*	4 hr cycling at 70% IAT in young, healthy men.	CD42b- CD42b-/CD62E+	11,000 g for 2 min	16 predefined time points during and after finishing cycling.	No change
Jenkins et al. *(68)*	Exercise of 70% of VO_2peak until 598 kcal was expended in recreationally, healthy active men.	CD31+/CD42b-CD62E+	Double centrifugation of 1,500 g for 20 min at room temperature	- Pre-meal - 1, 2, 3 and 4 hr post-prandial	↓CD31+/CD42b- ↓CD62E+ Unaffected by high fat meal
Guiraud et al. *(54)*	Single session of HIIE: 15-second intervals at 100% of PPO and 15-second passive recovery intervals or isocaloric MICE in men with coronary heart disease.	CD31+ CD62E+ CD42b− CD42b+	1,500 g for 15 min followed by a single centrifugation at 13,000 g for 2 min	- 10 min pre-ex - 20 min post-ex - 24 hrs post-ex - 72 hrs post-ex	No change

Ross et al. *(57)*	3 sets of 6 resistance exercises at 15 RM w/o rest in young, trained men.	CD144+ CD146+ CD105+	1,500 g for 15 min followed by 13,000 g for 2 min	- Pre-ex - 10 min post-ex - 2 hr post-ex - 24 hr post-ex	No change
Wahl et al. (*61*)	1. HVT; 130 min at 55% PPO; 2. 4x4 min at 95%PPO; 3. 4x30 sec all-out in healthy male, triathletes.	CD31+/CD42b- CD31/CD42b CD14/CD16	1.861 g for 10 min at 4°C	- Pre-ex - Immediately post-ex - 60 min post-ex - 180 min post-ex	↓CD31+/CD42b- ↓CD31/CD42b ↓CD14/CD16
Chanda et al. *(55)*	Acute strenuous exercise (treadmill running VO_2peak) vs. moderate (75% HR_{max} for 45 min) in healthy females.	Total EVs CD41a	2,500 rpm for 5 min at 4°C	- Pre-ex - Immediately post-ex - 45 min post-ex	↑Total EVs ↑CD41a Only after strenuous ex; No moderate ex change
Durrer et al. *(66)*	1. HICE-20 min cycling @ just above VT. 2. HIIE 10 X 1-min @ *90% peak aerobic power) in young, overweight, inactive males and females.	CD62E+ CD31+/CD42b-	Double centrifugation of 1,500 g for 15 min at room temperature	- Pre-ex - Immediate post-ex - Morning post-ex	↑CD62E+ (females only) No change CD31+/CD42b- ↓CD62E+ (males only) ↓CD31+/CD42b-

Lansford et al. *(63)*	Acute bout at 60-75% VO_2peak in healthy, active individuals	CD62E+, CD34+ CD31+/CD42b-	Double centrifugation of 1,500 g for 20 min at room temperature	- Pre-ex - Post-ex	↑CD62E+ in men ↑CD34+ in women No change CD31+/CD42b-
Wilhelm et al. *(59)*	1 h of moderate (46 ± 2% VO_2peak) or heavy (67 ± 2% VO_2peak) intensity semi-recumbent cycling in healthy, young men.	CD62E+ CD41+	17,500 g for 1 hr at 4°C	- Pre-ex - Post-ex	↑CD41+ following heavy exercise No CD62E+ change
Bei et al. (*108*)	Exercise stress test in middle-aged, overweight men and women.	CD63+	1,000 g for 10 min and 2,500 g for 15 min at room temperature	- Rest - Peak-ex - Recovery (15 min after completion	↑EV count
Whitham et al. *(60)*	1-hr (30 min at 55%, 20 min at 70% and ~10 min at 80% of VO_2peak) in healthy males.	ACTN4, ADAM10, ALIX, ANAX11, CD81	20,000 g for 2x at 60-min	- Pre-ex - Exercise - 4-hr post-ex	↑EV count

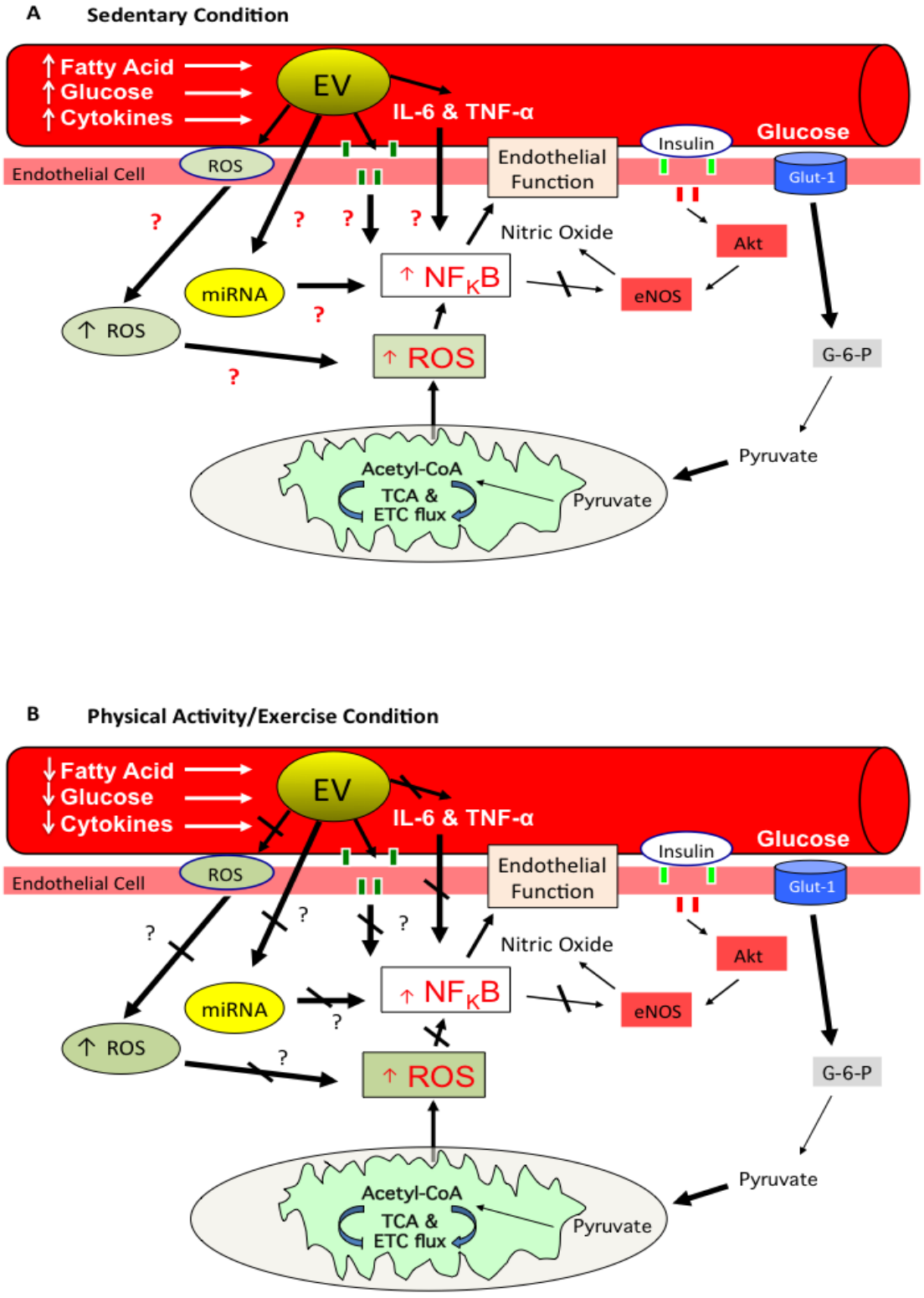
**Abbreviations: Individual anaerobic threshold (IAT); High Intensity Interval exercise (HIIE); Moderate intensity continuous exercise (MICE); Repetition Max (RM); High volume training (HVT); Peak power output (PPO); High Intensity Continuous Exercise (HICE); Ventilatory threshold (VT).*

Table 3: Chronic effects of exercise on circulating Extracellular Vesicles.

Author (Year)	***Exercise Dose***	***Subtype EV***	***Pre-Analytical Phase***	***Collection Time***	***Response***
La Vignera et al. *(81)*	150 min aerobic activity/wk for 3 mos in individuals with and without erectile dysfunction.	CD45-/CD34-/CD144+	Specifics not reported	- Baseline - 3 mos	↓CD45-/CD34-/CD144+
Babbitt et al. *(84)*	24 wk aerobic training, 3x/wk, 40 min at 65% VO_2peak in sedentary, middle-aged African American adults.	CD62E+	2,000 g for 20 min at 4∘ C	- Baseline - 6 mos	↓CD62E+
Kretzschmar et al. *(83)*	6 mos aerobic training, 3x/wk, 40 min at 65% VO_2peak in pre- and postmenopausal African American women.	CD62E+ CD31+/CD42b-	Double centrifugation at 1500g for 20 min at 24°C	- Baseline - 6 mos	↓CD62E+ ↓CD31+/CD42b- in premenopausal group only
Bruyndonckx et al. *(80)*	10 months of 3 supervised sessions/wk combined with diet intervention in obese children between the ages of 12-18.	CD31+/CD42b-	Double centrifugation of 1,525 g for 20 min	- Baseline - 5 mos - 10 mos	↓CD31+/CD42b-
Kim et al. *(82)*	3 days/wk for 6 mos of 40 min at 65% of predicted HR_{peak} in adults with prehypertension.	CD31+/CD42b-, CD62E-	Double centrifugation of 1,500 g for 20 min	- Baseline - 6 mos	↓CD31+/CD42a− ↓CD62E+

Pitha et al. *(85)*	6 mos of supervised training on cycle ergometer in renal transplant recipients.	CD34+ CD45+	ELISA	- Baseline - Median of 6 mos	No change
Van Craenenbroeck et al. *(89)*	12 wk, 3x/wk aerobic continuous training (70–75% HR_{peak}) or aerobic interval training (four 4-min intervals 90–95% of HR_{peak} with 3-min recovery at 50–70% of HR_{peak}) adults with coronary artery disease.	CD31+/CD42b-	Double centrifugation at 1,550 g	-Baseline - 12 wks	No change

Figure 1.


A
Sedentary Condition
↑ Fatty Acid
↑ Glucose
↑ Cytokines
EV
IL-6 & TNF-α
Glucose
Insulin
Endothelial Cell
ROS
Endothelial Function
Glut-1
Nitric Oxide
Akt
miRNA
↑ NFKB
eNOS
↑ ROS
↑ ROS
G-6-P
Pyruvate
Acetyl-CoA
TCA &
ETC flux
Pyruvate
B
Physical Activity/Exercise Condition
↓ Fatty Acid
↓ Glucose
↓ Cytokines
EV
IL-6 & TNF-α
Glucose
Insulin
Endothelial Cell
ROS
Endothelial Function
Glut-1
Nitric Oxide
Akt
miRNA
↑ NFKB
eNOS
↑ ROS
↑ ROS
G-6-P
Pyruvate
Acetyl-CoA
TCA &
ETC flux
Pyruvate

CHAPTER 3: AIM I (Pilot Work)

This work has been previously published in Physiological Reports as pilot data for Aims 2 and 3.

LOW CARDIORESPIRATORY FITNESS IS ASSOCIATED WITH HIGHER EXTRACELLULAR VESICLE COUNT IN ADULTS WITH OBESITY"

ABSTRACT

BACKGROUND: Low cardiorespiratory fitness (CRF) is associated with cardiovascular disease (CVD) independent of obesity. Extracellular vesicles (EVs) are a novel target of CVD, however, it is unknown if obese individuals with very poor fitness (VPF) have elevated EVs versus people with poor fitness (PF). Thus, we tested whether VPF was associated with greater EV subtypes in obese adults. **METHODS**: Subjects with VPF (n=13, VO_2peak: 15.4±0.6 ml/kg/min, BMI: 34.1±1.7 kg/m^2) and PF (n=13, VO_2peak: 25.9±3.0 ml/kg/min, BMI: 32.1±1.2 kg/m^2) were compared in this cross-sectional study. After an overnight fast, AnnexinV (AV) +/- platelet (CD31+/CD41+), leukocyte (CD45+/CD41-) and endothelial EVs (CD105+, CD31$^+$/ CD41$^-$) were analyzed from fresh platelet poor plasma via imaging flow cytometry. Body fat, blood pressure (BP) and glucose tolerance (OGTT) were also tested. **RESULTS**: Body weight, BP, and circulating glucose were similar between groups, although VPF subjects were older than PF (64.0±2.1 vs. 49.8±4.2 yr; $P<0.05$). People with VPF, compared with PF, had higher total AV- EVs ($P=0.04$), AV- platelet EVs (CD31+/CD41+; $P=0.006$) and AV- endothelial EVs (CD31+/CD41-; $P=0.005$) independent of age and body fat. Higher AV- platelet and endothelial EVs were associated with lower VO_2peak (r=-0.56, $P=0.006$ and r=-0.55, $P=0.005$, respectively). Endothelial derived AV-/CD31+/CD41-EVs were also related to pulse pressure (r=0.45, $P=0.03$), while AV-/CD105 was linked to postprandial glucose (r=0.41, $P=0.04$). **CONCLUSION**: VPF is associated with higher AnnexinV- total, endothelial and platelet EVs in obese adults, suggesting that subtle differences in fitness may reduce type 2 diabetes and CVD risk through an EV related mechanism.

INTRODUCTION

Low cardiorespiratory fitness (CRF) is characterized by reduced peak oxygen consumption (i.e. VO2peak) and is a strong independent predictor of all-cause mortality and cardiovascular disease (CVD) (3, 4, 28, 47) in normal weight and obese adults (47). In fact, obese individuals with at least moderate levels of CRF have lower rates of CVD when compared with normal weight individuals with poor fitness (47). Improvements in fitness from "unfit" to "fit" have also been shown to provide health benefit, suggesting that subtle fitness differences play a cardio-protective role in obese adults (3). However, the mechanism that accounts for such health benefit remains unclear.

Diminished fitness has traditionally been linked to CVD by biomarkers including, but not limited to: C-reactive protein, low- and high-density lipoprotein (LDL and HDL), and hyperglycemia (33). However, the American Heart Association indicates that these biomarkers explain only 40-60% of future CVD risk in both healthy and non-healthy individuals (21). Extracellular vesicles (EVs) have emerged as a potential novel biomarker and/or mediator of CVD risk (5). EVs are believed to range in cell size of 100-1000nm and have been associated with several disease states including type 2 diabetes and hypertension (1, 9, 36). EVs may reflect these metabolic disease states as they are products of cell activation, apoptosis or injury from various sources, including the endothelium, platelets and leukocytes (2, 13). Indeed, recent work suggests that EVs may mediate inflammatory and oxidative stress to promote endothelial and metabolic dysfunction (23). Given that elevated fitness is associated with improved endothelial function and reductions in hyperglycemia as well as inflammation (26, 31, 32), it would be reasonable to expect fitness to relate to lower EV levels in obese individuals. Although previous literature has suggested that endothelial EVs may play an important role in predicting exercise-mediated aerobic fitness (43), no study has systematically assessed the relationship between various subtypes of EVs using advanced imaging flow cytometry (13, 14) with fresh plasma to identify potential clinical relevance. In particular, the current literature

has focused mainly on AV+ EV cell types. This is a major knowledge gap since some suggest that AV- EVs may carry a different or even greater clinical relevance than AV+ derived EVs (14). In addition, previous literature has extensively quantified endothelial EVs (5, 7-9, 23) in relation to vascular health and disease, however, other subtypes such as platelet and leukocyte-derived EVs may also regulate health via inflammatory mechanisms (11, 12). Subsequently, no work exists characterizing the role of very poor aerobic fitness (VPF) compared with poor fitness (PF) on AV- or AV+ EV subtypes to understand how subtle differences in fitness relate to less CVD risk. Thus, we tested the hypothesis that obese adults with VPF would have higher levels of total, endothelial, platelet and leukocyte derived EVs when compared to people with PF. We also hypothesized that elevated EVs would correlate with increased CVD risk.

METHODS

Subjects

In this retrospective cross-sectional analysis of obese individuals who were part of two studies conducted in our lab, 26 of 39 subjects were ranked based VO_2peak and divided into tertiles, such that only the upper and lower tertiles were included in this analysis to test effects of CRF on EVs (***Table 1***). The average fitness levels of the group, taking into account both sex and age, were within published guidelines for PF and VPF; however, it is important to note that not all individual subjects within each group (i.e. n=3 (VPF) and 4 (PF)) met published guideline criteria for VPF and PF (2). Subjects were excluded from participation if they were physically active (> 60min/wk), smoking, on hormone replacement therapy, diagnosed with type 1 or 2 diabetes as well as metabolic syndrome. Subjects were also excluded if on medications known to influence insulin sensitivity (e.g. metformin, GLP-1 agonist, etc.), endothelial function (beta blockers, ACE-inhibitors, etc.). All subjects underwent physical examination and biochemical testing to ensure safety in study participation. A resting and exercise 12-lead EKG was also performed to assess cardiac arrthymia.

All subjects provided verbal and written informed consent as approved by our Institutional Review Board.

Metabolic Control

Subjects were instructed to refrain from strenuous exercise, caffeine, or alcohol consumption for 48-hr prior to testing. Subjects were also asked to refrain from taking any medications or dietary supplements 24-hr prior to reporting to the Clinical Research Unit. Subjects recorded habitual dietary intake 3-d before testing to confirm mixed meal consumption and were then instructed to consume 250 g/d of carbohydrates on the day before testing. There was no difference between habitual diet and food consumed prior to testing, so the 4 days were averaged for analysis.

Cardiorespiratory Fitness

VO_2peak was determined using a continuous progressive exercise test on a cycle ergometer with indirect calorimetry (Carefusion, Vmax CART, Yorba Linda, CA). Heart rate and blood pressure were obtained at rest and heart rate was continuously monitored using a 12-lead EKG. The power output was increased by 25 watts every 2 minutes until the subject met volitional exhaustion, RER was >1.1 and the cadence dropped below 60 rpm.

Body Composition

Following an approximate 4-hr fast, body weight was measured to the nearest 0.01 kg on a digital scale with minimal clothing and without shoes. Subjects were instructed to wipe their hands and feet with an anti-bacterial cloth prior to measurement to enhance electrical conductivity. Percent body fat and fat-free mass were measured using InBody 770 Body Composition Analyzer (InBody CO, Cerritos, CA) (16). Waist circumference was measured 2cm above the umbilicus (30).

Oral Glucose Tolerance Test (OGTT) and Arterial Stiffness

After an approximate 10-12-hr fast, subjects reported to the Clinical Research Unit. Subjects were then instructed to lay supine undisturbed for at least 5 minutes to determine resting heart rate

and blood pressure, which was averaged over three measurements for data analysis. Additionally, pulse pressure (defined as systolic-diastolic blood pressure) and mean arterial pressure (((2*diastolic)+systolic)/3) were calculated. Blood samples were drawn from an antecubital vein after placement of an indwelling catheter. Blood lipids and white blood cells were analyzed using enzymatic colorimetric-based assays via our University Medical Laboratories. A 75 g OGTT was then performed to assess glucose tolerance by determining plasma glucose every 30 minutes up to 120 minutes and then 180 minutes. Plasma glucose samples were analyzed using the YSI 2300 StatPlus Glucose Analyzer System (Yellow Springs, OH). Augmentation index (AI) was measured using the SphygmoCor® system, (AtCor Medical, Itasca, IL) for determination of arterial stiffness at minutes 0, 60, 120 and 180 minutes. AI was corrected to a heart rate of 75 bpm using the manufacturer's software. Total area under the curve (tAUC) was calculated using the trapezoidal model.

***EV* Isolation and Labeling**

Fresh blood prior to the OGTT was collected in citrate vacutainers and processed within 120 minutes of collection for the measurement of platelet (CD31+/CD41+), leukocyte (CD45+/CD41-), endothelial (CD105 and $CD31^{+}$/ $CD41^{-}$) EVs as described previously by Erdbrüegger et al. (13). Annexin V (AV) was used as a membrane dye. Platelet poor plasma was obtained by centrifugation (Sovall RC 5B Plus Centrifuge: Rotor SS-34 Fixed Angle Rotor) at 5000g at room temperature for 15 minutes. An EV pellet was obtained from platelet poor plasma by a second centrifugation spin (Centrifuge: 524/5424 R-Rotor FA-45-24-11) at 17,000 g for 10 minutes, which was then washed with PBS+0.5% BSA, repelleted, and resuspended with 1 X Annexin V buffer (1 x AVb) (BD Parmingen, San Diego, CA). 100µL of each washed EVs were split into 7 microfuge tubes. For the samples, 20µL of the antibody mix was added to one tube, while nothing was added to the second tube. For the controls, 20 µL of the antibody mix was added to 100µL of the AV buffer. For compensation controls, 1µL of each individual fluorescence (FITC (Annexin V, Biolegend®), PE

(CD105, Biolegend®), PacBlue (CD41, Biolegend®), BV510 (CD 45, Biolegend®), AF647 (CD31, Biolegend®) was added to 1 tube. The samples were then vortexed 3 times, for 5 seconds and then incubated 45-60 minutes in the dark at room temperature. After this time period, 1 mL of 1x AV buffer was added to all 7 tubes. The pellet EVs were spun for 10 minutes at 17,000xg at room temperature. The supernatant was removed, leaving approximately 10-20 µL in the tube. Between 30 and 40 µL of the 1x AV Buffer was added to all 7 tubes again, leaving approximately 50 µL of solution in each tube. The samples were vortexed 3 times for 5 seconds. Upon completion, EVs in the sample were concentrated 2 fold.

EV Phenotyping with Imaging flow cytometry and cryo-Electronmicroscopy

Imaging flow cytometry was used to isolate and determine the source and count of the EVs as described previously by our group (13, 14). The flow cytometer ImageStream®^{X} MKII (Amnis, Seattle, WA) (ISX) was utilized, as it combines the capabilities of conventional flow cytometry (FCM), with high-resolution imaging at the single cell level to accurately depict EV sub-types. All lasers of the ImageStream®^{X} MK II are set to full power, including the scatter laser. Magnification was set to 60X and core size reduced to 7 µm. Samples were loaded and acquired for 2 minutes (or specific fixed time for all samples). Acquisition gates were set on low scatter signals that are 2-3 decades lower than speed beads (***Figure 1a, 1b***). We used several controls, including compensation controls as single stained EV samples, Buffer only controls (collected for 2 minutes after filtering with a 0.1µm filter, Buffer plus reagents control (to rule out that antibodies by itself do not mimic appearance of EVs e.g. by aggregation (***Figure 1c***) and an unlabeled EV control saEVle (to establish the gating of subpopulations (***Figure 1d***)). EV counts were measured by a volumetric method provided by the software of ISX. The acquired raw data was then analyzed using IDEAS software. FCS Express6 DeNovo TM software was then used to create the histogram and dotplots.

Cryo-electron Microscopy to Image EVs

Purified samples were verified by standard methods for cryo-electron microscopy (cryoEM) to determine EV morphology (49). In brief, an aliquot (3 ml) was applied to a glow-discharged, perforated carboncoated grid (2/2-4C C-flats), blotted with filter paper, andrapidly plunged into liquid ethane. Low-dose images were recorded at a magnification of 29,0003 on a FEI Tecnai F20 Twin transmission electron microscope operating at 120 kV, with a nominal underfocus ranging from 3.5 to 5 mm and a pixel size of 0.388 nm at the specimen level. All images were recorded with a Gatan 4K 3 4K pixel CCD camera. The grids were stored in liquid nitrogen, and then maintained in the microscope at 2180_C using a Gatan 626 cryo-stage. Samples were also prepared for scanning electron microscopy (***Figure 2a***).

EV Size Detection

Tunable Resistive Pulse Sensing (TRPS) was performed with a gold qNano instrument (Izon Ltd) mounting a polyurethane nanopore membrane NP200 (range 85-500 nm) and NP400 (range 125-1100 nm) (Izon Ltd). Multi pressure at 4,5 and 8 mBar respectively was applied to determine the particle concentration. Electrolyte solution was made of PBS supplemented with 0.03 % (v/v) Tween-20 filtered with Minisart® high flow hydrophilic 0.1 µm syringe filter (Sartorious). Current pulse signals were collected using Izon Control Suite 3.2software (Izon Ltd). EV pellet after differential centrifugation was solubilized in 50 µl of filtered electrolyte solution. Polystyrene particle standards (SPK200B and CPC400B;IzonLtd.) were employed for calibration. Both uEVs pellet and particle standards were measured with a minimum of 1000 blockades (***Figure 2b***). Nanosight Tracking Analysis (NTA) was carried out using the Zetaview pmx110 multiple parameter particle tracking analyzer (particle metrix, Meerbusch, Germany) in size mode using zetaview software version 8.02.28. Plasma samples were diluted in 1x pbs to the working range of the system. The system was calibrated using 105 nm polystyrene beads and then plasma vesicle profiles were recorded and analyzed at 11 camera positions with a 2 second video length, a camera frame rate of 30 fps and a temperature of 21˚C (***Figure 2c***).

EV Protein detection by Western Blotting

Protein quantification of EVs was performed by Coomassie micro assays. EV pellets were solubilised in 40 µl of solubilisation buffer made of 5% (w/v) sodium dodecyl sulphate (SDS), 40 mM Tris-HCl pH 6.8, 0.5 mM ethylenediaminetetraacetic acid (EDTA), 20 % (v/v) glycerol without and with 50 mM dithiothreitol (DTT) respectively. Samples were denaturated overnight at room temperature. Proteins were separated by hand cast SDS-PAGE gradient gels (Resolving gel T= 5-20 % (w/v); C=2.6 %; Stacking gel T= 3.5 % (w/v); C=2.6 %) in 25 mMTris, 192 mM glycine and 0.1 % (w/v) SDS buffer and either stained with silver staining or transferred onto a 0.45µm nitrocellulose membrane (Amersham™ Protan™ 0.45µm NC, GE Healthcare) in a wet transfer system buffer made of 25 mMTris, 192 mM glycine and 20 % (v/v) methanol. Nitrocellulose membranes were saturated with Odyssey blocking buffer (LI-COR Biosciences) and incubated in polyconal rabbit anti TSG101 (Sigma, T5701-200UL) and monoclonal mouse anti CD9 (HansaBiomed; HBM-CD9-100) overnight at room temperature (RT= 23-24°C) in an Odyssey blocking buffer diluted 1:1 with PBS and 0.15 % (v/v) Tween-20 at concentration of 1.0 µg/ml. After 3x10 minute washes in PBS-Tween (0.15%, v/v), membranes were incubated with anti rabbit anti mouse dye-coupled secondary antibody 0.1 µg/ml(LI-COR Biosciences) in an Odyssey blocking solution diluted at 1:1 with PBS and 0.15 % (v/v) Tween-20; 1-hr at RT. Acquisition of the fluorescent signal was performed by Odyssey infrared imaging system with resolution set at 169µm (LI-COR Biosciences) (***Figure 2d***). We have submitted all relevant data of our experiments to the EV-Track knowledgebase (EV-TRACK ID: EV180013) (44).

Statistical Analysis

Data were analyzed using SPSS v24 (Armonk, NY). Data were log-transformed in the event normal distribution was not achieved. VPF and PF group differences were coEVared using independent, two-tailed *t*-tests. Sex differences were coEVared utilizing the Fischer exact test. Given that age and body fat percentage were statistically significant between groups, we conducted an

analyses of covariance to test independent effects of fitness on EVs. We also conducted a sub-analysis including women only to test sex effects based on current mixed literature (19, 26, 41). Pearson correlation was used to assess associations. Statistical significance was set at $P \leq 0.05$. Adjusted means are presented throughout the manuscript and data are reported as mean ± SEM.

RESULTS

Subject Demographics

Body weight, waist circumference, blood pressure, plasma lipids and circulating glucose were similar between VPF and PF (***Table 1***). There was no significant difference between groups in total calories (VPF: 2020.8±165.2 vs. PF: 2343.9±188.0, *P*=0.21), carbohydrates (VPF: 48.9±3.7 vs. PF: 48.5±2.2%, *P*=0.93), protein (VPF: 16.7±1.4 vs. PF:16.3±0.8%, *P*=0.80), or fat (VPF: 36.7± 2.7 vs. PF: 35.4±2.1%, *P*=0.70). However, individuals with VPF by study design had a lower VO_2peak than those with PF (*P*<0.01). Subjects with VPF also had more women (*P*=0.09), higher age (*P*=0.007), and increased body fat (*P*<0.001; ***Table 1***). All but two women (both in PF) were post-menopausal.

Characterization of EV

We report a heterogeneous size of EVs (***Figure 2a***), ranging from 80 to 600nm in size. Importantly, the size of EVs were similar, regardless of measurement by TPRS or NTA (***Figures 2b and 2c***). Using two different membrane sizes of 200 and 400nm for the TRPS analysis, with 2 different pressures, the mean size of EVs was 182nm and mode size was 142nm, whereas the mean size of NTA was 168nm and mode size was 138nm. We also verified by Western blot, that our method was appropriate for detecting vesicles by comparing 2 established proteins, namely tetraspanin CD9 and ESCRT, an endosomal sorting complex required for transport component marker TSG101. While CD9 was detected at its own molecular weight at 20-25 kDa, TSG101 presented a set of bands at molecular weight higher than the nominal one at 46kDa which can be

attributed to multiple ubiquitination site (Tal, a Tsg101-specific E3 ubiquitin ligase, regulates receptor endocytosis and retrovirus budding) and/or ISGylation (***Figure 2d***).

EV Subtype Phenotyping

Total AV- EVs were significantly elevated in people with VPF compared with PF (*P*=0.02). In addition, individuals with VPF had higher platelet EVs (AV-/CD31+/CD41+, *P*=0.03) and endothelial EVs (AV-/CD31+/CD41-, *P*=0.002) when compared with those with PF independent of age and body fat percentage ***(Figure 3a)***. However, there was no significant difference between VPF and PF in leukocyte-derived AV-/CD45+/CD41- EVs (*P*=0.70) or the endothelial EV AV-/CD105+ *(P*=0.28). There were no significant group differences in any AV+ EV subtype when stratified based on fitness level (***Figure 3b***), however, the endothelial derived EV AV+/CD31+/CD41- trended to be higher in the VPF group (*P*=0.07**;** ***Figure 3b*****)**. Sub-analysis of women verified higher total AV- EVs in VPF than PF (3.8±0.09 vs. 3.4±0.12, *P*=0.04). Moreover, AV-/CD31+ (2.96±0.14 vs. 3.5±0.10, *P*=0.01), AV-/CD31+/CD41+ platelet (2.8±0.2 vs. 3.4±0.1, *P*=0.006) and AV-/CD31+/CD41+ endothelial EVs (1.9±0.2 vs. 2.7±0.1, *P*=0.005) were also higher in VPF women independent of age and body fat.

Correlations

Low VO_2peak was significantly associated with higher AV-/CD31+ (r=-0.52, *P*=0.009), AV-/CD41+ (r=-0.57, *P*=0.003), AV-/CD31+/CD41+ platelet EVs (r=-0.55, *P*=0.005) and AV/CD31+/CD41- endothelial EVs (r=-0.56, *P*=0.006). Elevated AV-/ CD31+/CD41- endothelial EVs also correlated with increased pulse pressure (r=0.45, *P*=0.03; ***Figure 4a***). Moreover, endothelial EV AV-/CD105+ was positively related to 120-minute glucose (r=0.41, *P*=0.04; ***Figure 4b***) following the OGTT. High AV-/CD45+/CD41- leukocyte EVs tended to correlate with decreased HDL cholesterol (r=-0.43, *P*=0.06) and increased arterial stiffness (r=0.40, *P*=0.06). Increased arterial stiffness (measured as $tAUC_{180}$) was also associated with decreased fitness (r=-0.47, *P*=0.02).

No other significant correlations were found between AV- EV subtypes and body fat, BMI, blood pressure, LDL, total cholesterol or triglycerides (data not shown).

DISCUSSION

The major finding of the present study using detailed characterization of EVs is that individuals with VPF have elevated levels of both AV- platelet and endothelial-derived EVs when compared with individuals with PF (***Figure 3a***). These findings may be of clinical relevance since elevated platelet and endothelial EVs are in concordance with increased T2D and CVD risk (1, 36). Subtle reductions in cardiorespiratory fitness by just 1-MET is related to increased mortality risk by 13% (24), suggesting that even slight improvements in fitness may be cardio-protective. To date, the mechanism by which subtle differences in fitness promote this health benefit remains largely unknown. Van Craenenbroeck et al. previously suggested that baseline endothelial EVs were inversely correlated with increases in VO_2peak and endothelial function following a 3-month exercise intervention in older adults with coronary artery disease and poor fitness (43). In line with these observations, Navasiolava et al. reported that only 7 days of physical inactivity raised endothelial-derived EVs in association with reduced basal flow and endothelium-dependent vasodilation in healthy male adults (34). Taken together, our work extends these findings by showing for the first time that subtle elevations in aerobic fitness may be categorized by lower EV counts of platelet and endothelial, but not leukocyte, and confer cardio-metabolic health in obese individuals.

There are several possible roles by which fitness may be related to endothelial and/or platelet EV subtypes in these obese individuals. Previous work from our group has shown subcutaneous and abdominal visceral fat to be elevated in obese women with PF (22). This is potentially problematic because obese adults have elevated fat-derived hormones that promote inflammation and reduce insulin sensitivity and cardiometabolic health (29). Since inflammation is a purported mechanism involved in the regulation of endothelial EV release (12), it would be reasonable to expect that differences in body fat could relate to elevations in endothelial EV subtypes. However, although

people with VPF had higher body fat when measured with bioelectrical impedance when compared with PF, our results indicate that AV- platelet (CD31+/CD41+) and endothelial (CD31+/CD41-) EVs remained significantly elevated in people with VPF when compared with PF individuals after controlling for body fat. These findings suggest that fitness is related to differences in EVs independent of total body fat.

Inflammatory processes may drive atherosclerosis and CVD risk independent of total adiposity and help explain the impact of fitness on platelets EVs (20). Although previous literature has shown VO_2peak to be inversely related to CRP and inflammation (25, 27), no previous work has examined the relationship of fitness with platelet EVs and inflammation. Our results therefore fill this knowledge gap, as we observed significantly lower platelet EVs in people with PF compared with VPF. The clinical relevance of these elevated platelet EVs, however, remain unclear, as we did not find any significant correlation with platelet EVs. This may be due to the underlying pathophysiology of our specific clinical population, as even WBCs, a clinical marker of inflammation was unrelated to platelet EVs. Thus, future studies should consider examining more specific measures of inflammation, including CRP, IL-6, or TNFα since they have been suggested to impact EV release (37).

Another possible factor related to subtle differences in fitness contributing to lower EV levels may relate to vascular function. Indeed, circulating EVs play an important physiologic role in vascular physiology (12) and elevated endothelial EVs correlate with reduced endothelium-dependent vasorelaxation (48) and flow-mediated dilation (15), as well as increased arterial stiffness (46). Interestingly, we observed that elevated VO_2peak was significantly correlated with lower arterial stiffness. This finding is consistent with others showing that cardiorespiratory fitness is associated with improved endothelial function and lower blood pressure (35). Thus, it would be expected that elevated levels of circulating endothelial EVs would correlate with increased blood

pressure through a fitness related mechanism. Interestingly, we report that elevated AV- endothelial EVs (CD31+/CD41-) correlated with increased pulse pressure (***Figure 4a***), suggesting that endothelial EVs may play a role in blood pressure and CVD risk (39). Although the present study was not designed to test how fitness modifies endothelial EVs, we speculate that the higher levels of shear stress with physical activity in people with PF, compared with VPF, may counteract EV release (6, 40). Indeed, our data are consistent with *in vitro* work demonstrating that endothelial EVs promote vascular dysfunction by impairments in nitric oxide release and/or increased apoptosis of endothelial progenitor cells (38).

Cardiorespiratory fitness contributes to improved insulin sensitivity that in part explains lower blood glucose levels and type 2 diabetes risk (30). Recent work by Burger et al. assessed the effect of high glucose exposure to HUVEC cells on endothelial EVs and reported that hyperglycemia increased endothelial EV count, promoted a greater pro-coagulant activity, elevated reactive oxygen species, and blunted endothelial relaxation (10). This is line with previous work that suggested high glucose conditions increased NADPH oxidase activity in endothelial EVs, thereby promoting vascular inflammation (23). Consistent with these recent *in vitro* studies, we report that high 2-hr plasma glucose concentrations were directly correlated with endothelial EV AV-/CD105 (***Figure 4b***). These findings suggest that hyperglycemia may be an important modifier of vascular function that contributes to fitness related adaptation that lower risk of type 2 diabetes. Interestingly, this is consistent with recent work showing that endothelial EVs are higher in people with prediabetes when compared to adults with normal glucose tolerance (18). Whether prospective exercise interventions in people with prediabetes can alter EVs in relation to vascular adaptation remains to be determined.

Leukocyte EVs were not associated with fitness in the present study, although they did tend to correlate with both increased arterial stiffness and decreased HDL cholesterol. These observations suggest that leukocyte EVs may have clinical relevance in obese adults. In fact, high leukocyte-derived EVs were previously related to higher inflammation (indicated by hs-CRP) in people with

metabolic syndrome when compared to healthy counterparts (11). We speculate that we did not see a difference in leukocyte EVs in the present study due to a lack of difference in WBC counts between groups. Nonetheless, the interplay between leukocyte EVs and arterial stiffness and HDL cholesterol may be physiologically meaningful and additional studies are needed to definitively determine the impact of fitness on leukocyte EVs to understand their clinical relevance.

This study has several limitations that may impact our interpretation. This was a cross-sectional and correlation analysis does not imply causation. Additionally, this was a relatively small sample size and we cannot generalize these findings across race, which has been documented to relate to EVs (7). There were males in the PF group whereas the VPF group was entirely female, thereby raising questions on the role of sex explaining differences in EV subtypes between groups (26, 41). Although sex differences have been reported in EVs, this difference is not consistently reported across all EV subtypes (25, 38). Our sub-analysis including females only demonstrate that there are still significant fitness related EV differences. Thus, sex is unlikely to influence our present findings; however we are likely underpowered to definitively determine if sex differences exist and future research is warranted. We also observed that individuals with VPF were older than the PF participants, as well as had a higher percentage of body fat that could collectively explain why higher EV levels were observed between these cohorts. However, after including both of these covariates in our ANOVA model, we still saw that those individuals with lower fitness levels had higher levels of EVs when compared to individuals with slightly higher levels of fitness. Additionally, no direct relationships were observed between any AV- EV subtypes and age or body fat % (data not shown), suggesting that fitness is potentially an important modifier of EVs. Diet is another factor that may impact our results (8, 17, 23, 42). Although we did not strictly control for macronutrient intake proceeding measures in this study, there no statistical difference in caloric intake between groups, suggesting that diet is unlikely to influence our fitness mediated results. Nevertheless, a major strength of the present study is the use of fresh blood samples combined with imaging flow

cytometry to more accurately assess EV counts (45) across subtypes (13). In fact, our approach used EV-Track (www.evtrack.org) reporting and highlights not only EV origin (flow cytometry) but also EV size (TRPS and NTA), morphology (cryo EM) and proteins (Western blotting). These EV-Track guidelines are a recent attempt to improve transparency of methodology within the field of extracellular vesicles, as great heterogeneity of both isolation and characterization of extracellular vesicles exists within the field (44). As we are in accordance with these guidelines, these data add to the literature and strengthen our claim that EVs have clinical and aerobic fitness related relevance. Indeed, this study is the first to report significant findings between various clinical outcomes and AV- EVs, as previous literature has reported mostly about AV+ EV subtypes, which is likely due to the limitation of conventional flow cytometry capabilities in measuring smaller EV sizes of about 200-400 nM depending on the flowcytometer used (13,14).

In conclusion, VPF is associated with higher AV-, endothelial and platelet EVs in obese adults, suggesting that subtle differences in fitness may induce cardio-protection, in part, through a EV-subtype related mechanism. Moreover, we identified that EVs were significantly correlated with lower arterial stiffness and blood glucose, thereby highlighting potential connections with development of hypertension and type 2 diabetes. Indeed, these results support the need to examine both AV+/- EVs, as well as smaller vesicles, such as exosomes (40-100nm), in future clinical work to better understand the etiology of cardio-metabolic disease. Overall, these fitness related findings suggest that vascular and metabolic adaptations to physical activity/exercise elicit cell-specific EV responses, and future work is warranted to elucidate the mechanism by which EVs-induce cardio-metabolic health differences before after exercise interventions to optimize prevention and/or treatment of chronic disease.

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TABLE LEGENDS

Table 1**:** Very poor fitness (VPF) and poor fitness (PF) demographics**.** Data presented are mean ±SEM. BMI = body mass index. WC= waist circumference. tAUC = total area under the curve. PG= plasma glucose. LDL = low density lipoprotein. HDL = high density lipoprotein. TG= triglycerides. MAP = mean arterial pressure. AI = augmentation index.

FIGURE LEGENDS

Figure 1. EV Phenotyping with Imaging Flow Cytometry. Gating strategy based on low scatter (a) and Annexin V intensity positivity (b) on intensity histogram according to our previous published methods, (c) and (d) Controls: Buffer with only reagents, no EVS (c) unlabeled EVs without reagents (d). Example of dot plots of EV labeling: (e): CD31/Annexin V density plot, (f) CD105/Annexin V density plot.

Figure 2. Characterization of EV size, concentration and morphology. Cryo-Electron microscopy images of EVs of different sizes (<100nm to 1000nm) (a); Tunable resistive pulse sensing (TRPS, qNano® by Izon, using 200nm and 400nm with 2 pressures). Concentration of EV particles in this example using a 200nm pore size for qNANO with 2 pressures at 4A and 8A is 5.7e9 Particles/ml. Mean size is 161nm and mode size is 116nm taking average of both pressure measurement. Concentration of EV particles in the same example using a 400nm pore size for qNANO with 2 pressures at 5A and 8A is 4.5e9 Particles/ml. Mean size is 203nm and mode size is 170nm taking average of both pressure measurements (b). Particle tracking of EVs with Nanosight tracking analysis (Zetaview® by Particle Metrix). Concentration of EV particles in this example is 5.1e9 particles/ml. Mean size is 142 nm and mode size is 130nm (c); Western blotting of vesicle proteins. *A* (protein pattern) and *B* (Western blotting) show vesicle protein TSG101 in reducing condition and *C* (protein pattern) and *D* (Western blotting) show vesicle protein CD9 in non-reducing condition at expected band length (d).

Figure 3. Comparison of Annexin V+ (a) and Annexin V- EV subtypes (b) in obese individuals with very poor fitness (VPF) and poor fitness (PF). EV data were log-transformed. Subtypes: CD41

(platelets), CD105 (S-endoglin, endothelial), CD31 (PECAM, platelet endothelial cell adhesion molecule). Age and body fat were included as covariates.

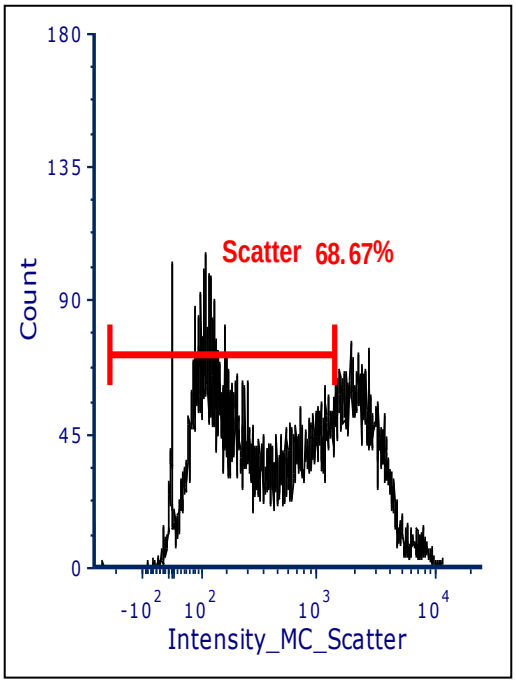
Figure 4. Correlation between CD31+/CD41- endothelial EVs and pulse pressure (a) and CD105+endothelial EVs with 2-hr glucose (b). EV data were log-transformed.

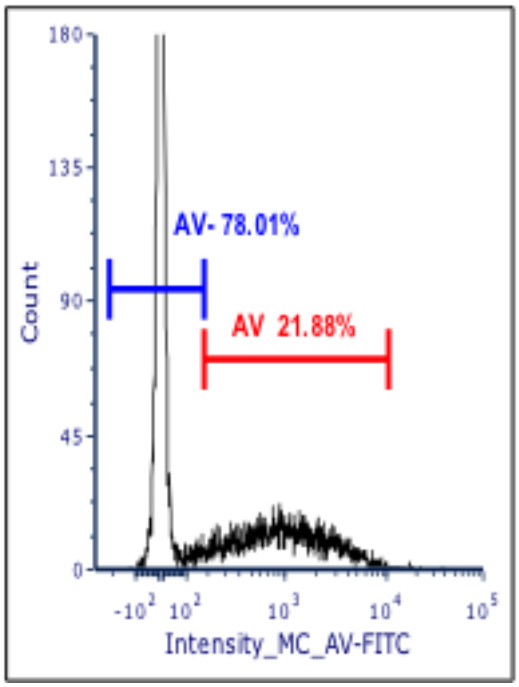
***Table 1*:** Very poor fitness (VPF) and poor fitness (PF) demographics**.**

	VPF (range)	***PF (range)***	***P-value***
N (M/F)	13 (0/13)	13 (4/9)	0.09
Age (yr)	64.0±2.1 (50-74)	49.8± 4.2 (19-70)	0.007
Body Weight (kg)	91.5± 4.8 (62.4-121.35)	90.6±3.7 (59.9-105.8)	0.87
BMI (kg/m^2)	34.1±1.7 (25.2-44.6)	32.1±1.2 (25.1-39.0)	0.36
WC (cm)	105.1±3.8 (89.6-130.0)	103.4±3.5 (84.8-122.2)	0.73
Body Fat Mass (kg)	45.3±3.5 (24.7-64.9)	34.6±2.7 (22.5-51.2)	0.02
Body Fat Percent (%)	48.7±1.4 (38.7-54.2)	38.1±2.0 (26.7-52.0)	0.001
Fat Free Mass (kg)	46.3±1.6 (37.3-59.5)	55.8±2.7 (36.2-71.1)	0.006
Cardiorespiratory Fitness			
VO_2peak (L/min)	1.4±0.09 (1.0-2.13)	2.3±0.1 (1.4-2.9)	<0.001
VO_2peak (ml/kg/min)	15.4±0.6 (11.1-18.0)	25.9±3.0 (22.7-33.1)	<0.001
Cardiovascular Risk Factors			
Systolic BP (mm/Hg)	130.1±6.3 (101.0-184.0)	125.6±3.0 (107.0-142.0)	0.53
Diastolic BP (mm/Hg)	72.8±4.0 (57.0-111.0)	71.5±2.4 (55.0-84.0)	0.75
MAP (mmHg)	91.9±4.7 (73.5-135.3)	89.4±2.3 (75.3-102.0)	0.47
Pulse Pressure (mmHg)	57.4±3.0 (41.3-78.5)	54.3±2.7 (35.0-72.0)	0.47
AI fasting (%)	32.8±3.7 (14.0-54.0)	28.7±4.7 (-4.0-66)	0.50
AI tAUC (%*180min)	4839±360 (2880.0-7080.0)	4140±787 (-2190.0-10530.0)	0.43
Fasting PG (mg/dl)	103.3±2.8 (91.3-122.0)	100.2±2.6 (86.0-114.0)	0.42
2-hr PG (mg/dl)	148.8±10.1 (99.5-217.0)	130.3±10.6 (77.3-185.0)	0.22
Glucose $tAUC_{180}$ (mg/dl*min)	26002.2±1424.4 (18415.5-36090.0)	23998.3±1523.6 (15413.3-34207.5)	0.35
TG (mg/dl)	131.7±19.0 (56.0-271.0)	115.6±23.9 (57-386.0)	0.61
LDL (mg/dl)	128.8±14.7 (67.0-259.0)	116.8±6.3 (86.0-156.0)	0.44
HDL (mg/dl)	51.6±4.5 (40.0-95.0)	50.1±3.6 (31.0-77.0)	0.79
Total Cholesterol (mg/dl)	202.2±16.8 (134.0-346.0)	186.1±8.0 (144.0-239.0)	0.39
White Blood Cell (k/ul)	5.6±0.4 (3.75-8.30)	5.8±0.3 (4.23-7.80)	0.66

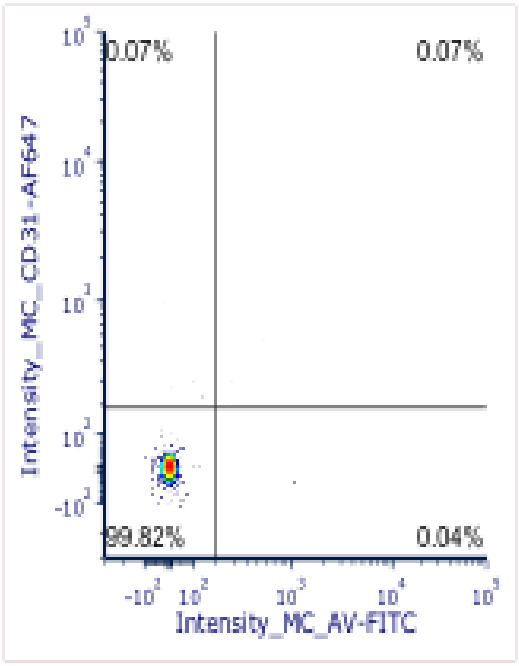
Figure 1

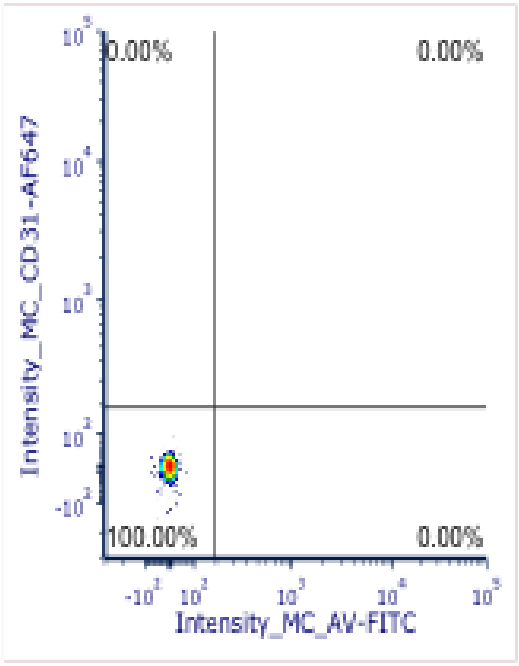
A & B.





C & D.





E & F.

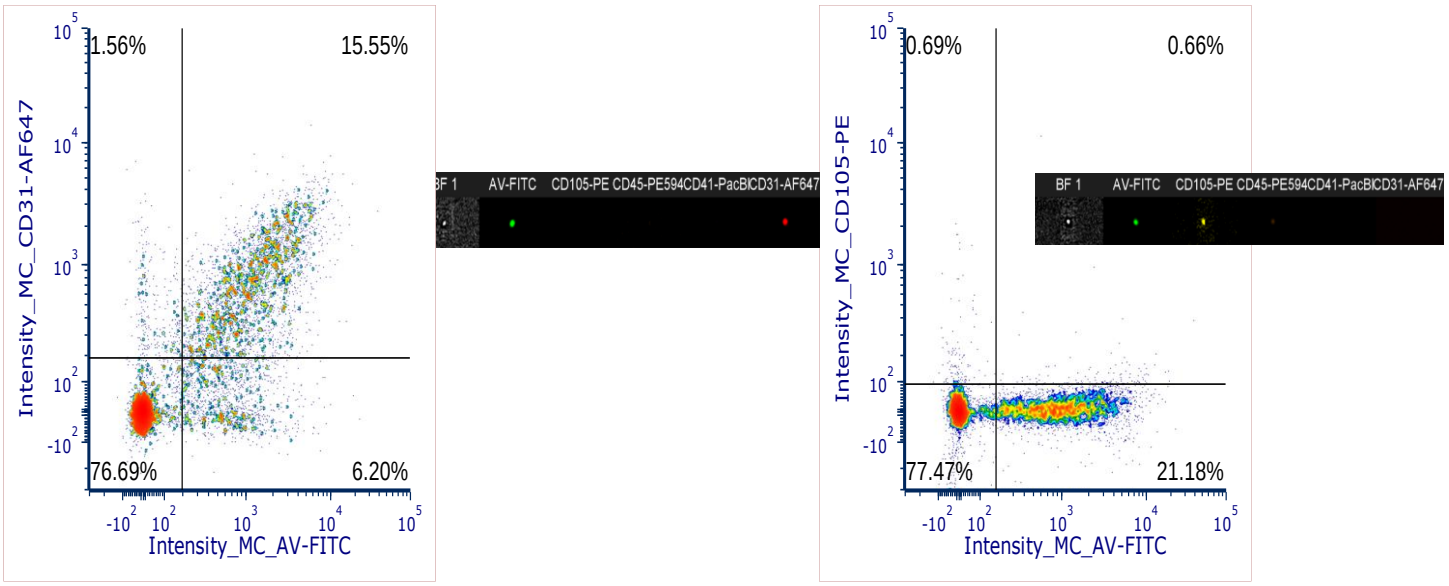
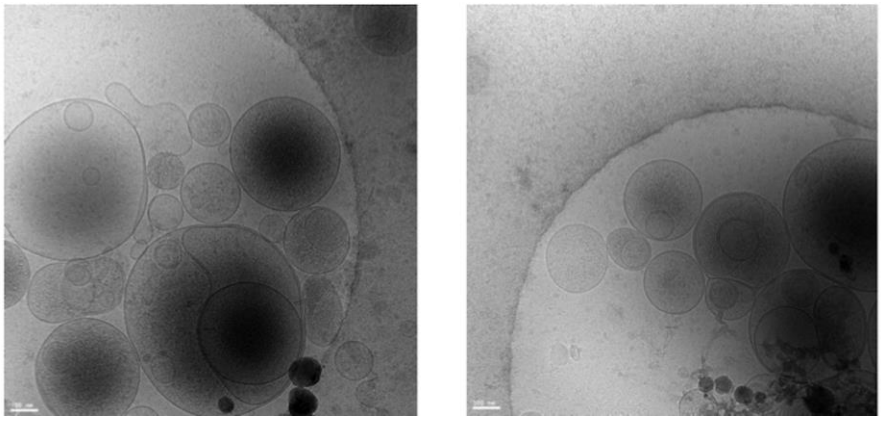
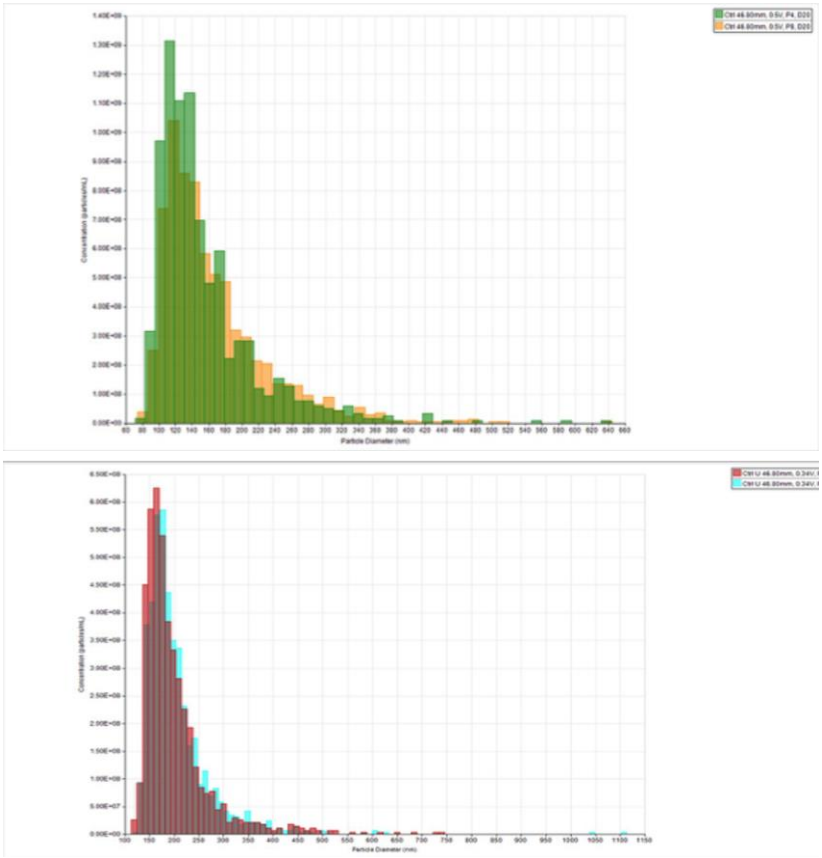

1.56%
15.55%
76.69%
6.20%
Intensity_MC_CD31-AF647
Intensity_MC_AV-FITC
BF 1
AV-FITC
CD105-PE CD45-PE594CD41-PacBlCD31-AF647
0.69%
0.66%
77.47%
21.18%
Intensity_MC_CD105-PE
Intensity_MC_AV-FITC
BF 1
AV-FITC
CD105-PE CD45-PE594CD41-PacBlCD31-AF647

Figure 2

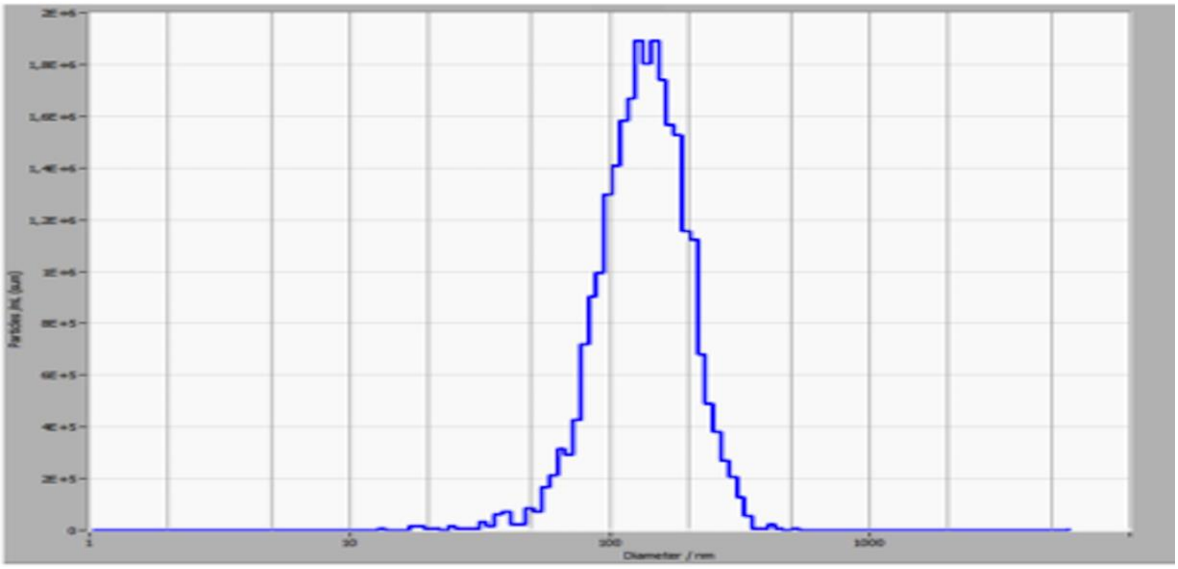
A.



B.



C.


Particles /mL (sum)
Diameter / nm
1
10
100
1000

D.

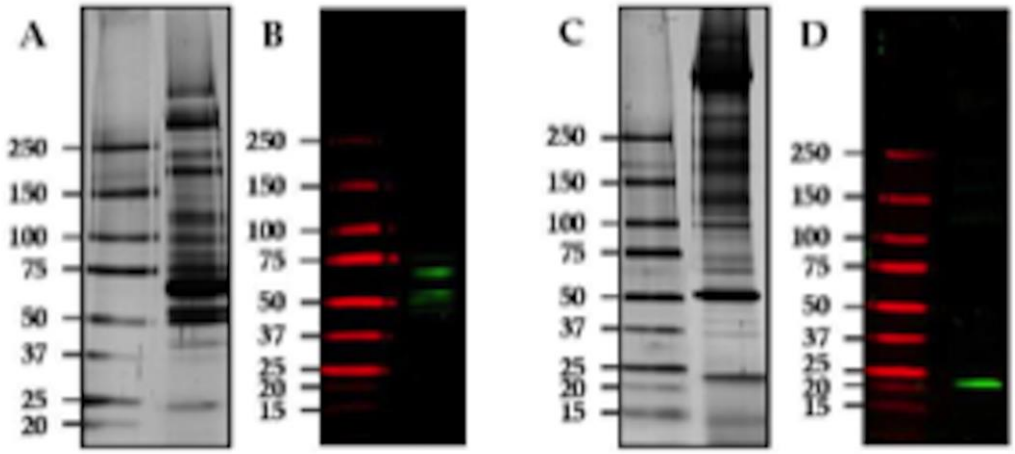

A
B
C
D
250
150
100
75
50
37
25
20
15

Figure 3

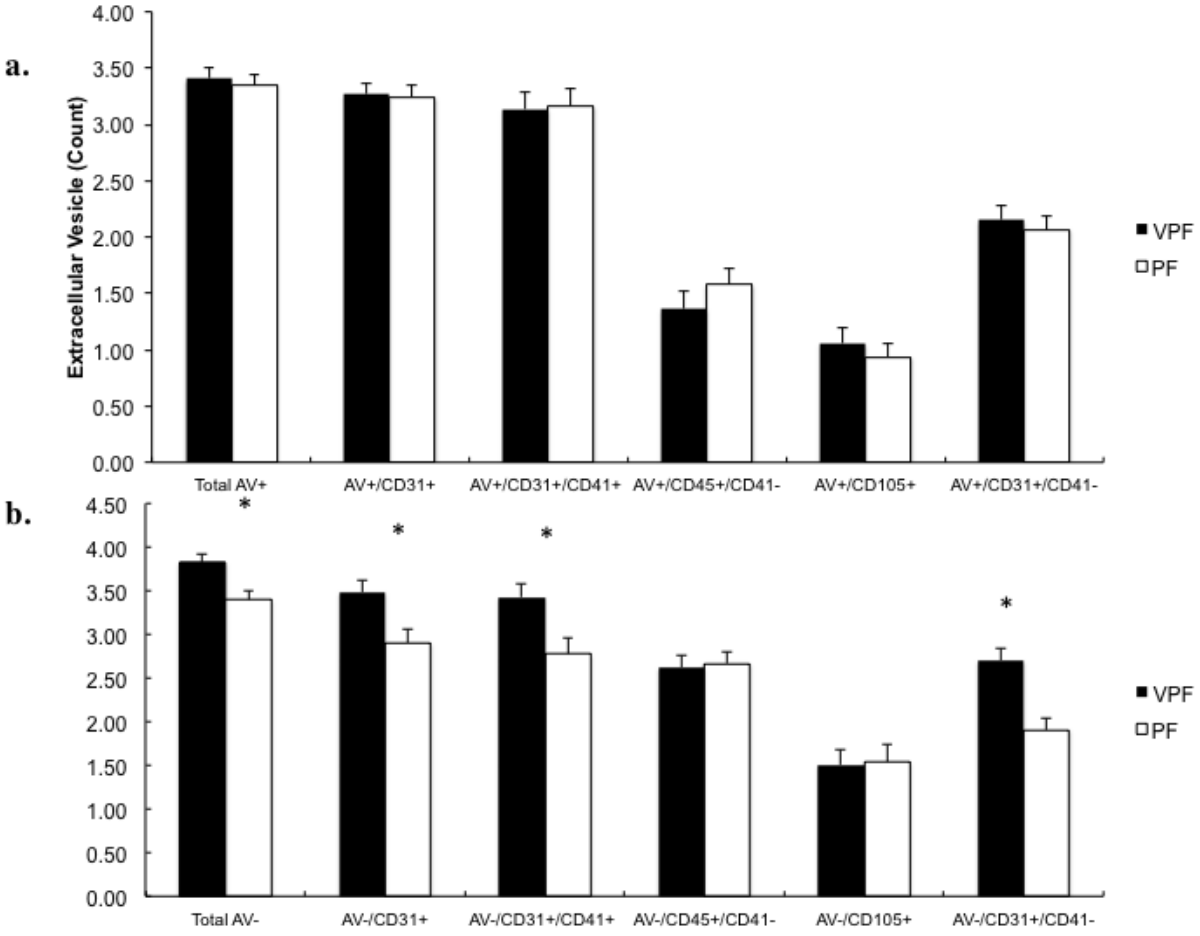
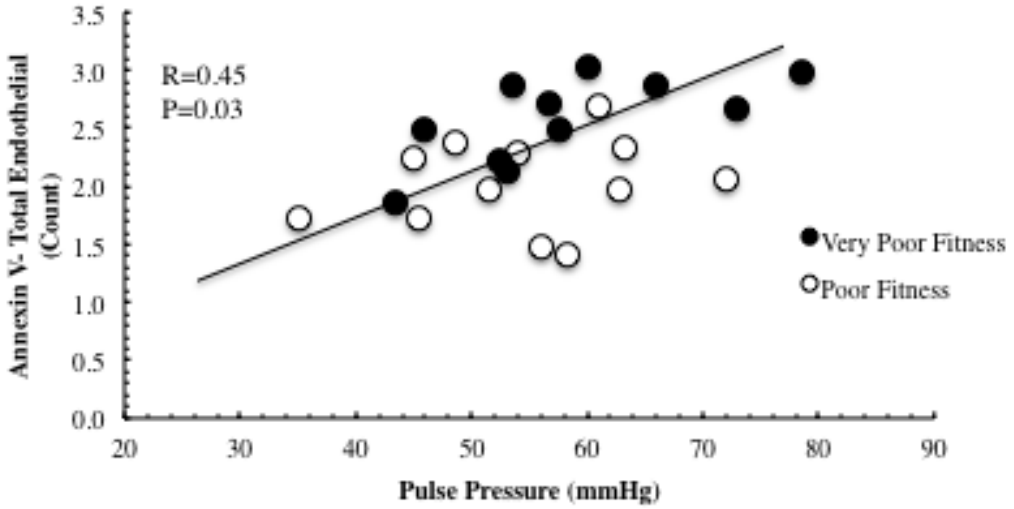
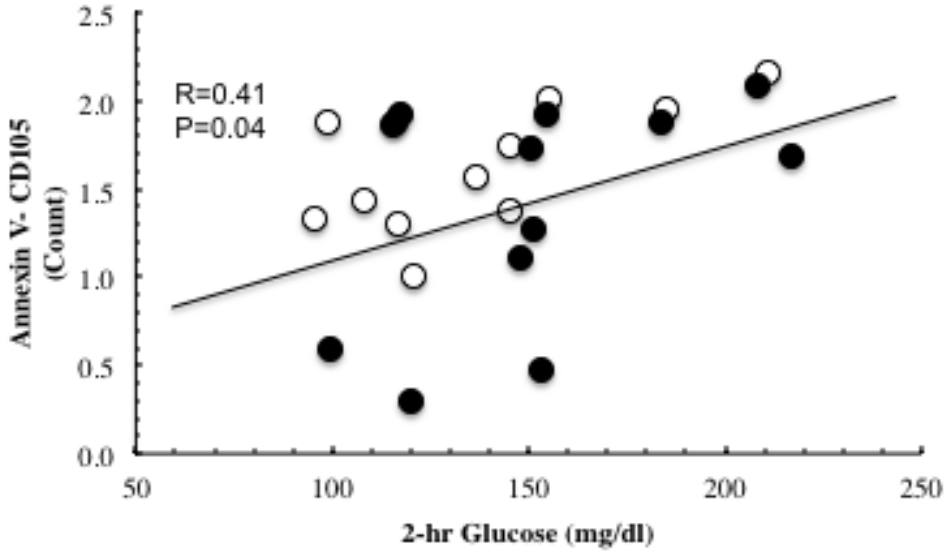

a.
Extracellular Vesicle (Count)
4.00
3.50
3.00
2.50
2.00
1.50
1.00
0.50
0.00
Total AV+
AV+/CD31+
AV+/CD31+/CD41+
AV+/CD45+/CD41-
AV+/CD105+
AV+/CD31+/CD41-
VPF
PF
b.
4.50
4.00
3.50
3.00
2.50
2.00
1.50
1.00
0.50
0.00
*
Total AV-
AV-/CD31+
AV-/CD31+/CD41+
AV-/CD45+/CD41-
AV-/CD105+
AV-/CD31+/CD41-
VPF
PF

Figure 4

A.


R=0.45
P=0.03
Annexin V- Total Endothelial (Count)
Pulse Pressure (mmHg)
Very Poor Fitness
Poor Fitness
0.0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
20
30
40
50
60
70
80
90

B.


R=0.41
P=0.04
Annexin V- CD105 (Count)
2-hr Glucose (mg/dl)
0.0
0.5
1.0
1.5
2.0
2.5
50
100
150
200
250

CHAPTER 4: AIM II

AN ORAL GLUCOSE LOAD DECREASES POSTPRANDIAL EXTRACELLULAR VESICLES IN OBESE ADULTS WITH AND WITHOUT PREDIABETES

ABSTRACT

BACKGROUND: Extracellular vesicles (EVs) are a novel mediator and/or biomarker of cardiovascular disease (CVD) and type 2 diabetes. *In-vitro* data has suggested hyperglycemia to modify EVs in relation to CVD risk, but the effects of hyperglycemia on EVs *in-vivo* is unknown. We tested the hypothesis that a 75g oral glucose tolerance test (OGTT) would promote changes in EVs linked to CVD risk. **METHODS**: Twenty-five obese adults (Age: 52.4±3.2y, BMI: 32.5±1.2kg/m^2) were screened for prediabetes using ADA criteria (75g OGTT and/or HbA1c). Eight were normal glucose tolerant (NGT) and 17 had prediabetes. Body composition (bioelectrical impedance) was measured. Arterial stiffness (augmentation index; AI) was collected at 0, 1- and 2-hr while insulin, glucose and free fatty acids were collected every 30 min during the OGTT to assess CVD risk. Annexin V+ (AV+) and Annexin V- (AV-) total EVs, platelet EVs (CD31$^+$/CD41$^+$), leukocyte EVs (CD45$^+$), platelet endothelial cell adhesiom molecule (PECAM) (CD31$^+$) and endothelial EVs (CD41$^+$; CD 31$^+$/CD41$^-$) were also collected at these times and analyzed from fresh plasma via imaging flow cytometry. **RESULTS**: There were no statistical differences in age, BMI, or body fat (all P>0.63) between NGT and PD, although fasting and 2-hr glucose as well as insulin were higher in prediabetes vs. NGT (both: P≤0.03). However, glucose and insulin increased comparably during the OGTT (both time: P≤0.001), while arterial stiffness decreased by about 6.9% (time: P=0.06). Fasting EVs were not different between groups. Total EVs, AV+ CD31$^+$ and AV+ CD31$^+$/CD41$^-$ EVs decreased after the OGTT (P≤0.04), while AV- CD31$^+$ (P=0.08) and AV- CD31$^+$/CD41$^+$ (P=0.10) trended. Increased circulating insulin at 2-hr correlated with elevated post-prandial AV+ CD105 (r=0.45, P=0.06) and AV- CD45$^+$ (r=0.48, P=0.04), while arterial stiffness was associated with reduced total EVs (r=-0.49, P=0.03) and AV+CD41$^+$ (r=-0.52, P=0.02). **CONCLUSION**: An oral glucose load lowers post-prandial total, platelet and endothelial MPs in

obese adults with NGT and prediabetes. Further work is required to examine EV content in order to gain mechanistic insight for optimizing type 2 diabetes and CVD risk management.

INTRODUCTION

Approximately 1 in 3 adults in the United States have prediabetes, and that number is expected to increase in the upcoming decades (1). This is concerning since these individuals are at an increased risk for type 2 diabetes (T2D), cardiovascular disease (CVD) and all-cause mortality when compared to individuals with normal glucose tolerance (NGT) (2). Prediabetes is defined as having either impaired fasting glucose and/or impaired glucose tolerance following a 75g oral glucose tolerance test (OGTT). This is clinically relevant as postprandial elevations in blood glucose are a better predictor of CVD when compared to fasting glucose alone (3, 4). However, the exact mechanism by which postprandial hyperglycemia confers increased CVD risk remains unclear.

Extracellular vesicles (EVs) have emerged as novel biomarkers of T2D and CVD (5, 6). In fact, addition of endothelial-derived EVs as a parameter to the Framingham risk score significantly improved prediction of future CVD events in high-risk patients (7). Elevated levels of endothelial-EVs have even been reported in individuals with prediabetes when compared to individuals with NGT (8), suggesting that even hyperglycemia is related to EV release. This is consistent with in-vitro work suggesting that EVs alter glucose homeostasis and insulin resistance (9-11), as well as promote inflammation (12, 13), endothelial dysfunction (6, 14, 15) and vascular stiffening (16, 17). Given these observations are all associated with CVD, it stands to reason that EVs may altered during the postprandial state (18). Interestingly, hyperglycemia is considered to be a key factor that impacts EV release, phenotype and function (19). In-vitro work has shown hyperglycemia to increase endothelial-derived EV formation when compared to controlled glucose conditions, which also resulted in greater EV oxidative stress and pro-coagulant activity (20). In vivo data also support the notion that EV release may be modulated by carbohydrate feeding, as recent report indicated that

endothelial EVs are lowered following carbohydrate restriction in adults with type 2 diabetes (21). Taken together, while various stimuli (e.g. lipids and inflammation) have been suggested to impact EV release (18, 22), the literature suggests that glucose availability is likely a key factor. However, no study has specifically examined the effect of a high glucose load in vivo on circulating EVs from endothelium, platelets and leukocytes in people at risk for T2D and/or CVD. This is an important knowledge gap as platelet, leukocyte and endothelial EVs each have distinct physiological effects (6, 23, 24). Therefore, the purpose of the present study was to determine if adults with prediabetes have elevated levels of total, endothelial, platelet and leukocyte derived EVs in response to a 75 g OGTT versus people with NGT. Based on previous in-vitro work, we hypothesized that EVs in individuals with prediabetes would increase more so than those individuals with NGT and that these elevations in EVs would correlate with increased CVD risk.

METHODS

Subjects

Twenty-five adults with obesity were classified as either having prediabetes (n=17) or NGT (n=8) using a standard 2-hr 75g OGTT according to the American Diabetes Association criteria (fasting glucose: 100-125 mg/dl, 2-hr glucose 140-199 mg/dl, or HbA1c 5.7-6.4%). Subjects were excluded from participation if physically active (>60 min/wk), smoking, or diagnosed with type 2 diabetes (determined by HbA1c), cardiac dysfunction, cardiopulmonary disorders, cancer within the last 5 years and/or liver dysfunction. Subjects were also excluded if on medications known to influence insulin sensitivity (e.g. metformin, GLP-1 agonist, etc.) or endothelial function (beta-blockers, ACE-inhibitors, etc.). All individuals underwent physical examination that included a resting and exercise 12-lead EKG, as well as biochemical and urine testing to rule out disease. Subjects provided verbal and written informed consent as approved by our Institutional Review Board.

Metabolic Control

Prior to admission to our Clinical Research Unit (CRU), subjects were instructed to refrain from strenuous exercise or alcohol consumption within 48-hr of testing. Subjects were also instructed to refrain from taking any medications or dietary supplements 24-hr prior to reporting to the CRU. Subjects were instructed consume approximately 250 g/d carbohydrate on the day before testing to minimize differences in insulin action. Three-day food logs, including two weekdays and one weekend day, were also used to assess *ad libitum* food intake. Participants selected these days and were provided with reference guides that displayed serving sizes of beverages and food. Data was analyzed using ESHA (Version 11.1, Salem, OR) and averaged for analysis.

Body Composition and Aerobic Fitness

Following an approximate 4-hr fast, body weight was measured to the nearest 0.01 kg on a digital scale with minimal clothing. Height was measured using a stadiometer for estimations of body mass index. Body fat and fat-free mass were measured using the InBody 770 Body Composition Analyzer (InBody CO, Cerritos, CA). Waist circumference was obtained 2cm above the umbilicus twice using a plastic tape measure and averaged. VO_2peak was used to assessed aerobic fitness and was determined using a continuous progressive exercise test on a cycle ergometer with indirect calorimetry (Carefusion, Vmax CART, Yorba Linda, CA).

Oral Glucose Tolerance Test (OGTT)

Following an approximate 10-12-hr fast, subjects reported to the CRU. Subjects were then instructed to lay supine undisturbed for at least 5 minutes to determine resting heart rate (HR) and blood pressure, which was averaged over three measurements for data analysis. Additionally, pulse pressure (defined as systolic-diastolic blood pressure) and mean arterial pressure [((2*diastolic)+systolic)/3] was calculated. An intravenous line was placed in the antecubital vein and blood samples of glucose and insulin were collected. A 75 g OGTT was then performed to assess postprandial glucose tolerance and insulin sensitivity. Plasma glucose, insulin and FFA during the

OGTT were tested at 30, 60, 90, 120 minutes. Additionally, measures of post-prandial systolic and diastolic blood pressure were obtained at 60 and 120 minutes. Total area under the curve (tAUC) was calculated using the trapezoidal model. HOMA-IR was used to assess hepatic insulin resistance as previously described (25). Adipose insulin resistance was also calculated as the product of plasma insulin and FFA tAUC at 120 minutes. Whole body insulin sensitivity, which mostly reflects peripheral glucose metabolism, was assessed by the Matsuda Index (26).

Arterial Stiffness

Fasting and postprandial augmentation index (AI) was measured by aplanation tonometry using the SphygmoCor® system (AtCor Medical, Itasca, IL) at 0, 60 and 120 minutes of the OGTT while resting quietly semi-supine in a temperature controlled room. AI was corrected to a heart rate of 75 bpm using the manufacturer's software.

EV preparation

Fresh blood prior to the OGTT and at 120 minutes during the OGTT was drawn using a BD Insyte Autoguard 22G, collected in citrate vacutainers, and processed at room temperature within 120 minutes of collection for the measurement of platelet ($CD31^+/CD41^+$), leukocyte ($CD45^+/CD41^-$; $CD45^+$), platelet endothelial cell adhesion molecule (PECAM) ($CD31^+$) and endothelial (CD105 and $CD31^+/CD41^-$) EVs. Annexin V (AV) was used as membrane dye and platelet poor plasma was obtained by centrifugation (Sovall RC 5B Plus Centrifuge: Rotor SS-34) at 5000 g at room temperature for 15 minutes. An EV pellet was obtained from platelet poor plasma by a second centrifugation spin (Centrifuge: 524/5424 R-Rotor FA-45-24-11) at 17,000 g for 10 minutes and subsequently washed with AV buffer (1xAVb) (BD Parmingen, San Diego, CA). Samples and controls were processed as previously described by our group (27). Upon completion, EVs in the sample were concentrated two-fold.

EV origin by imaging flow cytometry

The imaging flow cytometer ImageStream® MKII (Amnis, Seattle, WA) (ISX) was used to isolate and determine the source and count of the EVs (27, 28). EV counts were measured by a volumetric method provided by the software of ISX. The acquired raw data were then analyzed using IDEAS software.

EV image by cryo-electron microscopy

Purified samples were verified by standard methods for cryo-electron microscopy (cryoEM) to determine EV morphology (29). In brief, an aliquot (3 ml) was applied to a glow-discharged, perforated carboncoated grid (2/2-4C C-flats), blotted with filter paper, andrapidly plunged into liquid ethane. Low-dose images were recorded at a magnification of 29,0003 on a FEI Tecnai F20 Twin transmission electron microscope operating at 120 kV, with a nominal underfocus ranging from 3.5 to 5 mm and a pixel size of 0.388 nm at the specimen level. All images were recorded with a Gatan 4K 3 4K pixel CCD camera. The grids were stored in liquid nitrogen, and then maintained in the microscope at 2180_C using a Gatan 626 cryo-stage. Samples were also prepared for scanning electron microscopy.

EV size by Nanosight Tracking Analysis

Tunable Resistive Pulse Sensing (TRPS) was performed with a gold qNano instrument (Izon Ltd) mounting a polyurethane nanopore membrane NP200 (range 85-500 nm) and NP400 (range 125-1100 nm) (Izon Ltd). Multi pressure at 4,5 and 8 mBar respectively was applied to determine the particle concentration. Electrolyte solution was made of PBS supplemented with 0.03 % (v/v) Tween-20 filtered with Minisart® high flow hydrophilic 0.1 µm syringe filter (Sartorious). Current pulse signals were collected using Izon Control Suite 3.2software (Izon Ltd). EV pellet after differential centrifugation was solubilized in 50 µl of filtered electrolyte solution. Polystyrene particle standards (SPK200B and CPC400B;IzonLtd.) were employed for calibration. Both uEVs pellet and particle standards were measured with a minimum of 1000 blockades. Nanosight Tracking Analysis (NTA) was carried out using the Zetaview pmx110 multiple parameter particle tracking

analyzer (particle metrix, Meerbusch, Germany) in size mode using zetaview software version 8.02.28. Plasma samples were diluted in 1x pbs to the working range of the system. The system was calibrated using 105 nm polystyrene beads and then plasma vesicle profiles were recorded and analyzed at 11 camera positions with a 2 second video length, a camera frame rate of 30 fps and a temperature of 21˚C.

EV and non-EV protein by Western Blotting

Protein quantification of EVs was performed by Coomassie micro assays. EV pellets were solubilised in 40 µl of solubilisation buffer made of 5% (w/v) sodium dodecyl sulphate (SDS), 40 mM Tris-HCl pH 6.8, 0.5 mM ethylenediaminetetraacetic acid (EDTA), 20 % (v/v) glycerol without and with 50 mM dithiothreitol (DTT) respectively. Samples were denaturated overnight at room temperature. Proteins were separated by hand cast SDS-PAGE gradient gels (Resolving gel T= 5-20 % (w/v); C=2.6 %; Stacking gel T= 3.5 % (w/v); C=2.6 %) in 25 mMTris, 192 mM glycine and 0.1 % (w/v) SDS buffer and either stained with silver staining or transferred onto a 0.45µm nitrocellulose membrane (Amersham™ Protan™ 0.45µm NC, GE Healthcare) in a wet transfer system buffer made of 25 mMTris, 192 mM glycine and 20 % (v/v) methanol. Nitrocellulose membranes were saturated with Odyssey blocking buffer (LI-COR Biosciences) and incubated in polyconal rabbit anti TSG101 (Sigma, T5701-200UL) and monoclonal mouse anti CD9 (HansaBiomed; HBM-CD9-100) overnight at room temperature (RT= 23-24◦C) in an Odyssey blocking buffer diluted 1:1 with PBS and 0.15 % (v/v) Tween-20 at concentration of 1.0 µg/ml. After 3x10 minute washes in PBS-Tween (0.15%, v/v), membranes were incubated with anti rabbit anti mouse dye-coupled secondary antibody 0.1 µg/ml(LI-COR Biosciences) in an Odyssey blocking solution diluted at 1:1 with PBS and 0.15 % (v/v) Tween-20; 1-hr at RT. Acquisition of the fluorescent signal was performed by Odyssey infrared imaging system with resolution set at 169µm (LI-COR Biosciences) (27).

Clinical Biochemical Analysis

Plasma glucose samples were analyzed immediately using the YSI 2300 StatPlus Glucose Analyzer system (Yellow Springs, OH). All other samples were centrifuged for 10 minutes at 4°C and 3000 rpm, aliquoted, and stored at -80°C until later analysis. Plasma insulin and FFA was placed in vacutainers containing EDTA and the protease inhibitor aprotonin. Insulin was analyzed using an enzyme-link immunosorbent assay kits (Millipore, Billerica, MA) and circulating FFAs were analyzed using enzymatic colorimetric method assay (Wako Diagnostics, Richmond, VA). Fasted ICAM and VCAM were measured using ELISA (R&D Systems, Minneapolis, Minnesota) by the UVA Ligand & Analysis Core lab.

Statistical analysis

Data were analyzed using SPSS v24 (Armonk, NY). Normality was assessed using Shapiro-Wilk tests. The EV data was log-transformed due to non-normality. Independent, two-tailed *t*-tests were used to determine baseline group differences between NGT and prediabetes. A repeated measures ANOVA was utilized to compare group differences following the OGTT. Given that aerobic fitness was significantly different between NGT and prediabetes, we conducted a secondarily analysis in which we included VO_2peak as a covariate. Pearson correlations were used to test associations between EVs and clinical outcomes. Statistical significance was set at $P \leq 0.05$, and trends are accepted at P=0.05-0.10. Data are reported as mean ± SEM.

RESULTS

Subject Characteristics

Body weight and body fat percent were similar between individuals with NGT and prediabetes (***Table 1***). However, adults with prediabetes had a lower relative VO2peak compared to those with NGT (P<0.02). There were no significant group differences in resting HR in those with NGT and prediabetes (74.8±2.1 vs. 70.9±2.3 bpm, P=0.31, respectively), nor in HDL (P=0.71), LDL

(*P*=0.37), total cholesterol (*P*=0.28) or triglycerides (*P*=0.44). There were no significant differences between total calories (*P*=0.70), carbohydrate (*P*=0.21), fat (*P*=0.66) or protein (*P*=0.99) between NGT and prediabetic adults in ad-libitum diet consumption (***Table 1***).

Glucose Tolerance

By design, there was a significant difference between fasting glucose (*P*=0.006) as well as 2-hr glucose (*P*=0.006; ***Table 3***). However, HbA1c did not differ between groups (NGT= 5.4±0.09 vs. prediabetes= 5.6±0.07%, *P*=0.11). As expected, glucose increased in response to the 75 g OGTT but was not statistically higher in those with prediabetes (time effect: *P*=0.005; gxt: *P*=0.13). However, adults with prediabetes had higher $tAUC_{120}$ glucose when compared to those with NGT (*P*=0.05) (***Table 3***).

Insulin, FFA and Insulin Sensitivity

As anticipated, people with prediabetes had higher fasting insulin (*P*=0.03) than those with NGT. Baseline FFA was not different between groups (*P*=0.92). Insulin significantly increased in response to the OGTT at 120 minutes, while FFA decreased (both P<0.001). Insulin sensitivity, as assessed by the Matsuda Index, was higher in NGT versus those with prediabetes (*P*=0.02) and both HOMA-IR and adipose IR were significantly lower in NGT compared to those with prediabetes (both *P*<0.03).

Blood Pressure and Inflammation

There was no difference for baseline blood pressure between individuals with NGT and prediabetes (***Table 2***). However, SBP, DBP and MAP were significantly elevated at 120 minutes following administration of the OGTT (time effect: all *P*<0.02), while PP was reduced (*P*<0.001) in both NGT and prediabetes. Although ICAM was significantly elevated in adults with prediabetes compared to those with NGT (178.4±12.6 vs. 226.5±12.7 ng/mL, *P*=0.04), VCAM was similar between the two groups (592.6±41.5 vs. 697.0±50.7 ng/mL, *P*=0.25).

Arterial Stiffness

Although there was no baseline AI difference between groups, AI at 2 hr decreased in people with NGT and prediabetes following the 75g glucose load (time effect: P=0.06) (***Table 2***).

Extracellular Vesicles

Fasting EVs were not different at baseline between NGT and prediabetes. Total EVs, EV AV+ $CD31^+$ (PECAM), as well as platelet EVs AV+ $CD41^+$, and AV+ $CD31^+/CD41^+$, all decreased at 120 minutes during the OGTT (time effect: all $P \leq 0.04$). AV- $CD31^+$ (PECAM) and AV- $CD31^+/CD41^-$ (endothelial) also tended to be lowered following the glucose load, but did not reach statistical significance (time effect: $P \leq 0.10$). Leukocyte-derived EVs and endothelial EV CD105 were not different from baseline at 120 min during the OGTT (gxt effect: P=0.14, 0.92, respectively). When cardiorespiratory was included as a covariate in the ANOVA model, the postprandial response for total EV (P=0.11), PECAM $CD31^+$ (P=0.14), and platelet $CD31^+/CD41^+$ (P=0.28) EVs were mitigated.

Correlations

Greater elevations in postprandial insulin correlated with larger increases in AV- $CD31^+$ EVs following a 75g OGTT in obese adults with and without prediabetes (***Table 3A***). Increased circulating insulin at 120 minutes correlated with elevated post-prandial AV+ CD105 (r=0.45, P=0.06) and AV- $CD45^+$ (r=0.48, P=0.04). Additionally, those with greater insulin sensitivity as assessed by $Matsuda_{120}$ saw a blunted response of leukocyte derived AV- $CD45^+$ (r=-0.45, P=0.06) and AV- $CD41^+/CD45^+$ (r=-0.47, P=0.05) in response to the glucose load. Moreover, postprandial changes in total EVs (r=0.46, P=0.07), AV+ $CD45^+$ (r=0.46, P=0.05) and AV+ CD105 (r=0.55, P=0.02) were all positively related to adipose insulin resistance. Increased hepatic insulin resistance, as assessed by HOMA-IR, was associated with increased baseline leukocyte AV+ $CD45^+$ EVs (r=0.38, P=0.08). Changes in AI were also associated with reduced total EVs following the OGTT (r=-0.49, P=0.03) (***Figure 3B***). Fasted LDL (r=0.49, P=0.07) and triglycerides (r=0.57, P=0.03) were related to baseline levels of AV+/-$CD45^+$ and higher baseline FFA (r=0.42, P=0.10) and

cholesterol (r=0.56, *P*=0.03) were related to elevated post-prandial AV+CD45^{+}. Aerobic fitness was correlated to lower insulin levels at 120 minute of the OGTT (r=-0.41, P=0.07) as well as Matsuda Index (r=0.35, P=0.10).

DISCUSSION

The primary observation of this study is that a single oral glucose load lowers total, platelet and PECAM EVs in obese adults with NGT and prediabetes. Although the reduction in EVs in response to an oral glucose load was associated with insulin sensitivity and arterial stiffness, when VO2peak was included as a covariate, the effect of an oral glucose load on EVs was weakened. Collectively, our observations suggest that EVs may be a potential link to postprandial CVD risk. To date, there are no human studies examining the acute effect of carbohydrate feeding in humans. Prior work by Francois et al. suggested that carbohydrate restriction for 4 days in T2D lowered EVs, although this did not relate to improved endothelial function (21). However, it is possible that these changes were also related to a significant decrease in total calories consumed, as individuals were given three meals equating to 500 kcal/meal over four days. Similarly, Wekesa et al. also report significant decreases in the endothelial EV CD31^{+}/CD41^{-} following 24 weeks of a low-carbohydrate diet in obese women (30). In contrast, individuals in the present study were instructed to make no changes in their ad-libitum diet 3 days prior to collection of EVs and there were no differences in ad-libitum diet between NGT and adults with prediabetes, suggesting these present results are independent of energy or carbohydrate restriction. The present study tested the specific effect of dietary glucose on EVs, whereas Francois et al. report only total carbohydrates. Taken together, these results suggest that there may be differential effects of carbohydrates (i.e. glucose only vs. total carbohydrates) on EV responses. Either way, these findings are also in contrast to previous literature that reported a significant increase in endothelial EVs in response to a single high glucose load in-vitro (20). It is important to note, however, that this prior study treated HUVECs with a glucose load

equivalent to approximately 450 mg/dl. In the present study, adults with prediabetes reached an average of only 136.8 mg/dl at 2-hr following administration of the 75 g OGTT. Therefore, it is possible that our glucose stimulus was not enough to elicit the same response reported in the in-vitro data (20). Additionally, the in-vivo effects of insulin in this present cohort cannot be discounted, as insulin has previously been shown to be intimately related to EVs in-vivo (9). As such, it is possible that our results differ due to the in-vivo response of insulin to the high glucose load, compared to the independent effects of glucose on EVs published by Burger et al (20).

The reductions in EV following a glucose load may be explained by different potential mechanisms in adults with obesity. Previous work from our group suggested that aerobic fitness is associated with lower fasting platelet and endothelial EVs (27). Therefore, it would be reasonable to expect that VO2peak may in part, influence the postprandial EV response as well. Indeed, our results support this notion, as accounting for differences in aerobic fitness weakened the effect of an oral glucose load on decreasing total EV (P=0.11), PECAM $CD31^+$ (P=0.14), and platelet $CD31^+/CD41^+$ (P=0.28). The reason fitness attenuates the effect of the OGTT on EVs is beyond the scope of this study, but improved aerobic fitness is related to insulin sensitivity (31) and we report in the present study that VO2peak was associated with insulin concentrations. Consistent with this notion, recent work demonstrates that EVs derived from macrophages (9) and adipocytes (32) reduce insulin-stimulated glucose uptake in the liver (10) and skeletal muscle (11). Moreover, macrophage derived EVs interfere with GLUT-4 translocation in human adipocytes by decreasing Akt-phosphoyrlation (9). The reason for this decreased insulin signaling was proposed to be mediated by NFkB, suggesting that inflammation plays a role in EV-induced insulin resistance (9). The results herein are in line with this previous in-vitro work, as we report that platelet derived (AV- $CD31^+/CD41^+$) EVs and the PECAM EV AV- $CD31^+$ are significantly related to the postprandial insulin response during an OGTT (***Figure 3A***) as well as higher total, leukocyte (AV+ $CD45^+$) and endothelial EVs (AV+CD105) correlating with adipose insulin resistance. Taken together, our findings suggests for

the first time that fitness related insulin sensitivity may modulate EV responses to an oral glucose load in obese adults with or without prediabetes. Further work is warranted to understand if feeding impacts EV cargo and function, given work highlighting that insulin resistance increases EVs secretion and alters circulating leukocyte function (33).

Another factor by which the postprandial response may relate to EVs is through vascular function. Circulating endothelial EVs are thought to play a key physiologic role in vascular physiology (6, 22) and have recently been associated with reduced endothelium-dependent relaxation (14), reduced flow-mediated dilation (17), increased PWV (17), and increased carotid intima-media thickness (16). In response to this high glucose load, AI, an index of systemic arterial stiffness, was lowered at 120 min of the OGTT. This is consistent with prior work (34), suggesting that feeding lowers arterial stiffness for increased nutrient delivery. Interestingly, reductions in EVs in our study were associated with preservation/elevations in arterial stiffness (***Figure 3B***). Our finding suggests that postprandial total EVs may relate to impaired vascular function following a high glucose load. It is possible that this decrease in postprandial EVs relates to less ability for the blood vessel to vasodilate by a nitric oxide, as previous work has shown circulating EVs to carry eNOS (35). Future work should consider the specific mechanism by which these various EV subtypes respond to varying levels of CVD risk.

Vascular inflammation and inflammation related to elevations in body fat may also modulate EV release in the present study, as inflammation is a known modulator of EV release (6). As there were no significant relationships between body fat, baseline EVs or the postprandial EV response, we believe that obesity had little effect on the decrease in AV+ total, platelet and PECAM EVs we report (***Figure 1***). However, we are limited by the fact that this is a relatively homogeneous group of individuals and we did not design the present study to definitively determine the impact of obesity on postprandial EV response. Additionally, measures of vascular inflammation such as VCAM were not related to baseline EVs, or predictive of the postprandial EV response in individuals with NGT or

prediabetes. Despite higher levels of ICAM in adults with prediabetes, we still report no difference in EV response to the glucose challenge. It is important to note, however, that we did not characterize other markers of inflammation, such as IL-6 or CRP, that are also known to modulate EV release (36). Therefore, it is possible that the postprandial response of EVs to hyperglycemia may relate to inflammation through a mechanism not characterized in our study.

In this present study, leukocyte derived EVs were not lowered in response to the OGTT as were PECAM ($CD31^+$) and platelet-derived ($CD31^+/CD41^+$) EVs (***Figures 1, 2***). This raises the possibility that leukocyte EVs may be more intimately related to another stimuli, such as postprandial dyslipidemia than endothelial or platelet-derived EVs, as our group (27) and others (23) have shown leukocyte EVs are inversely related to HDL concentrations as well as participate in atherosclerosis progression (23). Herein, we expand this prior work and report significant relationships between fasted LDL, triglycerides and leukocyte derived EV $CD45^+$ in this present cohort. Additionally, previous in-vitro data has suggested lipotoxicity modulates the release of EVs (37) and we now add to the literature by showing that fasting FFA and cholesterol concentrations as well as hepatic and adipose insulin resistance also relate to postprandial AV+ $CD45^+$ responses. These findings are also independent of body fat given that individuals with NGT or prediabetes had similar BMI status. Collectively, these data suggest that circulating lipids, not glucose, may be an important modulator of leukocyte-derived EVs. Additional work is needed to determine if low versus high fat meals alter the postprandial leukocyte-derived EV responses in relation to CVD risk.

This study has a relatively small sample size and further work is required to understand if individuals across sex/race respond similarly. We tested the effects of a single oral glucose load on EVs, thereby limiting the generalizability of our results, and it is possible that a high fat or mixed-meal would elicit a differential EV responses. However, use of the OGTT provides clinical relevance and proof of concept for the effect of a glucose bolus relative to in-vitro data. Moreover, it is also possible that we were limited in our ability to differentiate prediabetes from the obese control.

Despite having elevated $tAUC_{120}$ for glucose, HbA1c not statistically different between the two groups. Future work is needed to determine the impact of hyperglycemia in relation to EV response in adults with more severe prediabetes, as well as type 2 diabetes. Finally, we only measured EVs at 2-hr post-oral glucose load. While this time-point corresponds to the 2-hr glucose criteria used to predict future CVD risk, it remains possible that EVs may respond to an acute nutrient load in a mono- or biphasic in nature as has been shown for circulating glucose (38). Interestingly, we report significant effects of hyperglycemia on AV+ EVs, with only some trends on AV- EVs. Previously, we have shown AV- EVs to be elevated in adults with lower cardiorespiratory fitness (27). Although it is beyond the scope of the present study to determine why we do not see significant effects of hyperglycemia on AV- EVs, we speculate that this may be due to difference in stimuli, as Connor et al. report the proportion of EVs that bound Annexin was dependent upon the agonist of EV release (24). Taken together, future work is needed to better understand the differing functions of AV+/- EVs, as our work suggests they may differentially respond to varying stimuli (i.e. fitness, feeding). Finally, associations do not equal causation and future work is needed to test how nutrients impact in vivo EV cargo (e.g. mRNA, proteins, etc.) and function (39) to illuminate roles these cells have on human physiology and disease risk.

In conclusion, a single oral glucose load lowers total, platelet, and PECAM EVs in obese adults with NGT and prediabetes. These findings may have clinical relevance, as the reductions in EVs were associated with insulin sensitivity and arterial stiffness. However, aerobic fitness may, in part, mediate the regulation of postprandial response of these EVs. Therefore, future research is warranted to examine whether training status and habitual dietary carbohydrate intake impacts EV subtypes in a cell-specific manner to reduce type 2 diabetes and CVD risk for optimization of disease care and management.

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TABLE LEGENDS

Table 1: Baseline demographics. Data are means±SEM. Significant baseline differences (*$P \leq 0.05$). Differences in fitness accounted for in the ANOVA model mitigated the postprandial response of EVs.

Table 2: Cardiovascular risk factors in adults with normal glucose tolerance (NGT) and prediabetes. Data are means±SEM. No significant baseline differences existed between groups for any outcome. Blood pressure (BP); mean arterial pressure (MAP); resting heart rate (RHR); augmentation index (AI). Time effect (*$P \leq 0.05$).

Table 3: Glucose Regulation in Adults with Normal Glucose Tolerance (NGT) and Prediabetes

Data are means±SEM. Significant baseline differences (*$P \leq 0.05$). Plasma glucose (PG); total area under the curve (tAUC); incremental area under the curve (iAUC); free fatty acid (FFA); insulin resistance (IR).

FIGURE LEGENDS

Figure 1: Comparison of changes (Δ; fed-fasted) in Annexin V+ (AV+) extracellular vesicle (EV) subtype count following an oral glucose tolerance test (OGTT) in normal glucose tolerance (NGT) versus prediabetes. No significant baseline difference between any EV subtypes. EV data were log-transformed. There were no group x time differences. *Denotes significant effect of time, P≤0.05.

Figure 2: Comparison of changes (Δ; fed-fasted) in Annexin V- (AV-) extracellular vesicle (EV) subtype count following an oral glucose tolerance test (OGTT) in normal glucose tolerance (NGT) versus prediabetes. No significant baseline difference between any EV subtypes. EV data were log-transformed. There were no group x time differences. ^Denotes trend for significant effect of time, ^$P \leq 0.10$.

Figure 3: Correlations between changes (Δ; fed-fasted) in extracellular vesicles (EVs), insulin (A) and arterial stiffness, as assessed by augmentation index (B). EV data were log-transformed. Open circles, normal glucose tolerant (NGT). Closed circles, prediabetes (PD).

Table 1: Baseline Demographics

	NGT	Prediabetes	P-value
N (M/F)	8 (1/7)	17 (4/13)	-
Age (yr)	50.1±5.1	53.5±4.1	0.63
Body weight (kg)	90.3±2.9	98.1±3.0	0.34
Body Mass Index (kg/m^2)	33.2±1.4	32.1±1.6	0.67
Body Fat (%)	42.3±2.9	44.1±5.3	0.64
Fat Free Mass (kg)	53.9±1.9	53.7±2.9	0.95
VO_2peak (L/min)	2.1±0.2	1.8±0.1	0.10
VO_2peak (ml/kg/min)	23.4±1.8*	18.6±0.9	0.01
HDL (mg/dL)	49.7±3.0	52.8±5.9	0.71
LDL (mg/dL)	111.3±7.0	131.7±15.8	0.37
Triglycerides (mg/dL)	103.0±22.2	136.7±28.8	0.44
Cholesterol (mg/dL)	178.0±8.4	207.1±18.4	0.28
Calories	2381.7±263.0	2261.7±263.0	0.70
CHO (g)	291.4±29.4	244.1±21.2	0.21
Sugar (g)	107.1±17.2	93.7±8.7	0.45
Total Fiber (g)	23.6±2.8	19.0±1.3	0.10
Fat (g)	97.7±14.6	91.4±26.5	0.66
Protein (g)	86.4±10.9	86.5±7.2	0.99

Data are means±SEM. Normal glucose tolerance (NGT); high density lipoprotein (HDL); low density lipoprotein (LDL); total carbohydrate (CHO). Significant differences (*$P \leq 0.05$). Differences in fitness accounted for in the ANOVA model mitigated the postprandial response of EVs.

Table 2: CVD Risk Factors in Adults with Normal Glucose Tolerance (NGT) and Prediabetes

	NGT		Prediabetes		ANOVA (P-value)	
	0 min	**120 min**	**0 min**	**120 min**	**Time**	**GxT**
Systolic BP (mmHg)	125.0±3.7	134.1±6.7*	129.2±4.8	137.6±4.4*	0.02	0.88
Diastolic BP (mmHg)	71.9±4.1	77.1±4.6*	71.3±3.3	81.7±2.7*	0.009	0.35
MAP (mmHg)	87.2±3.5	99.4±5.8*	91.4±3.7	99.4±3.3*	0.001	0.42
Pulse Pressure (mmHg)	51.6±2.8	41.9±3.2*	57.9±2.3	42.3±2.0*	<0.001	0.22
RHR (bpm)	66.8±2.0	66.2±2.0*	66.6±2.9	63.4±1.8*	0.02	0.44
AI (mmHg)	32.1±7.8	17.3±9.5	23.4±2.1	20.3±2.7	0.06	0.21

Data are means±SEM. No significant baseline differences existed between groups for any outcome. Blood pressure (BP); mean arterial pressure (MAP); resting heart rate (RHR); augmentation index (AI). Time effect (*$P \leq 0.05$).

Table 3: Glucose Regulation in Adults with Normal Glucose Tolerance (NGT) and Prediabetes

	NGT	Prediabetes	P-value
Fasting Plasma Glucose (mg/dL)	95.2±1.3	104.9±2.1*	0.006
120 Minute Glucose (mg/dL)	105.7±9.2	136.8±9.2	0.63
PG $tAUC_{120}$ (mg/dL*min)	15046.6±550.0	17593.8±1089.3*	0.05
Insulin (μU/ml)	13.8±2.2	12.8±2.4*	0.03
120 Minute Insulin (μU/ml)	55.3±11.3	107.0±11.8*	0.01
Insulin $iAUC_{120}$ (μU/ml*min)	8697.7±1718.6	10819.3±1079.4	0.29
Insulin $tAUC_{120}$ (μU/ml*min)	53.9±1.9	53.7±2.9	0.20
Fasting FFA (mEq/L)	0.52±0.07	0.53±0.03	0.92
120 minute FFA (mEq/L)	0.12±0.05	0.22±0.05	0.20
FFA $tAUC_{120}$ (mEq/L*min)	31.3±6.4	45.7±6.7	0.20
HOMA IR	2.4±0.4	4.5±0.7*	0.02
Adipose IR	19.2±3.9	35.9±6.0*	0.03
$Matsuda_{120}$	4.0±1.0	2.1±0.3*	0.02
HbA1c (%)	5.4±0.09	5.6±0.07	0.11

Data are means±SEM. Significant differences (*$P \leq 0.05$). Plasma glucose (PG); total area under the curve (tAUC); incremental area under the curve (iAUC); free fatty acid (FFA); insulin resistance (IR).

Figure 1: Comparison of changes (Δ; fed-fasted) in Annexin V+ (AV+) EV subtype count following an OGTT in NGT vs. prediabetes. EV data were log-transformed. *Effect of time, P≤0.05.

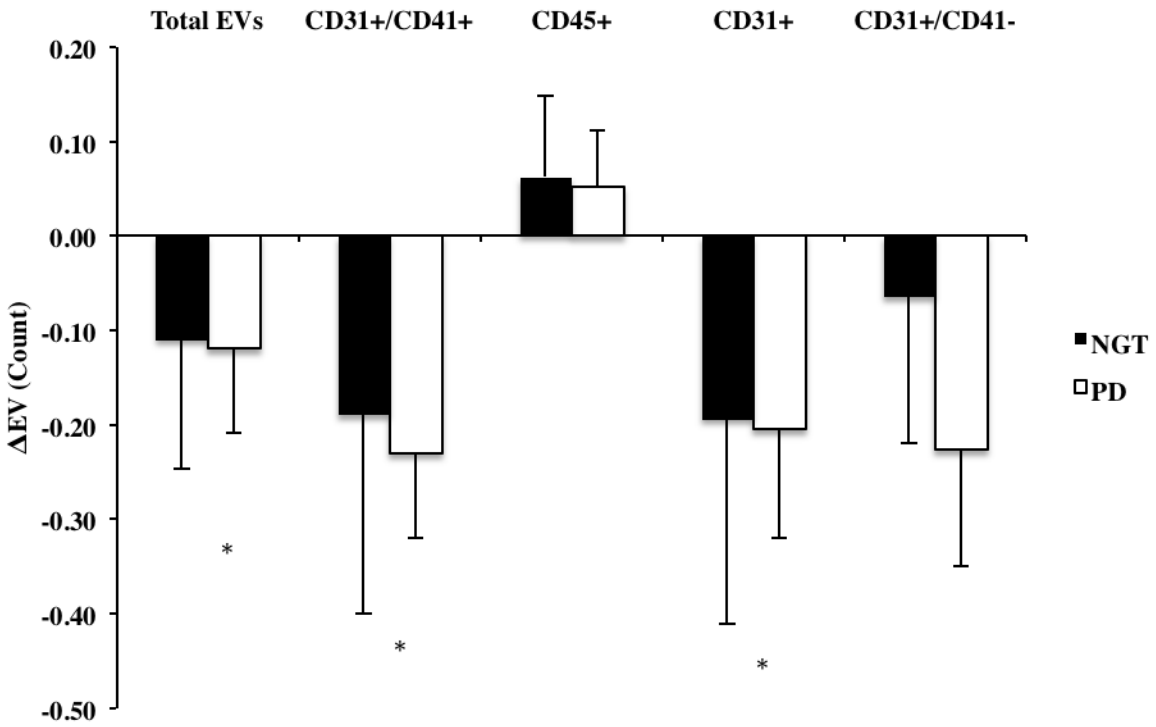


Figure 2: Comparison of changes (Δ; fed-fasted) in Annexin V- (AV) EV subtype count following an OGTT in NGT vs. prediabetes. EV data were log-transformed. Effect of time, ^P≤0.10.

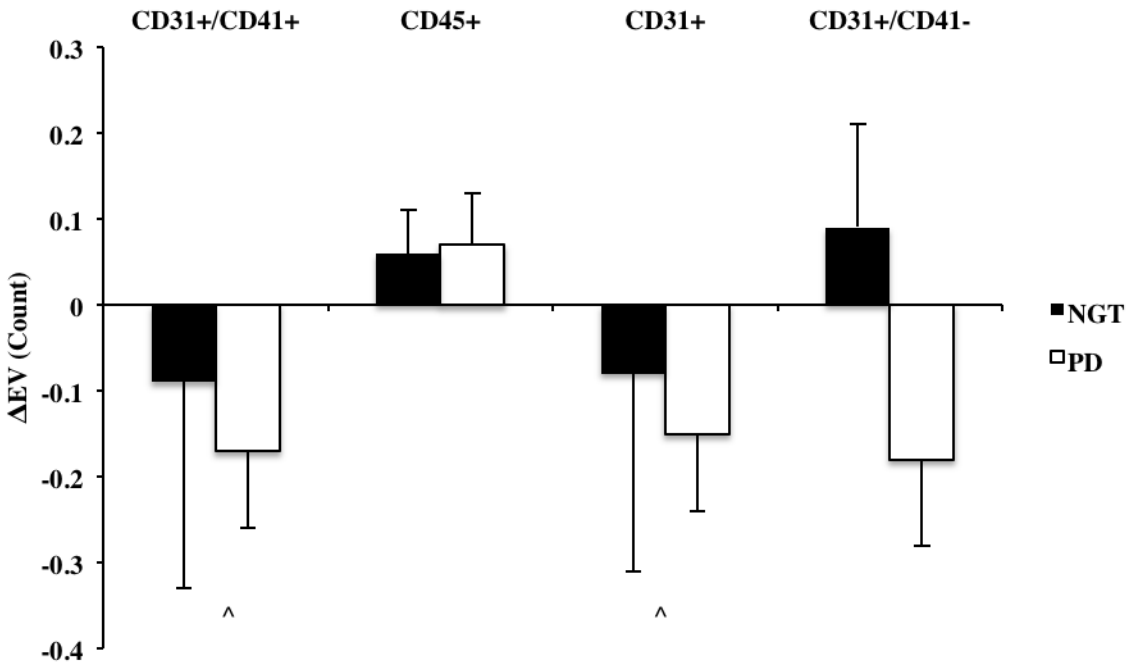
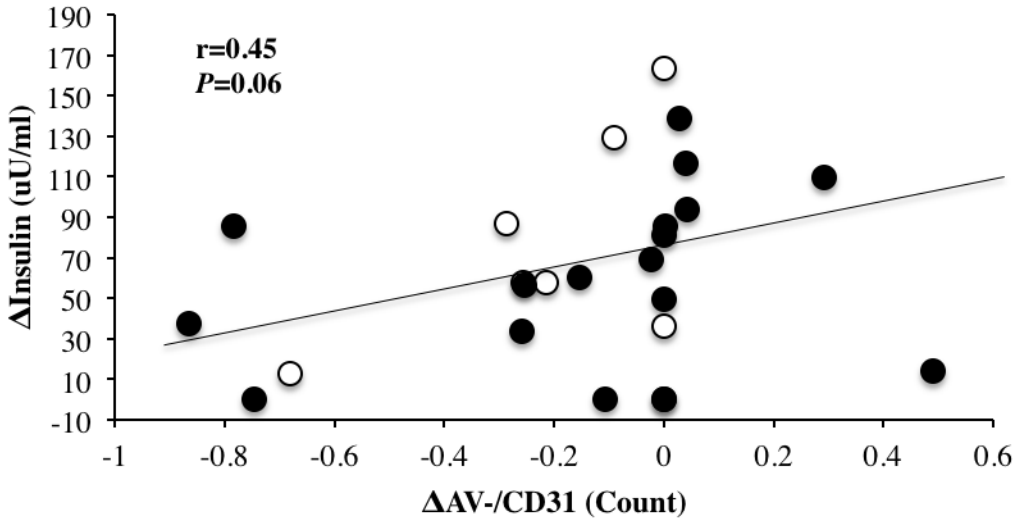
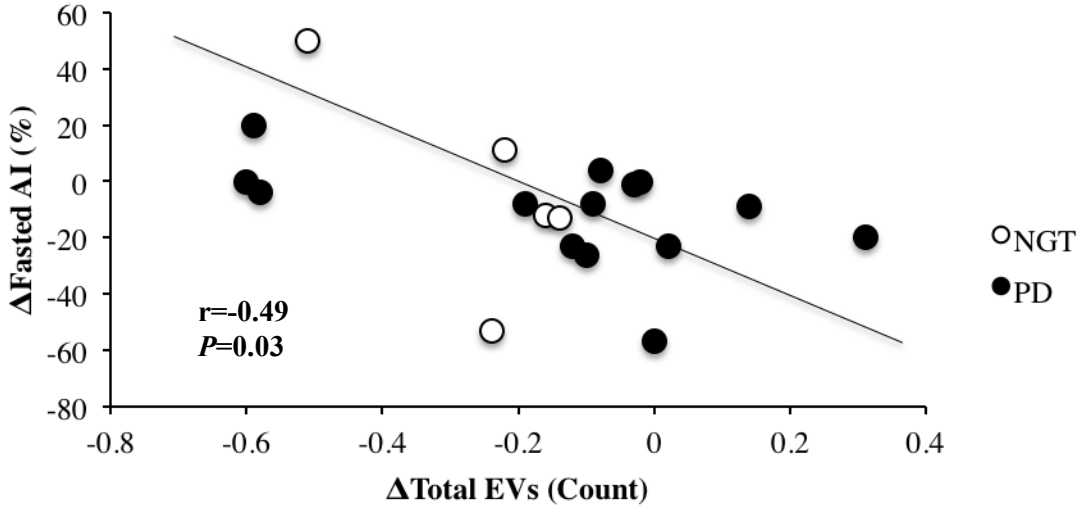


Figure 3: Correlations between changes (Δ; fed-fasted) in EVs, insulin (A) and arterial stiffness, as assessed by augmentation index (B) *EV data were log-transformed

A.



B.



CHAPTER 5: AIM III

INTERVAL EXERCISE REDUCES CIRCULATING ANNEXIN V- CD105 EXTRACELLULAR VESICLES IN ADULTS WITH PREDIABETES

ABSTRACT

BACKGROUND: Extracellular vesicles (EVs) are derived from platelet, leukocyte and endothelial cells and are purported to mediate type 2 diabetes (T2D) and cardiovascular disease (CVD). Lifestyle, including physical activity and diet, reduce disease risk, but no study has tested the effect of short-term training intensity on EV subtypes in people with prediabetes. We tested the hypothesis that short-term interval (INT) training would reduce EVs compared with continuous (CONT) exercise. **METHODS**: Eighteen obese adults (age: 63.8±1.5yrs BMI: 31.0±1.3 kg/m^2) were screened for prediabetes using ADA criteria (75g OGTT). Subjects were randomized to INT (n=10, 3 min intervals at 90% and 50% HR_{peak}) or CONT (n=8, 70% HR_{peak}) training for 12 supervised sessions over 2 wks for 60 min/d. Fitness (VO_2peak), weight (kg), as well as ad-libitum dietary intake were assessed and arterial stiffness (augmentation index; AI) was calculated using total AUC during a 75g OGTT. Total EVs, platelet EVs ($CD31^+/CD41^+$), endothelial EVs (CD105; $CD31^+/CD41^-$), platelet endothelial cell adhesion molecule (PECAM) ($CD31^+$) and leukocyte EVs ($CD45^+$; $CD45^+/CD41^-$) were analyzed from fresh plasma via imaging flow cytometry pre-/post- intervention. **RESULTS**: The interventions had no effect on weight loss, but INT exercise increased VO_2peak (P=0.04) and reduced fasted AI (trend: P=0.08) compared with CONT training. While training had no effect on platelet or leukocyte EVs, INT exercise decreased Annexin V- endothelial EV CD105 compared with CONT training (P=0.04). When accounting for dietary sugar intake, however, the intensity effect was lost (P=0.18). Increased ad-libitum dietary sugar intake following training was also linked to elevated AV+CD105 (r=0.49, P=0.06) and AV-$CD45^+$ (r=0.59, P=0.01). Nonetheless, increased VO_2peak correlated with decreased AV+ CD105 (r=-0.60, P=0.01). **CONCLUSION**: Independent of weight loss, exercise intensity decreases endothelial derived EVs in adults with prediabetes. Although increased sugar consumption may attenuate EV profiles following a short-term exercise intervention, cardiorespiratory fitness plays an important role in modulation of EV release.

INTRODUCTION

Exercise is cornerstone at reducing type 2 diabetes (T2D) and CVD risk. However, the precise biological mechanism(s) by which exercise confers beneficial effects remains unclear. Extracellular vesicles (EVs) have emerged as not only novel biomarkers of T2D and CVD risk (2, 3, 17, 26, 46), but also as potential mediators of exercise-induced cardiometabolic health (19, 55, 56). Importantly, smaller EVs (<100 nm) are typically derived from multivesicular bodies, whereas larger EVs (100-1000nm) known as microparticles, are released in response to cell activation, injury or apoptosis (1) from various types of cells, including the endothelium, platelets and leukocytes. EVs are suggested to not only be associated with blunted endothelial-dependent vasodilation (54) and increased arterial stiffness (22), but has also carry relevant cargo such as eNOS (31) and bioactive lipids (5) that may mediate insulin resistance (25). Our group and others have demonstrated that exercise improves cardiometabolic health by lowering lipids (11, 48), inflammation (23) and glucose (39), as well as improving insulin sensitivity (27) and endothelial function (30). Therefore, it stands to reason that exercise may work through an EV related mechanism.

The effects of exercise training on EVs are mixed. Several (4, 13, 37), but not all (47, 51) chronic exercise interventions report significant reductions in EVs. The reason for the opposing findings is unclear but it may relate to weight loss (45), time at which EVs are studied post-exercise (7, 38, 44, 55, 57), or differences in EV methodology and characterization (i.e. conventional flow cytrometry) (19, 43, 58). Previous cross-sectional work from our group demonstrated through use of advanced imaging flow cytometery that cardiorespiratory fitness is related to lower endothelial and platelet-derived EVs derived from “fresh” blood samples in obese adults. These findings may be of clinical relevance since lower EVs were associated with lower blood pressure and postprandial glucose (20). However, it is unknown if exercise affects EV subtypes using advanced imaging flow cytometry in individuals at risk for type 2 diabetes and CVD prior to clinical weight loss. Because some studies suggest that short-term interval exercise (INT) improves fitness (35) and endothelial

function (29) more than continuous training (CONT), we tested the hypothesis that INT training would lower EVs when compared with CONT exercise in relation to vascular function and/or insulin sensitivity in adults with prediabetes.

METHODS

Subjects

Eighteen obese adults (age: 63.8±1.5 yrs BMI:31.0±1.3 kg/m^2) were screened for prediabetes using ADA criteria (2-hr 75g oral glucose tolerance test (OGTT)) after an overnight fast. Subjects were excluded if physically active (> 60min/wk), smoking, had chronic disease (e.g. type 2 diabetes (as determined by HbA_{1c}), renal, cancer liver, etc.), and/or on medications known to influence insulin sensitivity (e.g. metformin, GLP-1 agonist, etc.) or vascular function (beta-blockers, ACE-inhibitors, etc.). All subjects underwent physical examination that included a resting and exercise 12-lead EKG, and biochemical analysis to rule out disease. Subjects provided verbal and written informed consent as approved by the University of Virginia Institutional Review Board.

Metabolic Control

Prior to testing, subjects were instructed to refrain from strenuous exercise or alcohol consumption within 48-hr of testing. Individuals were also instructed to refrain from taking any medications or dietary supplements 24-hr prior to reporting to the Clinical Research Unit. Subjects were instructed consume approximately 250 g/d carbohydrate on the day before pre-testing to minimize influence of diet on insulin action and were instructed to repeat this diet on the day prior to their post-testing. Three-day food logs, including two weekdays and one weekend day, were also used to assess *ad libitum* food intake before and after exercise training. Participants selected these days and were provided with reference guides that displayed serving sizes of beverages and food. Food intake data were averaged for analysis and analyzed using ESHA (Version 11.1, Salem, OR).

Cardiorespiratory Fitness and Body Composition

VO_2peak was determined using a continuous incremental exercise test on a cycle ergometer with indirect calorimetry (Carefusion, Vmax CART, Yorba Linda, CA) with standard criteria. Following an approximate 4-hr fast, body weight was measured to the nearest 0.01 kg on a digital scale with minimal clothing and body fat as well as fat-free mass was measured using bioelectrical impedance (InBody 770 model, CO, Cerritos, CA). Waist circumferences were obtained 2cm above the umbilicus twice using a plastic tape measure and averaged. A third measurement was obtained if the first two differed by more than 0.5 cm.

Oral Glucose Tolerance Test (OGTT)

Following an approximate 10-12-hr fast subjects reported to our Clinical Research Unit. Subjects were then instructed to lay supine undisturbed for at least 5 minutes to determine resting heart rate and blood pressure, which was averaged over three measurements for data analysis. An intravenous line was placed in the antecubital vein and blood samples of glucose, insulin and free fatty acids (FFA) were collected. A 75 g OGTT was then performed and plasma glucose and insulin were tested at 30, 60, 90, 120 and 180 minutes. Early and total phase glucose tolerance were calculated as total area under the curve (AUC) using the trapezoidal model. Insulin sensitivity was assessed by the Matsuda Index (41) and the simple index of insulin sensitivity (SI_{IS})OGTT was calculated as: 1/[log(G0+G30+G60+G120)+ log(I0+I30+I60+I120)] (6).

Arterial Stiffness

Fasting and post-prandial augmentation index (AI) was measured by aplanation tonometry using the SphygmoCor® system (AtCor Medical, Itasca, IL) at 0, 60, 120 and 180 minutes of the OGTT to assess vascular function. Subjects rested quietly semi-supine in a temperature-controlled room of the Clinical Research Unit. AI was corrected to a heart rate of 75 bpm using the manufacturer's software.

Exercise Interventions

Subjects completed 12 supervised, work-matched aerobic exercise sessions. Exercise was performed for 60 minutes/d over 2 wks. Appropriate submaximal exercise workload was determined from HRpeak obtained during the VO_2peak test. Continuous exercise (CONT) consisted of a constant session at 70% of their HRpeak, while interval training (INT) consisted of subjects completing alternating 3-min intervals at 90% and 50% of their HRpeak, respectively. Rating of perceived exertion (10) was obtained every 10 min. The last exercise bout was performed approximately 24-hr prior to cardiometabolic testing.

EV preparation

Fresh blood prior to the OGTT was drawn using a BD Insyte Autoguard 22G, collected in citrate vacutainers, and processed at room temperature within 120 minutes of collection for the measurement of platelet ($CD31^+/CD41^+$), leukocyte ($CD45^+/CD41^-$; $CD45^+$), platelet endothelial cell adhesion molecule (PECAM) ($CD31^+$) and endothelial (CD105 and $CD31^+/CD41^-$) EVs. Annexin V (AV) was used as membrane dye and platelet poor plasma was obtained by centrifugation (Sovall RC 5B Plus Centrifuge: Rotor SS-34) at 5000 g at room temperature for 15 minutes. Both were used given AV+ and AV- have independently been shown to relate to cardiometabolic health (8, 12, 16, 20). An EV pellet was obtained from platelet poor plasma by a second centrifugation spin (Centrifuge: 524/5424 R-Rotor FA-45-24-11) at 17,000 g for 10 minutes and subsequently washed with AV buffer (1xAVb) (BD Parmingen, San Diego, CA). Samples and controls were processed as previously described by our group (20, 21). Upon completion, EVs in the sample were concentrated two-fold.

EV origin by imaging flow cytometry

The imaging flow cytometer ImageStream® MKII (Amnis, Seattle, WA) (ISX) was used to isolate and determine the source and count of the EVs (27, 28). EV counts were measured by a volumetric method provided by the software of ISX. The acquired raw data were then analyzed using IDEAS software.

EV image by cryo-electron microscopy

Purified samples were verified by standard methods for cryo-electron microscopy (cryoEM) to determine EV morphology. In brief, an aliquot (3 ml) was applied to a glow-discharged, perforated carboncoated grid (2/2-4C C-flats), blotted with filter paper, andrapidly plunged into liquid ethane. Low-dose images were recorded at a magnification of 29,0003 on a FEI Tecnai F20 Twin transmission electron microscope operating at 120 kV, with a nominal underfocus ranging from 3.5 to 5 mm and a pixel size of 0.388 nm at the specimen level. All images were recorded with a Gatan 4K 3 4K pixel CCD camera. The grids were stored in liquid nitrogen, and then maintained in the microscope at 2180_C using a Gatan 626 cryo-stage. Samples were also prepared for scanning electron microscopy.

EV size by Nanosight Tracking Analysis

Tunable Resistive Pulse Sensing (TRPS) was performed with a gold qNano instrument (Izon Ltd) mounting a polyurethane nanopore membrane NP200 (range 85-500 nm) and NP400 (range 125-1100 nm) (Izon Ltd). Multi pressure at 4,5 and 8 mBar respectively was applied to determine the particle concentration. Electrolyte solution was made of PBS supplemented with 0.03 % (v/v) Tween-20 filtered with Minisart® high flow hydrophilic 0.1 µm syringe filter (Sartorious). Current pulse signals were collected using Izon Control Suite 3.2software (Izon Ltd). EV pellet after differential centrifugation was solubilized in 50 µl of filtered electrolyte solution. Polystyrene particle standards (SPK200B and CPC400B;IzonLtd.) were employed for calibration. Both uEVs pellet and particle standards were measured with a minimum of 1000 blockades. Nanosight Tracking Analysis (NTA) was carried out using the Zetaview pmx110 multiple parameter particle tracking analyzer (particle metrix, Meerbusch, Germany) in size mode using zetaview software version 8.02.28. Plasma samples were diluted in 1x pbs to the working range of the system. The system was calibrated using 105 nm polystyrene beads and then plasma vesicle profiles were recorded and

analyzed at 11 camera positions with a 2 second video length, a camera frame rate of 30 fps and a temperature of 21˚C.

EV and non-EV protein by Western Blotting

Protein quantification of EVs was performed by Coomassie micro assays. EV pellets were solubilised in 40 µl of solubilisation buffer made of 5% (w/v) sodium dodecyl sulphate (SDS), 40 mM Tris-HCl pH 6.8, 0.5 mM ethylenediaminetetraacetic acid (EDTA), 20 % (v/v) glycerol without and with 50 mM dithiothreitol (DTT) respectively. Samples were denaturated overnight at room temperature. Proteins were separated by hand cast SDS-PAGE gradient gels (Resolving gel T= 5-20 % (w/v); C=2.6 %; Stacking gel T= 3.5 % (w/v); C=2.6 %) in 25 mMTris, 192 mM glycine and 0.1 % (w/v) SDS buffer and either stained with silver staining or transferred onto a 0.45µm nitrocellulose membrane (Amersham™ Protan™ 0.45µm NC, GE Healthcare) in a wet transfer system buffer made of 25 mMTris, 192 mM glycine and 20 % (v/v) methanol. Nitrocellulose membranes were saturated with Odyssey blocking buffer (LI-COR Biosciences) and incubated in polyconal rabbit anti TSG101 (Sigma, T5701-200UL) and monoclonal mouse anti CD9 (HansaBiomed; HBM-CD9-100) overnight at room temperature (RT= 23-24◦C) in an Odyssey blocking buffer diluted 1:1 with PBS and 0.15 % (v/v) Tween-20 at concentration of 1.0 µg/ml. After 3x10 minute washes in PBS-Tween (0.15%, v/v), membranes were incubated with anti rabbit anti mouse dye-coupled secondary antibody 0.1 µg/ml(LI-COR Biosciences) in an Odyssey blocking solution diluted at 1:1 with PBS and 0.15 % (v/v) Tween-20; 1-hr at RT. Acquisition of the fluorescent signal was performed by Odyssey infrared imaging system with resolution set at 169µm (LI-COR Biosciences) .

Biochemical Analysis

Plasma glucose samples were analyzed immediately using the YSI 2300 StatPlus Glucose Analyzer system (Yellow Springs, OH). All other samples were centrifuged for 10 minutes at 4°C and 3000 rpm, aliquoted, and stored at -80°C until later analysis. Plasma insulin was placed in

vacutainers containing EDTA and the protease inhibitor aprotonin. Insulin was analyzed using an enzyme-link immunosorbent assay kits (Millipore, Billerica, MA) and circulating free fatty acids (FFA) were analyzed using enzymatic colorimetric method assay (Wako Diagnostics, Richmond, VA).

Statistical Analysis

Data were analyzed using SPSS v24 (Armonk, NY). Normality was assessed using Shapiro-Wilk tests. EV data were log-transformed to meet normality. Independent, two-tailed *t*-tests were used to determine pre-test group differences between CONT and INT groups. A 2-way repeated measures ANOVA was utilized to study intervention effects. There were significant pre-test differences in total, AV- CD31+ (PECAM) and AV- CD31+/CD41- EVs. Therefore, we co-varied for this in our ANOVA model. Given that age trended towards significant group differences as well, we added this in our ANOVA model. Pearson correlations were used to examine associations and multiple linear regression was used to determine the interaction amongst EVs, fitness and dietary intake. Statistical significance was set at $P \leq 0.05$ and data are reported as mean ± SEM.

RESULTS

Exercise Training Characteristics

Exercise session adherence was 96 and 98.9% for INT and CONT, respectively (P=0.12). The average percentage of HRpeak was 76.7±1.0% for INT and 75.5±2.1% for CONT training (P=0.45) and there was no difference between groups in rating of perceived exertion (11.9±0.6 vs. 12.5±0.3, P=0.63).

Cardiorespiratory Fitness and Body Composition

INT exercise increased VO_2peak compared to CONT training (P=0.04), although there were no significant changes in body weight (P=0.11) following the intervention. Fat free mass (FFM) was

significantly lower in both groups following the intervention (P=0.007), while exercise had no effect on fat mass (P=0.17).

Dietary Intake

Both INT and CONT decreased total caloric (P<0.01), CHO (trend: P=0.08) and fat consumption (trend: P=0.09) (***Table 2***). INT training decreased, while CONT increased, dietary sugar consumption (-27.7±7.6 vs. 27.4±17.3%, P=0.01).

Vascular Function

INT training reduced fasted AI compared to CONT training (trend: P=0.08). Exercise, regardless of intensity, significantly lowered 2-hr postprandial AI (P=0.05), and AI $tAUC_{180}$ (P=0.03). There was a trend for reductions in systolic blood pressure following INT training only (P=0.11), but no improvements in resting HR or diastolic blood pressure (***Table 1***).

Glucose Metabolism

Exercise training significantly reduced 2-hr glucose (P=0.04) and insulin $tAUC_{180}$ (P=0.05), independent of intensity. Exercise had no effect on fasting glucose (P=0.93), or insulin sensitivity assessed by as assessed by the Matsuda Index (P=0.22). However, (SI_{IS})OGTT did significantly increase following training (P=0.03) (***Table 1***).

EV Response

The intervention had no effect on AV+/- leukocyte ($CD45^+$ and $CD45^+/CD41^-$) or platelet-derived EVs ($CD31^+/CD41^+$), although there was a trend for increased platelet-endothelial cell adhesion molecule (PECAM) AV- $CD31^+$ (trend: P=0.11) following the exercise intervention. INT exercise decreased AV- CD105 compared to CONT training (Δ= -0.2±0.2 vs. 0.6±0.15 count; P=0.04) (***Figure 1A***). However, after AV-CD105 was co-varied for the changes in dietary sugar, and the effect of exercise intensity was attenuated (P=0.18). We report no significant effects of exercise on AV+ EVs (***Figure 1B***).

Correlation and Regression Analysis

Changes in endothelial AV+ CD105 and AV- CD105 (r=0.71, *P*=0.001), as well as changes in PECAM AV+ and AV- $CD31^+$ (r=0.61, *P*=0.007) was highly correlated, suggesting clinical relevance of both AV+ and AV- EVs. Increased VO_2peak correlated with decreased AV+ CD105 (r=-0.60, *P*=0.01) ***(Figure 2A***), while reductions in AV- $CD31^+$ was linked to decreased early phase glucose tolerance following exercise training (trend: r=0.40, *P*=0.10) (***Figure 2B***). Interestingly, increased PECAM AV+ $CD31^+$ (trend: r=-0.44, *P*=0.09) and platelet AV+ $CD31^+/CD41^+$ (trend: r=-0.42, *P*=0.11) were related to blunted insulin sensitivity (***Figure 4***) when assessed by $Matsuda_{120}$ following exercise training. Improvements in (SI_{IS})OGTT were not related to any changes in EVs in this present cohort. Reduction in dietary sugar intake after training was linked to decreased AV+CD105 (r=0.60, *P*=0.01) (***Figure 3A***) and AV-$CD45^+$ (r=0.61, *P*<0.01) as well as VO_2peak (r=-0.53, *P*=0.03) and fasted AI (trend: r =0.47, *P*=0.06). The results indicated that the model accounting for changes in fitness and changes in dietary sugar was a significant predictor of changes in AV+CD105 (F(2,14)= 8.662, P=0.004) and the model explained 48.9% of the variance (variance inflation factor= 1.38). While changes in fitness did significantly contribute to the model (β=-5.84, *P*=0.03), changes in dietary sugar did not (β=0.172, *P*=0.17). The final predictive model was: ΔAV+ CD105= 12.789+ (0.172*Δsugar) + (-5.84*ΔVO2peak).

DISCUSSION

The primary observation of this current study is that short-term INT exercise significantly lowered endothelial-derived EV AV- CD105 compared to CONT training, while leukocyte and platelet-derived EVs did not change. The change in EV CD105 was also directly related to increased fitness and reduction in dietary sugar. Together, these data suggest that EV responses to short-term exercise intensity may be subtype specific and diet mediated. To our knowledge, we are the first to report the short-term effects of exercise intensity on platelet, leukocyte and endothelial EVs in adults with prediabetes, thereby making it difficult to directly compare our work to others. However, our

findings are somewhat consistent with previous acute exercise studies, independent of intensity (53) that have reported decreases in endothelial EVs $CD31^+/CD42b^-$ (18, 53) and $CD62E^+$ (18) following a single bout of exercise. It is important to note, however, that these were not the same endothelial markers utilized in this present study (CD105 and $CD31^+/CD41^-$) and that conventional flow cytometry was used to quantify EV count. Additionally, many of these acute studies noted a temporal rise in EVs that return to baseline several hours following the last exercise bout (15, 18). Our work expands on these findings, as we collected EV data 24-hr following the last exercise bout, to minimize the residual effect of the last exercise bout on EVs. In comparison to our study, other long-term training interventions (e.g. 12 wks or more) report decreased endothelial EVs ($CD62E^+$, $CD31^+/CD42b^-$) as well (4, 13, 34), although none of the exercise protocols were high intensity. The only long-term study to directly compare interval (four 4-min intervals at 90-95% HRpeak, 3 min recovery at 50-70% HRpeak) and continuous (47 min at 70-75%HRpeak) aerobic training showed no change in EVs following 3 sessions/wk for 12-wks of training in adults with coronary artery disease (51). Interestingly, however, this same study reported baseline EVs to be predictive of fitness gains following the intervention (51). Taken together, these findings highlight that exercise intensity uniquely affects endothelial, but not platelet or leukocyte derived EVs in relation to cardiometabolic health.

There are several reasons that could explain why INT exercise lowered CD105 compared with CONT training in these adults with prediabetes. First, previous work from our group (20) suggests that improved aerobic fitness is related with lower EVs (platelet: AV- $CD31^+/CD41^+$ and endothelial: AV- $CD31^+/CD41^-$) in obese adults. If fitness improved more following INT than CONT exercise, then it would be expected to modulate EVs. Indeed, we report that the lowering of EVs following INT exercise was directly related to increased VO2peak (***Figure 1A***). This is consistent with others (51) highlighting that EVs modulate the response to exercise training in patients with CVD. However, although short-term exercise appears to modulate EV release, the influence of

dietary sugar on EVs warrants acknowledgment given that co-varying for changes in sugar intake attenuates the effect of the intervention on EVs. In part, these findings may suggest that diet eliminates the effect of exercise on EVs. To date, no study has been designed to specifically address this gap. Francois et al. reported that while a low-carbohydrate diet significantly lowered endothelial-derived EV, a low-carbohydrate combined with post-meal exercise lowered total EVs, as well as improved endothelial function (24) in adults with T2D, suggesting a synergistic effect of exercise and diet. We report herein that INT and CONT decreased total caloric ($P<0.01$) and CHO (trend: $P=0.08$) intake following exercise, but INT decreased sugar consumption (-27.7±7.6%) while the CONT group increased sugar consumption (27.4±17.3%, $P=0.01$) (***Table 2***). Interestingly, only the decrease in ad-libitum sugar consumption was directly related to decreases in endothelial AV+CD105 ($r=0.60$, $P=0.01$) (***Figure 3A***). Although this suggests that dietary sugar directly modifies EV levels, it is worth noting that increased dietary sugar intake was related to attenuated elevations in VO_2peak. We recognize that directionality cannot be inferred from our study design, but it is speculated that fitness is an independent factor modulating EVs since the linear regression model was strengthened by inclusion of dietary sugar and fitness compared with dietary sugar alone. As such, we speculate that increasing fitness, with and without diet change, may contribute to declines in endothelial EVs.

Glucose regulation may relate to EVs. Hyperglycemia has been reported to increase endothelial EV count, in addition to promoting greater coagulant activity, reactive oxidative species, and blunted endothelial constriction (14). Additionally, high glucose conditions increase NADPH oxidase activity in endothelial EVs, thereby promoting vascular inflammation (32). As exercise is known to improve glucose regulation (9, 27, 33), it is reasonable to expect that exercise may modulate EV release through improvements in glucose control (19). Indeed, we report significant reductions in postprandial glucose concentrations in the current study (***Table 1***). However, this improvement in glucose tolerance did not directly correlate with reduced endothelial EV profiles.

This suggests that a reduction in ambient plasma glucose may not directly mediate endothelial EVs per se. Interestingly, however, improved early phase glucose did tend to correlate with changes in PECAM AV- $CD31^+$ (***Figure 2b***) following our intervention. This highlights that improved glucose tolerance following exercise may mediate subtype-specific responses in EV profiles. To this extent, an alternative explanation for the reduction in CD105 following INT exercise may relate to increases in insulin sensitivity. In fact, EVs have been directly implicated in the development of insulin resistance, as they have been shown to reduce insulin-stimulated glucose uptake (42) by interfering with GLUT-4 translocation in human adipocytes (59). However, consistent with reductions in insulin AUC suggesting improved insulin sensitivity following the intervention, we report that increased EVs relate to blunted insulin sensitivity gains post-exercise. The reason we detected no increase in the Matsuda Index is difficult to reconcile, but we speculate it is likely due to reduced sample size given our recent work using the same exercise training intensity model (27). Thus, further work is required to understand how exercise improves glycemic control through EVs.

As EVs are intimately linked with vascular physiology (17), it remains possible that reductions in hypertension and/or arterial stiffness may be another potential factor by which exercise relates to changes in EVs. Indeed, we previously reported that fitness was related to EVs in part through reductions in pulse pressure (20). Despite reductions in fasted and postprandial arterial stiffness, as measured by AI, we report no significant effects of our treatments on blood pressure (***Figure 1***), and none of these improvements correlated with changes in the endothelial EV CD105. It is important to note in this present analysis, however, that we did not characterize other components of vascular function, such as endothelial-dependent vasodilation or responsiveness of vascular smooth cells in resistance arteries to vasoconstrictor stimuli, both of which has previously been shown to relate to EVs (40, 54). Also, on average individuals in the present study were normotensive, and it remains possible that individuals with hypertension would relate to exercise-mediated EV

effects. Future work is needed assessing other components of vascular function in relation to EV subtypes to definitively determine the role of exercise on vascular health.

The results of the present study suggest a subtype-specific EV response to exercise. Although we report no significant changes in leukocyte derived EVs following 2 wks of INT and CONT training (***Figures 2, 3***). There was a trend for increased PECAM AV- $CD31^+$ following the intervention, independent of intensity. These findings are consistent with our prior work showing that fitness has no relation to leukocyte derived EVs in obese adults (20). Wahl et al. recently speculated that EVs derived from different cell types undergo unique clearance mechanisms following exercise (52). While we did not directly measure clearance in the present study, the observation that endothelial compared with platelet EVs have differential responses to INT exercise would be consistent with this notion. Regardless of the exact mechanism, these data highlight the unique effect of exercise on cell-specific EV effects and warrant additional work to elucidate how exercise impacts EV function in relation to their lipid and protein cargo (55, 57).

This study is not without limitations. The present sample size is relatively small and associations do not equal causation. It is possible that we may have lacked statistical power to detect differences in both AV+ and AV- EVs, although we do report that the two were highly related in the present cohort, suggesting clinical relevance for both subtypes. Moreover, although sex differences have been reported in EV profiles in response to an exercise stimulus (18, 36, 38), we previously reported that sex had no influence on fitness related EV differences in obese adults (20). Thus, we do not believe sex influenced training responses in the current study. Although menstrual cycle may play a role in modulation of EV release (28, 50), all women in the present analysis were post-menopausal and the influence of sex hormone difference herein is minimal. We estimated insulin sensitivity in the present student and it remains possible that use of the euglycemic-hyperinsulinemic clamp would have revealed associations with EVs. Vasculature function was assessed by arterial stiffness. While arterial stiffness is an independent predictor of CVD, it remains possible that EVs

may have influenced large conduit/microcirculatory endothelial function. Finally, although our EV preps most likely contained exosomes, we stained for larger EVs with protein analysis before using the same approach as previously published (20). Nonetheless, future work is required to examine effects of exercise training on exosomes to elucidate roles in treating metabolic disease (7, 49).

In conclusion, short-term INT exercise training significantly lowered endothelial EVs compared to CONT training in adults with prediabetes, but there was no significant effect of intensity on platelet or leukocyte EVs. Interestingly, reductions in early phase glucose tolerance were related to lower platelet EVs, suggesting that EVs are related to glucose homeostasis. Further, ad-libitum sugar consumption appears to modify exercise training induced effects on endothelial and leukocyte EVs, although fitness remains an independent mediator of training-induced changes in EVs. Together, these data future work is needed to better understand how dietary and exercise interventions interact to impact EV physiology for the optimization of type 2 diabetes and cardiometabolic health treatment.

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TABLE LEGENDS

Table 1. Effect of INT or CONT exercise on body composition, fitness, and cardiovascular disease (CVD) risk in adults with prediabetes. Data are means ± SEM. Body mass index (BMI); total area under the curve (tAUC); free fatty acids (FFA). Differences in age between groups trended towards statistically different (P=0.07) and were accounted for in the ANOVA model to determine intervention effects. Time effect (*$P \leq 0.05$, $^{\$}P \leq 0.09$). Group x time interaction: (^$P=0.05$; $^{\dagger}P<0.11$).

Table 2. Effect of INT or CONT exercise on ad-libitum diet in adults with prediabetes. Data are means±SEM. No significant baseline differences existed between groups for any outcome. Time effect (*$P \leq 0.05$, $^{\$}P \leq 0.09$). Group x time interaction: (^$P=0.05$).

FIGURE LEGENDS

Figure 1. Changes in circulating Annexin V- (AV-) **(*A*)** and Annexin V+ (AV+) **(*B*)** extracellular vesicles (EVs) following 12 days of exercise training. EV data were log-transformed (^ Group x Test, $P \leq 0.05$).

Figure 2: Relationship between circulating Annexin V+ (AV+) CD105 extracellular vesicles (EVs) and fitness ***(A)***; circulating Annexin V- (AV-) CD31+ EVs and glucose tolerance ***(B)***. Open circles, interval training (INT). Closed circles, continuous training (CONT).

Figure 3: Relationship between changes in ad-libitum dietary sugar, Annexin V+ (AV+) CD105+ (A) and fitness (B). Open circles, interval training (INT). Closed circles, continuous training (CONT).

Figure 4: Relationship with AV+ CD31+ and insulin sensitivity as assessed by the Matsuda Index. Open circles, interval training (INT). Closed circles, continuous training (CONT).

Table 1: Subject Demographics and CVD Outcomes

	INT		CONT	
	PRE	**POST**	**PRE**	**POST**
N (M/F)	10 (2/8)	-	8 (2/6)	-
Age (yrs)	61.7±1.9	-	67.4±2.0	-
Body mass (kg)	86.8±4.4	86.1±4.5	91.5±7.1	91.2±7.0
BMI (kg/m^2)	30.9±1.4	30.7±1.4	31.3±2.6	32.4±2.3
Fat mass (kg)	35.6±2.9	35.6±2.8	38.9±4.4	39.4±4.3
Fat free mass (kg)	56.7±3.2	49.9±3.2	52.7±4.2	51.8±4.3
Waist Circumference (cm)	103.4±3.7	101.9±3.7	108.0±5.3	107.5±5.7
VO_2peak (L/min)	1.9±0.1	2.0±0.1	1.6±0.2	1.6±0.1
VO_2peak (ml/kg/min)	21.3±1.0	23.0±0.9^	18.2±1.3	17.8±1.0
Fasting glucose (mg/dL)	102.6±2.3	102.7±2.5	106.3±3.9	104.7±4.5
2-hr glucose (mg/dL)	137.7±13.4	125.5±12.0*	160.5±14.6	130.6±9.7*
$Glucose_{tAUC}$ (mg/dL*180min)	24378.8±1996.7	23744.1±1668.2	27818.0±1830.9	25190.6±1150.7
Fasting insulin (µU/mL)	7.9±1.5	8.7±1.9	10.3±1.2	13.1±2.8
2-hr insulin (µU/mL)	55.4±7.2	44.2±7.2	87.8±15.5	77.8±12.9
Matsuda Index	4.0±0.5	4.3±0.7	1.9±0.4	1.8±0.3
(Si_{is})OGTT	0.197±0.002	0.202±0.003*	0.189±0.004	0.191±0.003*
Fasting FFA (mEq/L)	0.48±0.04	0.57±0.06$	0.67±0.03	0.83±0.15$
2-hr FFA (mEq/L)	0.18±0.04	0.19±0.03	0.26±0.05	0.24±0.06
Resting HR (bpm)	63.7±2.2	61.4±2.1	67.3±2.6	70.1±3.0

Systolic BP (mmHg)	128.4±2.5	122.1±3.5†	120.1±3.5	123.1±2.0
Diastolic BP (mmHg)	70.9±2.2	66.6±2.1	66.0±2.3	64.5±2.1
Fasted AI (mmHg)	28.3±3.1	23.0±3.0†	26.8±3.6	30.4±3.1
2-hr AI (mmHg)	23.3±4.5	12.7±3.9*	25.9±3.0	20.4±1.5*
AI_{tAUC} (%*180min)	3771.0±443.4	2979.0±321.6*	4641.4±294.1	4242.9±190.1*

Data are means±SEM. Interval (INT); continuous (CONT); body mass index (BMI); total area under the curve (tAUC); free fatty acids (FFA); heart rate (HR); blood pressure (BP); augmentation index (AI); Differences in age and Matsuda Index between groups trended towards statistically different (P≤0.07) and were accounted for in the ANOVA model to determine intervention effects. Time effect (*P≤0.05, $^{\$}$P≤0.09). Group x time interaction: (^P=0.05; $^{\dagger}P$<0.11).

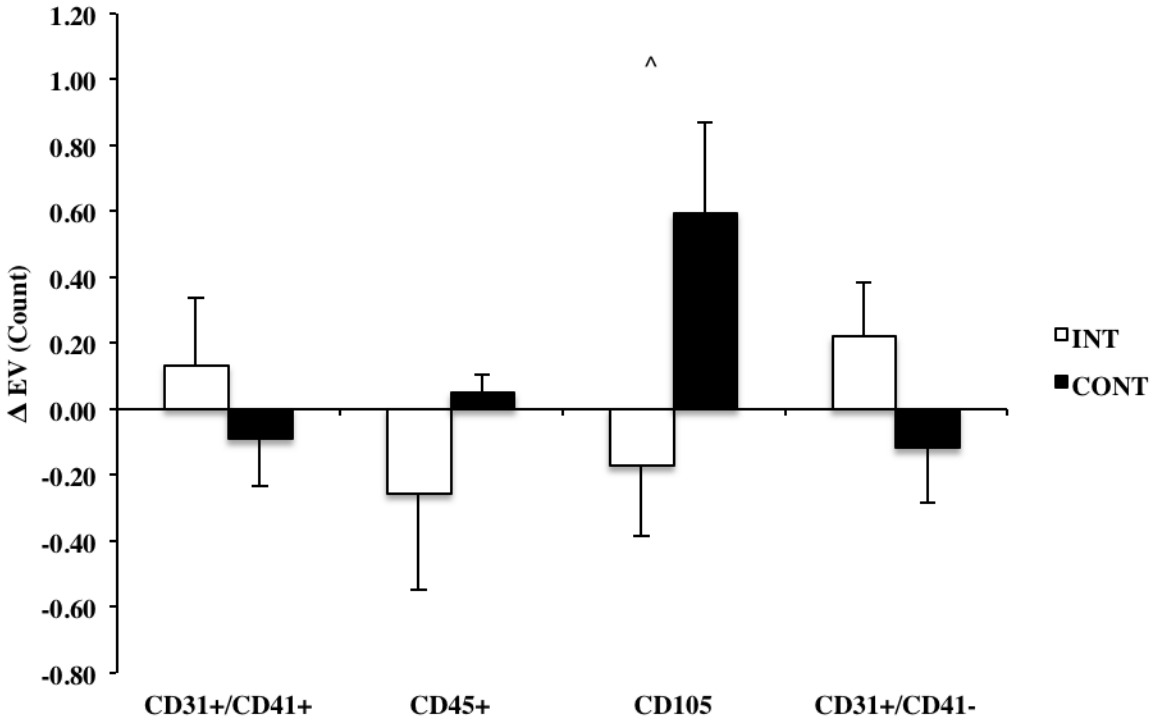
Table 2: Ad-libitum diet changes following 2 weeks of exercise training

	INT		CONT	
	PRE	**POST**	**PRE**	**POST**
Calories	2184.4±109.7	1995.4±160.2*	2448.3±242.6	1940.4±179.8*
Fat (g)	84.6±7.6	80.7±10.2$	101.2±18.3	72.0±10.2$
CHO (g)	270.8±18.5	244.4±16.2$	285.5±34.5	252.2±16.0$
Sugar (g)	130.0±14.0	95.1±7.4^	103.1±17.4	117.1±13.3
Total Fiber (g)	27.6±3.4	25.6±3.2	29.4±6.7	21.7±2.4
Protein	84.9±3.6	77.1±3.1	102.7±12.1	74.0±8.0

Data are means±SEM. Carbohydrate (CHO). No significant baseline differences existed between groups for any outcome. Time effect (*$P \leq 0.05$, $^{\$}P \leq 0.09$). Group x time interaction: (^$P = 0.05$).

Figure 1: Changes in circulating Annexin V- (A) and Annexin V+ (B) EVs following 12 d of training. EV data were log-transformed (^ Group x Test, P≤0.05)

A.



B.

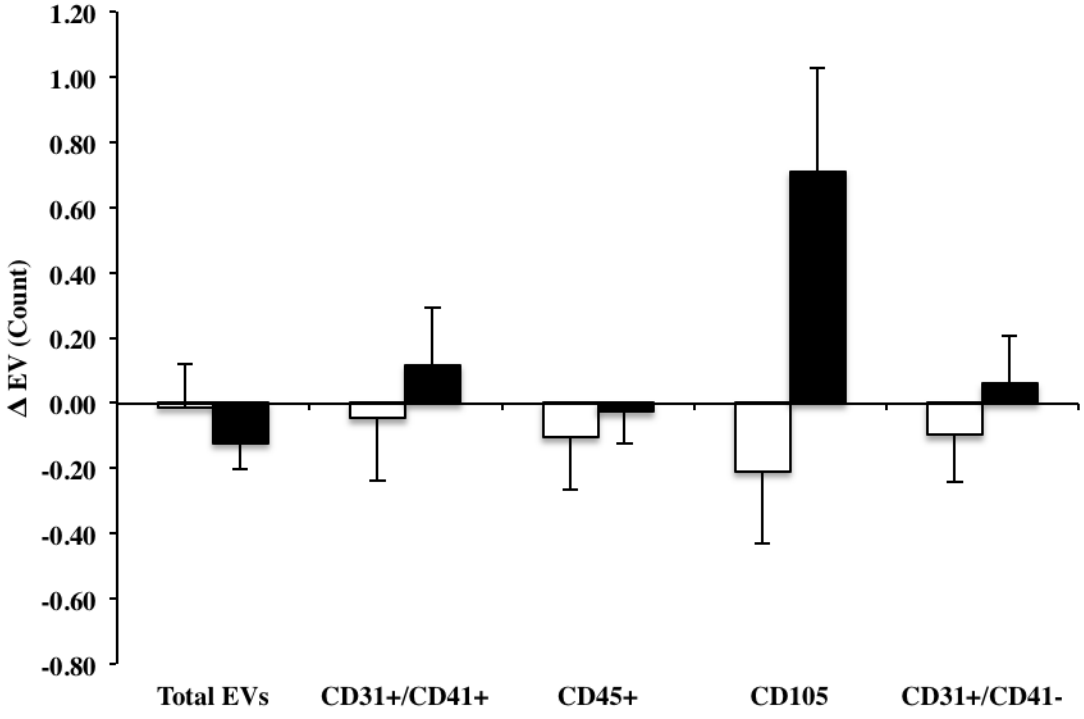
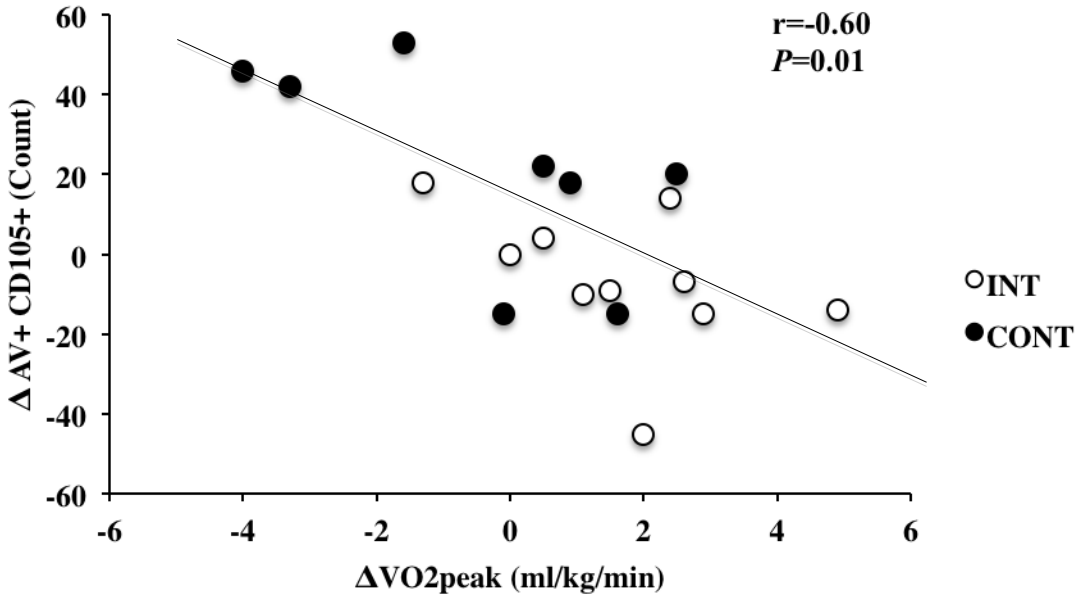


Figure 2: Correlation between circulating AV+ CD105 EVs and fitness (A); circulating AV- CD31+ EVs and glucose tolerance (B).

A.



B.

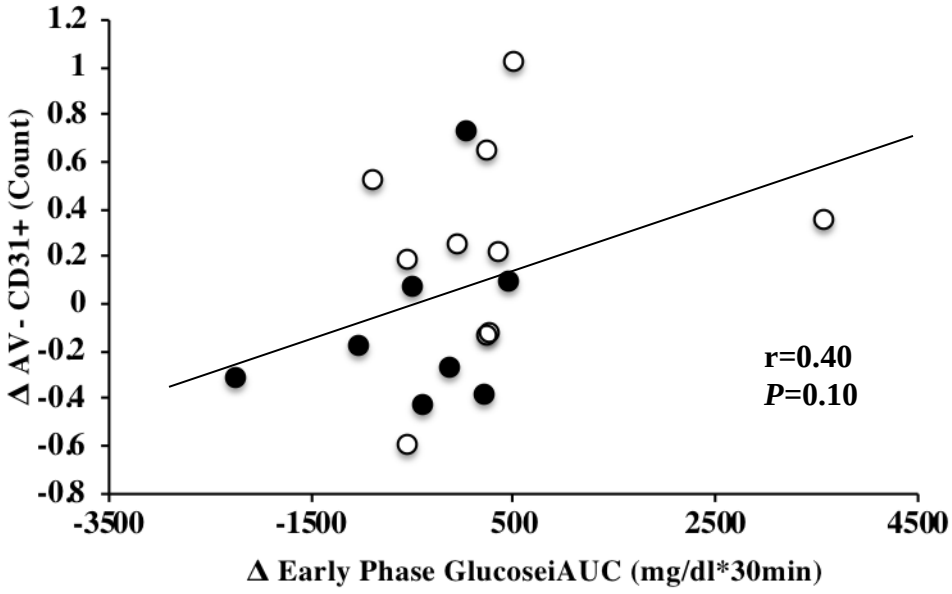


Figure 3: Correlation between changes in ad-libitum dietary sugar, AV+CD105+ (A) and fitness (B).

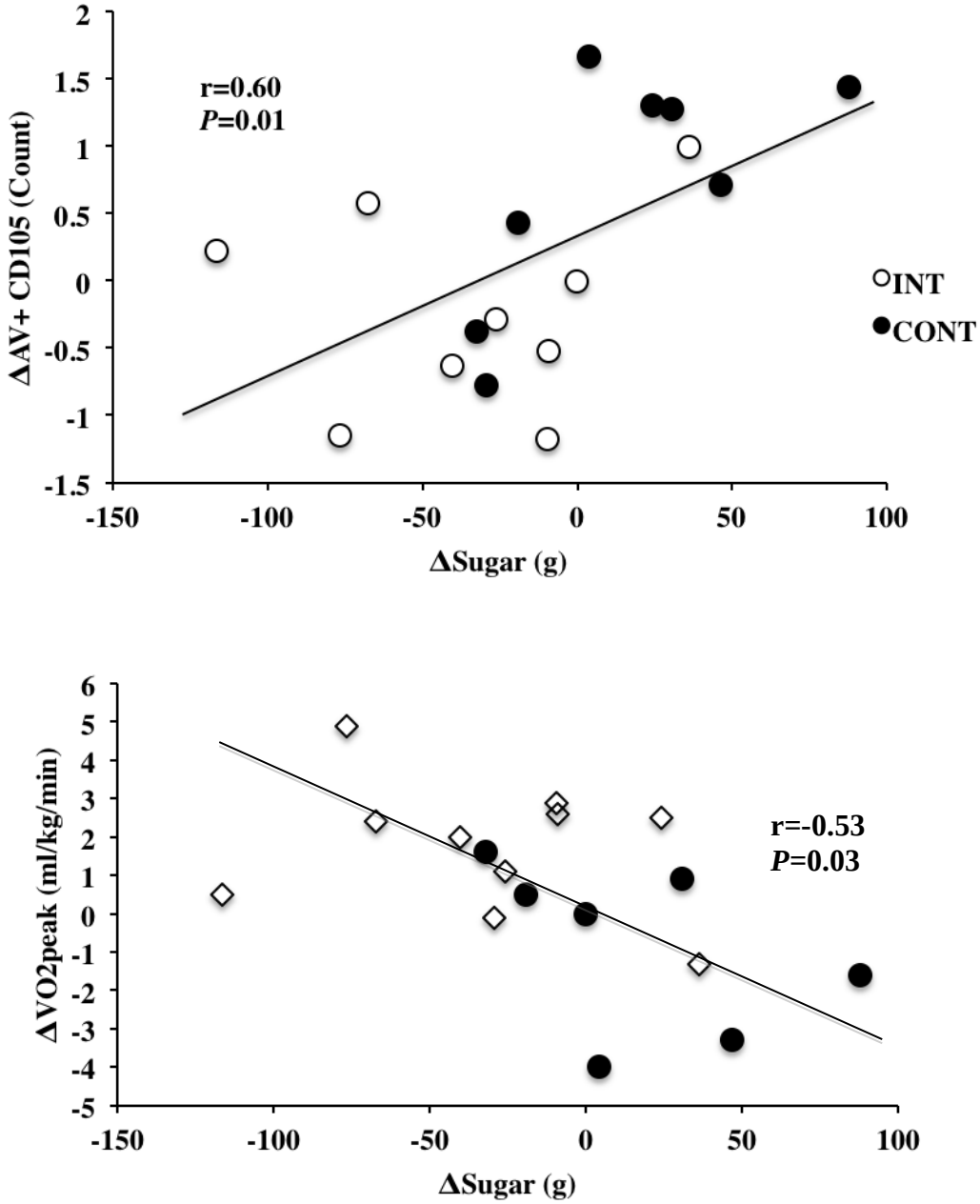
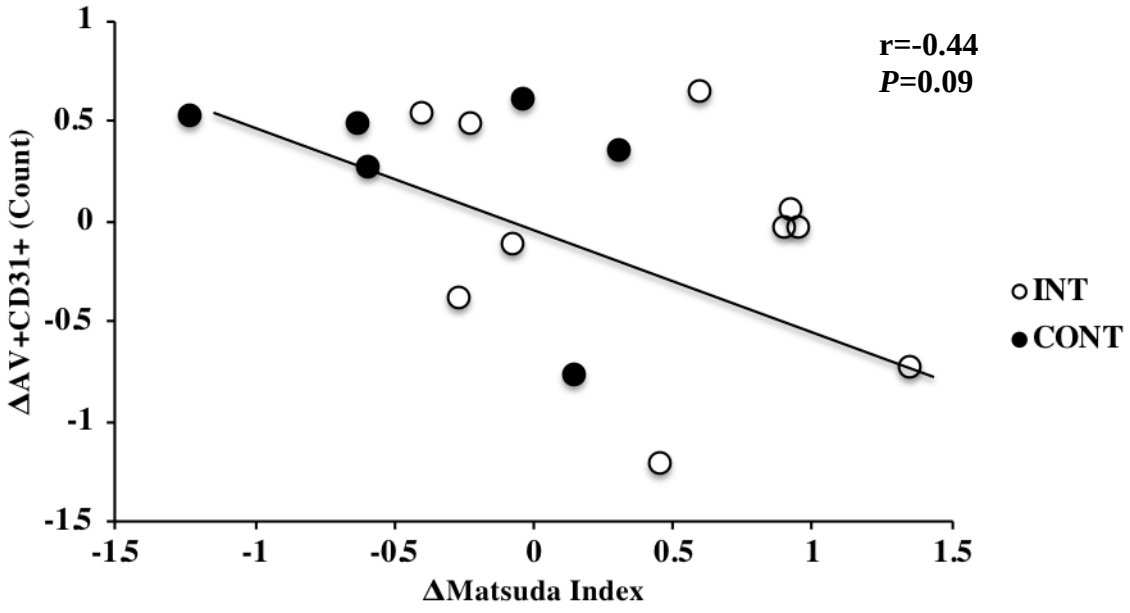


Figure 4: Correlation with AV+ CD31+ and insulin sensitivity as assessed by the Matsuda Index.



CHAPTER 6: FUTURE DIRECTIONS

Based on these summarized findings and the work of others, we believe that EVs hold value above and beyond a biomarker. However, the present work was not designed to determine the functionality of EVs and how EV functionality may change in response to interventions such as feeding or exercise. Ascertaining this information is clinically meaningful, as it may provide understanding of the precise mechanism(s) by which increased cardiorespiratory fitness, exercise and dietary interventions lower cardiovascular disease (CVD) risk. Some work in this area is already being conducted, as Wilhelm et al. (10) have shown that EVs from healthy young men immediately post-exercise enhanced endothelial proliferation, migration and tubule formation when compared to EVs obtained from the same men at rest. This suggests EVs may be a novel mechanism by which exercise promotes vascular health. Others have even suggested that EVs may be responsible for "tissue-crosstalk" during exercise (9). To better understand how EVs may exert this influence, however, investigators must look beyond EV count, designing studies aimed at understanding the effects of various interventions on EV subtype (i.e. endothelium, leukocyte, platelet, etc.), composition, and cargo (i.e. peptides, DNA, miRNA, mRNA). Specifically, more work is needed in relation to smaller EVs, exosomes, as they have been shown to help facilitate exchange of cargo between cells and tissues (7). The idea of "exersomes," exosomes enriched with exercise-induced peptides and nucleic acids from skeletal muscle and other various tissues, has been proposed to have therapeutic utility in the treatment of various metabolic diseases (6), although much more work is needed to better understand their therapeutic potential.

Herein we have shown Annexin V+ (AV+) and Annexin V- (AV-) EV count to be modulated by feeding and exercise interventions; these changes in AV+/- count also correlated with improvements in clinical outcomes such as insulin sensitivity and arterial stiffness. Interestingly, we report fitness and exercise to impact AV- EVs more substantially, while hyperglycemia appears to influence primarily AV+ EV count. Although it is important to acknowledge that these were

relatively small sample sizes, it brings up an interesting question of the physiological (and clinical) relevance of AV+ versus AV- EVs. This point is poorly understood, although some have suggested that the agonist of EV release is crucial in determining the ability of EVs to bind Annexin (3). To date, however, very little work is being done to determine differences in functionality between AV+ and AV- EVs. Our work supports the need for future research to consider AV+ and AV- EVs in relation to various diseases, as well as to better understand the relevant physiological differences between the two populations.

Other factors warrant consideration in future research. Previous work has proposed sex to be a potential mediator of EV release, mainly due to differences in sex-specific hormones. Indeed, various phases of the menstrual cycle has been shown to change circulating EVs in the same women (8). However, this difference is not consistently reported across the literature (4) and it may be dependent upon EV subtype (5). Demographic factors, such as age and post-menopausal status, must also be considered, as they mediate CVD risk (and potentially EV profile) (1, 2). A thorough characterization of all of these factors in relation to EV count and subtype, utilizing rigorous characterization of EVs is necessary. Therefore, future feeding and exercise trials including a large number of men and women across varying ages (accounting for menstrual status in women) are needed to account for these factors to promote more personalized assessments or treatments in the future.

Our results suggest that both feeding and exercise may differentially impact specific subtypes of EVs. Although leukocyte EVs were not related to hyperglycemia or exercise intensity in the present analyses, they were in fact, strongly related to dyslipidemia. Therefore, it stands to reason that a different acute feeding stimulus (i.e. high fat meal) may impact leukocyte-derived EVs. Future work should consider examining not only the acute effect of different "feeding conditions" on different EV subtypes, but also characterizing these EV subtypes in relation to habitual diet and long-term dietary interventions. Finally, as short-term exercise intensity may be important in modulating

the release of the endothelial EV CD105, we must also consider the impact of various types (i.e. cycling, running, resistance training), intensities, and durations (short vs. long term) of exercise on EV count, subtype, and function.

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CHAPTER 7: SUMMARY AND FINAL COMMENTS

The field of extracellular vesicles, feeding and exercise is promising. Herein we present novel data suggesting that feeding and exercise, in conjunction with changes in clinical outcomes such as arterial stiffness and insulin sensitivity, may differentially impact EV subtype and count in adults with obesity. In Aim 1, we provide pilot data that show elevations in Annexin V- platelet ($CD31^+/CD41^+$) and endothelial ($CD31^+/CD41^-$) derived EVs in adults with obesity and poor cardiorespiratory fitness compared to obese adults with only slightly higher levels of cardiorespiratory fitness. These results suggest that subtle differences in fitness, may in part, reduce CVD risk through an EV-mediated mechanism. This work also fills a gap in the present literature, as we present a very rigorous characterization of EVs, combined with methodology (fresh blood samples, imaging flow cytometry) that has been shown to greatly enhance the sensitivity of characterization of EV count. To our knowledge, no cross-sectional study of EVs in relation to clinical outcomes in obesity has utilized this methodology.

Aim 2 adds to the current literature by suggesting that hyperglycemia significantly lowers AV+ platelet and platelet endothelial cell adhesion molecule (PECAM) $CD31^+$ EVs, independent of glucose status, in adults with obesity. These reductions in EVs in response to this glucose load were also associated with increased circulating levels of insulin and decreased arterial stiffness, suggesting clinical relevance. EVs may help to better understand the mechanism by which postprandial hyperglycemia is a better predictor of CVD when compared to fasting glucose alone. However, more mechanistic work is needed to determine this potential effect. Interestingly, when accounting for group differences in fitness, the effect of the OGTT on postprandial EVs was no longer significant, suggesting that baseline levels of fitness may still play a crucial role in relation to postprandial physiology and EVs.

Finally, we are the first to report the impact of short-term exercise intensity on circulating EVs utilizing fresh blood samples and imaging flow cytometry. Exercise had no significant effect of

platelet or leukocyte derived EVs, however, we found that those who engaged in high intensity interval training saw lower circulating levels of the AV- endothelial EV CD105, whereas those who underwent continuous training saw increases in AV- CD105. As these decreases in CD105 were also related to greater improvements in fitness, and those individuals in the interval group saw greater improvements in fitness, we speculate that interval training may have mediated lowering of CD105 through a fitness-mediated mechanism. However, it is important to note, that increased sugar consumption blunted the effects of exercise intensity on EVs, suggesting that the interaction between EVs, exercise and ad-libitum dietary sugar intake may be relevant. Taken together, these data suggest that cardiorespiratory fitness, postprandial hyperglycemia and ad-libitum dietary sugar intake modulate EV release and that these changes are related to clinical outcomes. Future work should consider examining the impact of these stimuli beyond EV count, as changes in EV content (i.e. miRNA, etc.) may help elucidate the mechanism by which exercise and postprandial hyperglycemia confer beneficial and deleterious physiological effects, respectively.