

Original Research

Extracellular Vesicles as Predictors of Individual Response to Exercise Training in Youth Living with Obesity

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Abstract

Background: Exercise is associated with health benefits, including the prevention and management of obesity. However, heterogeneity in the adaptive response to exercise training exists. Our objective was to evaluate if changes in extracellular vesicles (EVs) after acute aerobic exercise were associated with the responder phenotype following 6-weeks of resistance training (RT). **Methods:** This is a secondary analysis of plasma samples from the EXIT trial (clinical trial#02204670). Eleven sedentary youth with obesity (15.7 ± 0.5 yrs, BMI ≥ 95 th percentile) underwent acute exercise (60% HRR, 45 min). Blood was collected at baseline [AT0 min], during [AT15–45 min], and 75 min post-recovery [AT120], and EVs purified using size exclusion chromatography from extracted plasma. Afterward, youth participated in 6-weeks RT and were categorized into responders or non-responders based on changes in insulin sensitivity. **Results:** We assessed EV biophysical profile (size, zeta potential, protein yield, and EV subtype protein expression) in a single-blind fashion. Overall, there was a general increase in EV production in both groups. Average EV size was larger in responders (~147 nm) vs. non-responders (~124 nm; $p < 0.05$). EV size was positively associated with absolute change in Matsuda index (insulin sensitivity) following RT ($r = 0.44$, $p = 0.08$). EV size distribution revealed responders predominantly expressed EVs sized 150–300 nm, whereas non-responders expressed EVs sized 50–150 nm ($p < 0.05$). At baseline, responders had ~25% lower TSG101, ~85% higher MMP2 levels. EV protein yield was higher in responders than non-responders at AT15 ($p < 0.05$). **Conclusions:** Our data suggest that youth with obesity that respond to RT produce larger EVs that are TSG101+ and CD63+, with increased EV protein yield during acute exercise.

Keywords: extracellular vesicles; youth; obesity; acute exercise; resistance exercise training; responders to exercise; insulin sensitivity

1. Introduction

Obesity, type 2 diabetes, and hypertension are common cardiometabolic diseases in adolescents worldwide [1,2]. In Canada, ~1-in-7 children/youth are overweight or obese [3,4]. With increasing rates of cardiometabolic diseases in youth/adolescents, understanding the impact of prevention strategies is needed, particularly, who responds well to specific interventions [5,6]. Exercise is fundamental in the management of cardiometabolic risk associated with obesity as it promotes visceral fat loss and cardiorespiratory fitness [7,8]. Despite the general effectiveness of exercise on cardiometabolic risk in youth, our group [9] and others have documented notable heterogeneity in the individual response to exercise training [10]. Within clinical trials, participants who display positive metabolic improvement post exercise-training are classified as exercise

responders, and make up 40–70% of youth in these trials [11]. Variable adaptation in the metabolic response to exercise, e.g., change in adiposity or hepatic triglycerides content, is linked with the differential increase in cardiorespiratory fitness with training [9]. As genetic factors account for ~30–50% of this response heterogeneity [12], it suggests that other physiological factors likely play a role in this variable response to physical activity.

Chronic exercise training activates transcriptional and non-transcriptional signalling cascades in skeletal muscle that lead to an increase in mitochondrial biogenesis and ultimately metabolic capacity [13]. This in turn promotes improved insulin signalling, weight loss, and enhanced cardiorespiratory fitness [14]. Foundational work by Pedersen and colleagues established the role of myokines in potentiating the systemic benefits to regular exercise training [15].



Myokines are proteins or peptides released by skeletal muscle into circulation at rest, and at elevated rates upon exercise [16]. Myokines, such as interleukin-6 (IL-6) and irisin, are important in mediating the whole-body metabolic response to physical activity and are power contributors to the adaptations observed with chronic exercise training [17–20]. In humans, IL-6 also increases lipolysis and free fatty acid oxidation in adipocytes, suggesting that IL-6 plays a significant role in fat metabolism [21]. Interestingly, IL-6 is involved in exercise training-induced uncoupling protein-1 (UCP1) expression in murine inguinal white adipose tissue (WAT), and thus, it participates in adipocyte browning [22]. Besides that, irisin influences adipogenesis and induce thermogenesis to promote energy expenditure [23,24], and despite most of its findings were based on rodent experiments, some studies have shown increased irisin levels in humans in response to exercise [25,26]. Myokines have been found packaged within extracellular vesicles (EVs) [27,28], however little information exists on the interaction between myokines, EVs and their role in regulating the physiological responses to exercise in youth living with obesity.

EVs are small membrane-bound vesicles produced by cells, containing molecular cargo representative of the cell of origin [29], and play a vital role in intracellular communication [30–32]. EVs are present in all biological fluids, and characterized according to size, density, biochemical composition or cell of origin [33]. EVs are traditionally divided into three main subtypes: (1) exosomes (30–150 nm), (2) microvesicles (100–1000 nm) and (3) apoptotic bodies (500–5000 nm). The biogenesis of EV subtypes, their size and biophysical characteristics, and molecular cargo are distinct from one another and affect recipient cells differentially upon vesicle uptake. Unless EV subcellular origin can be quantifiably established, according to the Minimal Information for Studies of Extracellular Vesicles (MISEV) guidelines, EVs should be classified as small EVs (<200 nm) or medium/large EVs (>200 nm) [34]. Recently, studies have shown that exercise promotes the release of EVs from skeletal muscle, platelets, endothelial cells, and leukocytes into the bloodstream [32,35–37]. There is a growing evidence supporting the role of EVs in the regulation of cardiometabolic adaptations to exercise training. We previously hypothesized that EVs may play a functionally important role in determining the systemic adaptations to training [32]. However, we do not know if the variance in the cardiometabolic response to exercise is associated with changes in EV subtypes and biophysical characteristics. To address this gap, we performed a secondary analysis of plasma samples from the EXIT study (Clinical Trial#02204670, [38]). The EXIT study investigated the influence of exercise-induced myokine, irisin, on the metabolic response to exercise training in youth with obesity [38]. Our main objective in the current study was to determine if EVs released during an acute bout of aerobic

exercise prior to 6-weeks of resistance training, were associated with the exercise responder phenotype. Within this secondary analysis of data from the EXIT trial, we hypothesized that youth with obesity that are exercise responders will be characterized by biophysically different EVs (by size, yield, and/or stability) as well as by molecular cargo, vs. youth who do not respond to exercise training.

2. Materials and Methods

2.1 Participants and Plasma

Plasma samples available at various time points from the EXIT study [Clinical Trial#02204670, [30]] from all sedentary youth with obesity (15.7 ± 0.5 yrs, BMI ≥ 95 th percentile, $n = 11$), were included. Participants underwent a baseline screening: medical history, an oral glucose tolerance test (OGTT) and a baseline assessment of body composition, fitness, physical activity level, and muscle strength. Exclusion criteria included: (1) previous diagnosis of Type 2 diabetes; (2) use of corticosteroids or atypical antipsychotics; (3) use of medications known to affect insulin sensitivity or its secretion within the last 30 d; (4) use of medications related to weight loss/gain; (5) use of anabolic steroids; (6) presence of orthopedic injury or a chronic illness that prevented from participating in the exercise testing/intervention; (7) underwent weight loss or participation in a weight loss program within 6 months of enrolment; (8) history of alcoholism or drug abuse and (9) be pregnant or planning to be pregnant. Following the first visit, youth completed an acute bout of aerobic exercise (AE, performed by cycling at 60% heart rate reserve for 45 min), during which blood draws were taken. As detailed before [38], blood was collected through a venous catheter from an antecubital vein by a registered nurse before AE [at time = 0 min (AT0)], during AE [AT15, 30, 45 min], and after AE+75 min recovery [AT120]. Blood was centrifuged and plasma collected and stored at -80°C for further analysis, including EV isolation. A week later an acute bout of resistance exercise was performed as detailed previously [38]. Following these two acute exercise sessions, youth participated in 6-week resistance exercise training program that consisted of 8 different exercises performed in three weekly sessions as described below. The study was approved by the University of Manitoba Biomedical Research Ethics Board and performed according to the Declaration of Helsinki. All participants and parents gave written informed consent and assent prior to the start of the study. We isolated and analyzed EVs from the plasma samples that were collected before, during, and after an acute bout of AE in a single-blind fashion. We did not assess the plasma samples from the acute bout of resistance exercise in the original study as the authors reported that irisin *only* increased during the acute aerobic exercise session, and it was this increase in plasma irisin that was associated with insulin sensitivity in the responders after training [38].

2.2 Resistance Exercise Training

The 6-week resistance training program has been described in detail previously [38]. Briefly, it consisted of three weekly sessions on non-consecutive days conducted at the local YMCA in Winnipeg. Participants warmed up on a cycle ergometer, treadmill or an elliptical for about 5–10 min before each session. Resistance training consisted of 8 different exercises: seated chest press, narrow grip latissimus pull down, seated leg curl, leg extension, shoulder elevation, arm curl, triceps extension, and plank. Each exercise was performed for 3 sets of 12–15 repetitions at 60–65% of the estimated one-repetition-maximum (1-RM) with 60 sec rest period separating each set [38].

2.3 EV Isolation

EVs were isolated using size exclusion chromatography [(SEC), qEV, Izon] according to manufacturer's instructions. Previous studies showed that SEC minimally alters the characteristics of isolated EVs, and is considered to be one of the best methods for separating exosomes from protein contaminants and co-precipitates [33,39]. We extracted EVs from plasma samples collected at baseline (AT0), during AE (AT15, 30 and 45) and during recovery (AT120) as shown in Fig. 1A. Briefly, samples were passed through a 0.22 μm filter (Merck Millipore) to eliminate cells and cellular debris. Next, 150 μL of each sample was loaded onto qEV columns and 12 \times 200 μL fractions (F) were collected with filtered PBS as the elution buffer. Each fraction (F1–12) was analyzed for EV size, protein yield and presence of EV-specific markers by Western blotting. In line with manufacturer recommendations, F7–10 were found to be EV-rich, plasma protein- and lipoprotein-poor, and were pooled to measure EV size, zeta potential, protein yield and presence of EV subtype markers by immunoblotting.

2.4 EV Characterization

EVs were stored at 4 $^{\circ}\text{C}$ for up to 24 hrs before being used to measure their hydrodynamic diameter and zeta potential using a NanoBrook ZetaPALS (Brookhaven Instruments, Holtsville, NY, USA) dynamic light scattering (DLS) instrument (Dr. Hagar Labouta, University of Manitoba, MB, Canada). Prior to characterization, 20 μL of EVs were diluted 1:75 (PBS) and kept on ice until analysis. EV size was measured as an intensity averaged multimodal distribution using a scattering angle of 90 $^{\circ}$, and size bins were used to represent total size intensity within a given size range. For zeta potential, each sample was loaded into a disposable cuvette, and an electrode inserted for phase analysis light scattering to carry out mobility measurements. Each sample underwent 5 runs, each run ~15 sec, with a dust cut-off set to 40. Values were averaged (irrespective of negative/positive charge) to calculate zeta potential using the Smoluchowski formula from mobility measurements [40]. All measurements were performed in PBS (pH 7.4, 25 $^{\circ}\text{C}$).

2.5 Protein Concentration and Quantification

To concentrate samples, F7–10 were centrifuged at 14,000 \times g , at 4 $^{\circ}\text{C}$ for 60 min (Amicon Ultra-4 3 kDa centrifugal filter device, EMD Millipore, Burlington, MA, USA). ~50 μL retentate was the final volume of concentrated EVs. Proteins were extracted using 1:1 Pierce RIPA solution with protease inhibitor tablet (Roche). Samples were vortexed for 5 sec, followed by two freeze/thaw cycles with liquid nitrogen, sonicated at 30 Hz 3 \times 3 sec on ice and centrifuged at 15,000 rpm (21,130 \times g , 15 min, 4 $^{\circ}\text{C}$). Proteins were quantified using the PierceTM MicroBCA Protein Assay kit (Thermo Fisher Scientific, Waltham, MA, USA) following manufacturer's instructions. Briefly, working reagent was prepared by mixing 25 parts of Reagent A, 24 parts of Reagent B and 1 part of Reagent C. Protein standards were prepared by serial dilution of a 2 mg/mL BSA ampule into clean vials using ultrapure water. 150 μL of each standard or sample was added to a 96-well microplate in duplicates, followed by addition of 150 μL of working reagent/well. Samples were incubated (37 $^{\circ}\text{C}$, 2 hrs) and absorbance measured at 562 nm using a microplate spectrophotometer (BioTek Epoch, Santa Clara, CA, USA).

2.6 Western Blotting

Western blotting was used to determine the expression of subtype specific protein expression in order to ascertain the subcellular origin of EVs. Samples were denatured by the addition of β -mercaptoethanol and 4 \times laemmli buffer, followed by incubation at 95 $^{\circ}\text{C}$ (5 min). 7 μg of total protein were loaded on 12% SDS-PAGE gels and electrophoresed at 90 mV (30 min), and then 120 mV (2 hrs). Proteins were transferred to polyvinylidene difluoride (PVDF) membrane using Trans-Blot[®] TurboTM (Bio-Rad, Hercules, CA, USA). Next, the membrane was incubated with blocking buffer [5% skim milk in Tris-buffered saline Tween-20 solution (TBST)] for 1 hr at room temperature, followed by incubation with primary antibodies in 1% skim milk overnight at 4 $^{\circ}\text{C}$: rabbit polyclonal anti-TSG101 (T5701, Sigma-Aldrich Co, 1:200), rabbit polyclonal anti-CD63 (SAB4301607, Sigma-Aldrich Co, 1:300), mouse monoclonal anti-CD81 (sc-166029, Santa Cruz Biotechnology, 1:200), rabbit polyclonal anti-Cytochrome-c (Cyt-c; AHP2302, Bio-Rad Laboratories, 1:500), mouse monoclonal anti-MMP2 (sc-13595, Santa Cruz Biotechnology, 1:200), and mouse monoclonal anti-Apolipoprotein-A1 (Apo-A1; 0650-0050, Bio-Rad Laboratories, 1:200). Membranes were washed 3 \times TBST and incubated with anti-mouse or anti-rabbit IgG secondary antibody (A16017, A16035, Thermofisher, 1:1000) for 1 hr at room temperature. ClarityTM or Clarity MaxTM ECL substrate (Bio-Rad) was applied to visualize bands via enhanced chemiluminescence and images captured using ChemiDocTM MP Imaging System (Bio-Rad, Hercules, CA, USA). Band intensity was quantified using Image lab software (Bio-Rad, Hercules, CA, USA) and corrected for loading using Coomassie blue dye staining.

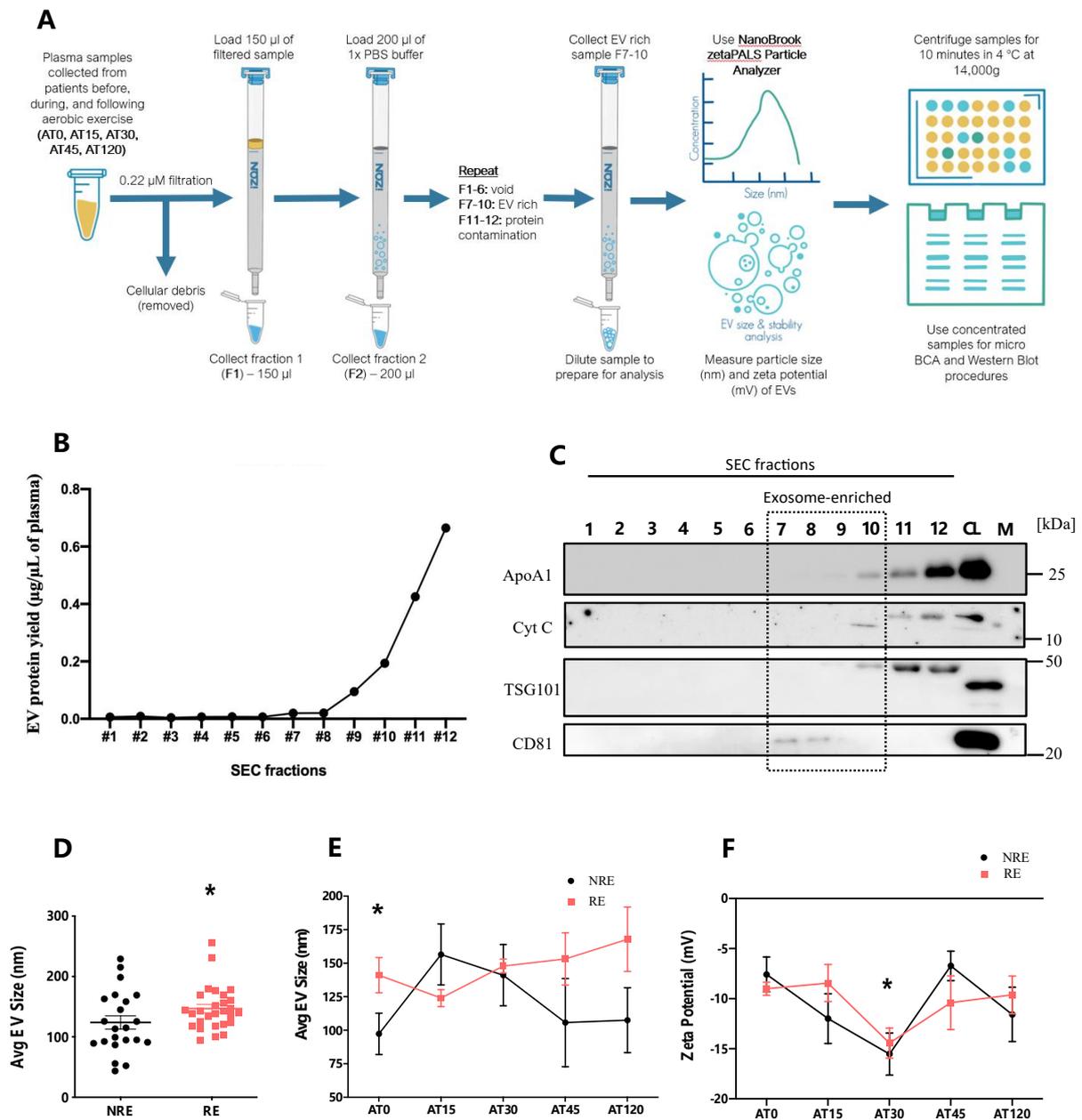


Fig. 1. Study design, SEC validation and biophysical characterization of EVs from EXIT trial participants. (A) Schematic depicts the study design. Plasma was collected from participants in the EXIT trial study, before acute exercise at time 0 (AT0), during (AT15, 30 and 45) and after recovery (AT120). EVs from non-responders (NRE) and exercise responders (RE) were isolated by SEC and analyzed for size and zeta potential using dynamic light scattering (NanoBrook ZetaPALS). (B) Protein yield was measured using Pierce™ MicroBCA Protein Assay kit and showed an increasing protein concentration from fraction 7 (F7) onwards to F12. (C) Western blotting was performed (12% SDS-PAGE) and Coomassie blue staining used as a loading control to measure proteins traditionally enriched in small vesicles (TSG101 and CD81), non-EV co-isolates [(Cyt-c) and lipoprotein marker (Apo-A1)]. F7 to F10 were considered small EV-rich while lipoprotein-poor. (D) Average EV size was larger in responders compared with non-responders overall, (E) and especially at AT0 (* $p < 0.05$). (F) Zeta potential was not different between the two groups. Both groups had the lowest zeta potential, i.e., most stable EVs at AT30 (* $p < 0.05$). SEC, size exclusion chromatography; CL, cell lysate; M, markers. Data were analyzed using multiple unpaired Student's *t*-test with * $p < 0.05$ considered as significant and expressed as mean \pm standard error (N = 5–6, where each N refers to a biological replicate). For (D,E,F): each biological replicate was measured 5 times in the NanoBrook ZetaPALS as detailed in the methods.

2.7 Definition of Responder or Non-Responder to Exercise Training

Participants performed an OGTT at baseline and post-intervention. Plasma glucose and insulin were used to determine the Matsuda index. Blood samples were taken at baseline, 30, 60, and 120 min following the ingestion of a 75-gram glucose drink. Matsuda index was calculated according to the formula: $10000 / \sqrt{(Go \times Io) \times (Gm \times Im)}$, where Go = fasting plasma glucose, Io = fasting plasma insulin, Gm and Im = mean plasma glucose, and mean plasma insulin concentration during OGTT. Following the intervention, participants were categorized into responders to exercise if change in insulin sensitivity was above the 50th percentile, or non-responders to exercise if change in insulin sensitivity was below the 50th percentile.

2.8 Statistical Analysis

Average EV size, zeta potential, size distribution, protein yield, and expression of protein markers at AT0, data were analyzed using multiple unpaired Student's *t*-test. A one-way ANOVA with Tukey post-hoc was used to analyze protein expression over time. Average EV size during the acute bout of AE, and absolute change in Matsuda Index post-training were analyzed using Pearson's Correlation Coefficient. All data were analyzed using PRISM software, version 8.4.2 (GraphPad Software, San Diego, CA, USA) with 95% confidence intervals. Significance was set at $*p < 0.05$ and data expressed as mean \pm standard error.

3. Results

3.1 Biophysical Characterization of Isolated EVs

Pooled EV-rich fractions (F7–10) isolated via SEC from responders and non-responders to exercise were concentrated, total protein yield determined, and markers of subcellular origin (small/large vesicles) measured by immunoblotting as illustrated in Fig. 1A. To validate EV isolation as per MISEV guidelines [34], we analyzed each fraction (F1–12) for EV size (data not shown), protein yield (Fig. 1B) and expression of proteins traditionally enriched in small vesicles (TSG101, CD81) or non-EV co-isolated proteins (Cyt-c and Apo-A1, Fig. 1C). Protein concentration increased nearly exponentially starting in F7 to F12 (Fig. 1B), and TSG101 and CD81 were enriched in F7–10 as reported previously (Fig. 1C) [41,42]. F10 was not completely free of contaminating Apo-A1, or Cyt-c normally found in large vesicles (Fig. 1C), however size analysis on F10 revealed it contained small vesicles sized 56.3 ± 17.8 nm. F11 was not included because it showed the likely presence of extremely large apoptotic bodies or aggregates or cellular debris (~ 7899 nm, data not shown) and significant contamination by non-exosome markers (Fig. 1C). Our results are compatible with other studies that reported similar findings when using SEC [41,42]. This established that F7–F10 are sufficiently enriched with TSG101+/CD81+ small EVs and were subsequently pooled for all further analysis.

Average EV size (all time points) was larger in the responder group (146.9 ± 6.8 nm) vs. the non-responders (124.1 ± 11.0 nm, $*p < 0.05$, Fig. 1D). EV size distribution showed larger EVs in the responder group at all time points except at AT15 (Fig. 1E). The groups were significantly different at AT0, demonstrating that the subjects were releasing EVs of different sizes even before initiating acute exercise ($*p < 0.05$, Fig. 1E). Zeta potential, indicative of EV stability in suspension [43], remained comparable between the two groups (non-responders: -10.7 ± 1.6 mV; responders: -10.4 ± 1.0 mV). Zeta potential was lowest at AT30 for both groups when compared to the other time points ($*p < 0.05$, Fig. 1F).

Overall size distribution across all-time points reaffirmed that participants that responded to exercise had a higher yield of medium/large EVs, between 150–300 nm vs. non-responders ($*p < 0.05$, Fig. 2A). In contrast, non-responders had more smaller EVs 50–150 nm ($*p < 0.05$, Fig. 2A). Analyses of EV size distribution at baseline (AT0), during exercise (AT15, AT45) and recovery (AT120) showed a similar pattern where the responder group expressed more medium/larger EVs than non-responders ($*p < 0.05$, Fig. 2B). This pattern was not observed at AT30 where EV size distribution between the two groups was virtually the same (Fig. 2B). There was a general increase in EV production with exercise and recovery in both groups, when results were expressed for each group separately (Fig. 2C). The pattern of increased expression of medium/large EVs in the responders, and small EVs in non-responders remained consistent irrespective of whether it was during exercise or recovery (Fig. 2C).

3.2 Western Blot Analysis of Proteins Enriched in EV Subtypes

EV protein yield from both groups was similar at baseline (AT0, Fig. 3A), and remained consistent across the acute exercise and recovery in responders. Non-responders showed a significant decrease in EV protein content at AT15 ($0.08 \pm 0.03 \mu\text{g}/\mu\text{L}$) vs. $0.25 \pm 0.03 \mu\text{g}/\mu\text{L}$ in responders ($*p < 0.05$, Fig. 3A). This difference in protein yield between groups diminished at AT30 and returned to similar levels at AT45 and AT120 (Fig. 3A). Western blot analysis was performed to assess expression of small and large vesicles-specific proteins in line with MISEV guidelines [34]. We examined the expression of CD63 and TSG101 as representative proteins enriched in small vesicles, and MMP-2 as a protein enriched in medium/large vesicles. Responders expressed $\sim 25\%$ lower TSG101 and $\sim 85\%$ higher MMP2 content, while CD63 levels remained unchanged between the groups at AT0 (Fig. 3B,C). The expression of CD63, TSG101 and MMP-2 did not change with acute exercise/recovery, likely due to the low sample size and individual heterogeneity (Fig. 3C–E).

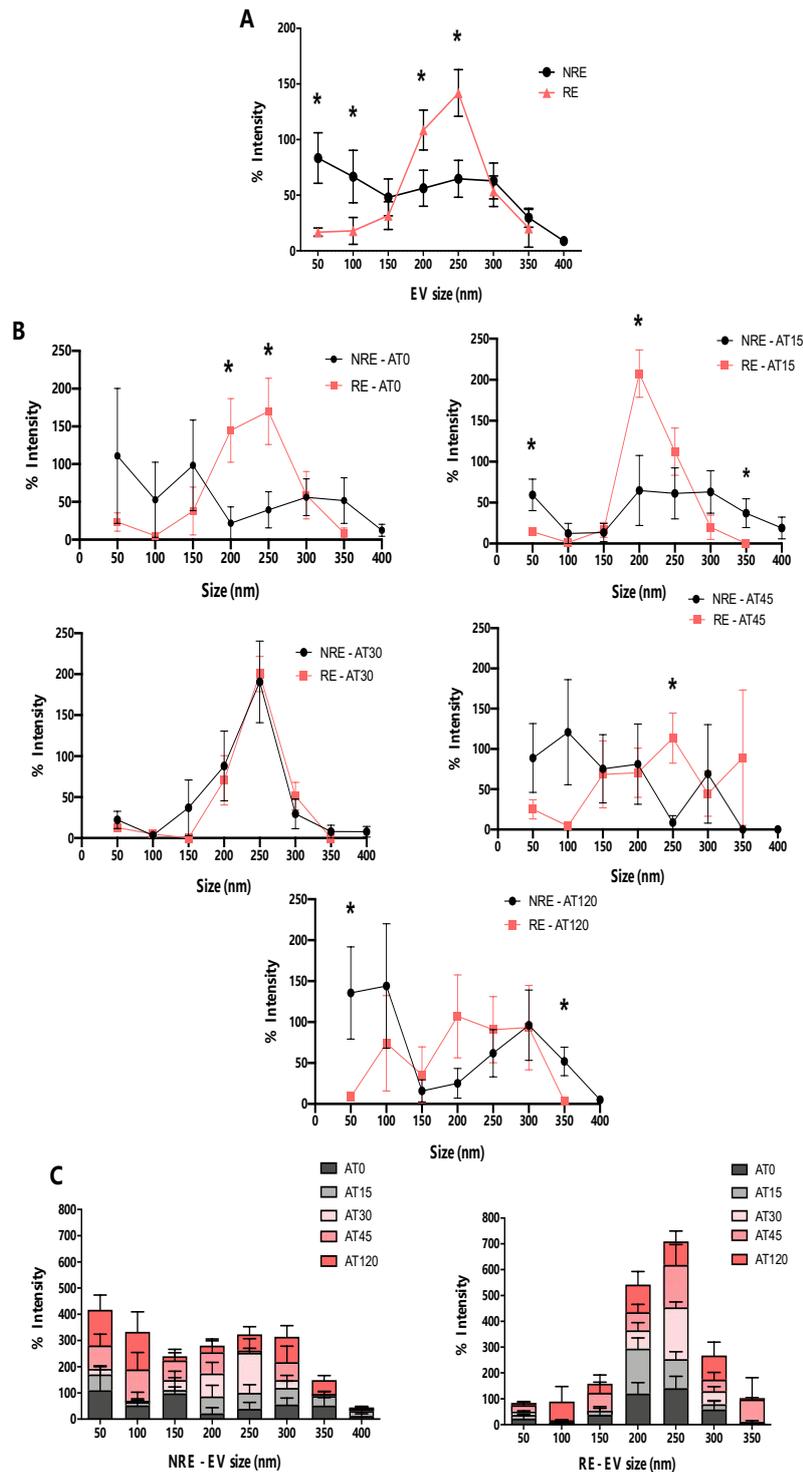


Fig. 2. EV size distribution profile in responders vs. non-responders to exercise. (A) EV size distribution analysis combined for all time points illustrated a higher yield of medium/large EVs in the responder (RE) group between 150–300 nm. Non-responders to exercise (NRE) expressed increased content of small EVs between 50–150 nm size range ($*p < 0.05$). (B) Analyses of EV size distribution by individual time points showed RE group with an increase in larger EVs at baseline (AT0) and during AE (AT15, 30 and 45), while NRE showed enhanced expression of small EVs at all time points except AT30 where it matched the RE group ($*p < 0.05$). (C) Size distribution demonstrated a general increase in EV production with time in both groups. Data were analyzed using multiple unpaired Student's *t*-test with $*p < 0.05$ considered as significant and expressed as mean \pm standard error ($N = 5-6$, where each N refers to a biological replicate). Each biological replicate was measured 5 times in the NanoBrook ZetaPALS as detailed in the methods for size and zeta potential measurements.

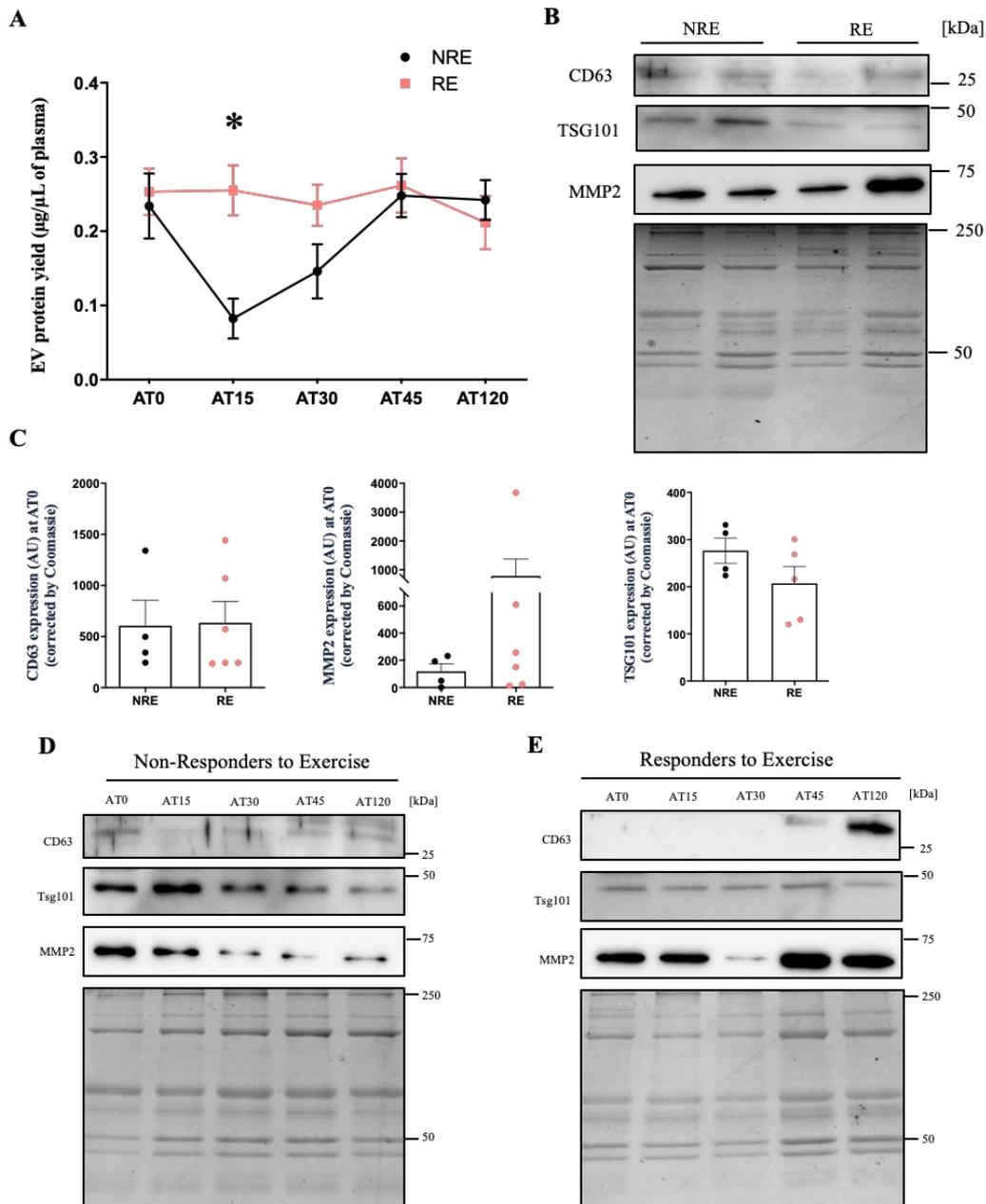


Fig. 3. Protein yield and expression of markers of EV subtypes AT0. (A) Non-responder (NRE) group showed a significant decrease in EV protein yield compared to responders (RE) at AT15 ($*p < 0.05$). (B,C) Equal amounts of EV protein ($7 \mu\text{g/mL}$) from both groups were subjected to SDS-PAGE (12%) and expression of proteins traditionally enriched in small vesicles: TSG101 (46 kDa) and CD63 (28 kDa), and medium/large vesicles: MMP2 (63 kDa) were quantified. Responder EVs expressed $\sim 25\%$ lower TSG101 protein, $\sim 85\%$ higher MMP2 content, while CD63 levels remained unchanged between the groups at AT0. Coomassie blue staining was used as a loading control. The same gel was stained with Coomassie after proteins were transferred onto a PVDF membrane. Data were analyzed using unpaired Student's *t*-test with $*p < 0.05$ considered as significant and expressed as mean \pm standard error ($N = 5-6$). (D) Representative blots from non-responders and (E) responders to exercise showing expression of TSG101, CD63 and MMP2 at baseline (AT0), during aerobic exercise (AT15–45), and after recovery (AT120). Coomassie staining (shown below the blots) was used as a loading control. No significant changes were observed for any protein. Data were analyzed using one-way ANOVA ($N = 3-4$, where each N refers to a biological replicate).

3.3 EV Size and Correlation with Insulin Sensitivity

To investigate any correlation between EV size and insulin sensitivity, we applied Pearson's correlation coefficient to our data. Our results suggested that there is a moderate positive correlation between average EV size released in circulation during an acute bout of aerobic exercise and absolute change in Matsuda index post-6 weeks of resistance training ($r = 0.4374$, $p = 0.08$, Fig. 4).

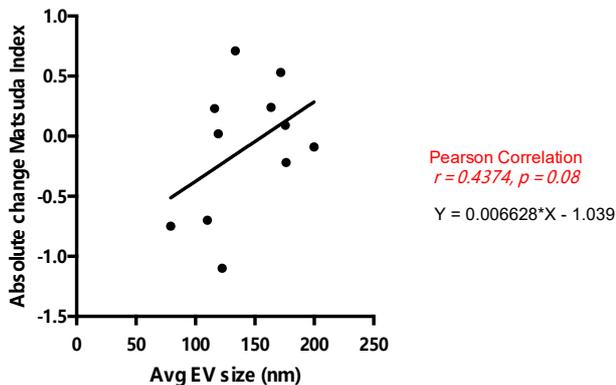


Fig. 4. Association of average EV size with absolute change in Matsuda Index. Pearson's Correlation analysis showed a moderate positive association between average EV size and absolute change in Matsuda index (indicative of insulin sensitivity) post 6-week resistance exercise training ($r = 0.4374$, $p = 0.08$).

4. Discussion

To the best of our knowledge, this is the first study to analyze EVs in youth with obesity in response to acute aerobic exercise and identify any association between EVs and exercise responder phenotype. The analysis was done in a single-blind fashion. The main findings of our study are: (1) acute aerobic exercise leads to a general increase in systemic EVs; (2) average EV size was larger in responders (150–300 nm) vs. non-responders (50–150 nm); (3) the size of EVs aligned with expression of markers enriched in EV subtypes, i.e., responder group released EVs that were TSG101+/CD63+, but with lower expression of proteins traditionally enriched in small EVs, e.g., TSG101 and higher expression of MMP-2, commonly found enriched in medium/large EVs, and (4) there was a mild positive correlation ($r = 0.4374$, $p = 0.08$) between overall average EV size and Matsuda index, meaning that the larger the EVs released during a bout of acute aerobic exercise, the more likely the participants were to respond to 6-weeks of resistance exercise training and improve insulin sensitivity. These results are important as they shed light on potential determinants of individual exercise response in youth with obesity at risk of Type 2 diabetes.

Previous studies have shown that circulating EV levels increase with obesity in mice and humans [44,45], how-

ever, the potential mechanisms that lead to higher EV release and the biological effects of the EV-subtypes in obesity are not yet known [46]. Our data showed larger EVs in responders (150–300 nm) vs. non-responders (50–150 nm). While both groups expressed protein markers of small and medium/large vesicles, we measured 25% lower TSG101 expression, and ~85% higher MMP2 content in the responders which is in line with the preponderance of larger EVs in this group. The protein expression data was not statistically significant, likely due to low sample size warranting further investigation. EV size is associated with EV subtypes and subsequently distinct transcriptomic, lipidomic and proteomic cargo, which in turn predicts the biological effects of EVs on recipient cells. Durcin *et al.* [47] showed that adipocytes released different sized EVs, with distinct protein expression: medium/large EVs encapsulated proteins/enzymes related to metabolic function, many of mitochondrial origin, leading to the hypothesis that they may play a role in metabolic regulation. This hypothesis is supported by our data where the responders produced larger EVs vs. non-responders. Interestingly, we also observed that responders had significantly higher EV protein yield than the non-responders, particularly at AT15 and AT30. This occurred in tandem with lowest zeta potential values (i.e., more stable particles) at these time points, irrespective of responder phenotype. The increase in EV protein yield is in line with previous work by Oliveira *et al.* [48] who showed that low, moderate and high intensity aerobic exercise significantly increased serum EV protein yield in rats. However, the physiological relevance and importance of this observation is not known. It may be that during acute exercise, responders release more proteins (related to metabolic regulation) packaged in medium/large EVs, which given the increase vesicle stability at the same time, can lead to beneficial metabolic adaptations vs. non-responders. This hypothesis is supported by our finding that *a priori* average EV size during an acute exercise bout is moderately associated with improved insulin sensitivity following 6-weeks of training. Hence, we propose that a differential effect of EVs, likely due to different biomolecular cargo based on the inherent differences in size, on recipient tissues in responders vs. non-responders may be an underlying mechanism for the variance in insulin sensitivity. To confirm, comprehensive analysis of the biomolecular EV cargo in responders is warranted. Further targeted experiments to specifically isolate temporal medium/large EVs secreted during exercise, and to determine their effects on metabolic adaptations are also needed.

EVs in circulation may originate from any cells capable of releasing EV systemically. Circulatory EVs released with exercise largely originate from platelets, endothelial cells, leukocytes, with skeletal muscle-derived EVs accounting for a small proportion of the whole [37,49]. Eichner *et al.* [50] showed that participants living with obesity and with very poor cardiorespiratory fitness presented

elevated counts of platelet and endothelial origin EVs vs. subjects with poor fitness, independent of age and body fat. The authors suggested that subtle differences in fitness may reduce type 2 diabetes and cardiovascular disease risk through an EV-related mechanism. Others have demonstrated that white adipose tissue-derived EVs possess immunomodulatory properties and can affect insulin signalling in muscle and liver cells [51]. Thus, the fitness and health status of subjects can affect EV production, and both EV size and cell of origin can modulate the downstream functional effects of EVs on recipient cells. Due to limited samples, we were unable to ascertain the origin of the EVs isolated in our study, but this research is necessary to understand the origin and possible downstream targets of EVs.

Our study demonstrated a general increase in EV production over time in both groups after aerobic exercise. This is in agreement with previous work that demonstrated an increase of EVs in young and middle-aged healthy [35,49], and overweight human subjects after aerobic exercise [31]. Whitman and colleagues [49] showed that one hour of cycling induced a significant increase in systemic EVs, while Frühbeis *et al.* [35] reported that the systemic EV concentration was higher immediately after a single exhaustive bout of resistance exercise. Another study in rats showed that acute aerobic exercise was associated with increased EV concentration and presence of differential miRNAs cargo [48]. In contrast, in adults with obesity, Rigamonti *et al.* [52] reported reduced EVs concentration immediately at the end of acute exercise and after 3 hrs and 24 hrs. Exercise intensity, time, and blood collection points in our study are different compared to Rigamonti *et al.* [52] who measured EVs immediately post-exercise, 3 hrs and 24 hrs later, and where participants exercised for 30 min at 60% VO₂ max. Other than the obvious difference in participant age, the levels of circulating EVs are dynamic and can be modulated by a number of factors including body weight [45], immune status, hormone levels, metabolic state, exercise duration, type and intensity, and even by the methodology used to measure EVs [37], which explains the data that appear to be in conflict with previous work. Further studies are needed to understand the temporal pattern of EV release with exercise in healthy adults and youth, as well as those with metabolism-related conditions such as obesity, type 2 diabetes and the metabolic syndrome. Careful consideration and control of exercise dose, collection time points, blood collection methodology, and EV isolation methodology are necessary to compare results from different studies. Indeed there is growing recognition of EVs as biomarkers of cardiometabolic diseases [53,54], with the corollary that endurance exercise-derived EVs can rescue metabolic diseases [32,55]. However, these findings need to be interpreted carefully due to the technical challenges associated with the different EV isolation and characterization techniques, and the heterogeneity of EV subtypes and their biological significance [34,47].

5. Conclusions

In summary, here we showed that youth living with obesity that are responsive to exercise training, produced larger EVs, with a higher protein yield, lower expression of proteins found in small vesicles and higher expression of large vesicles proteins, *apriori* to exercise training. We also documented a unique temporal pattern of EV release during exercise and immediately after recovery in both groups, and a positive correlation between EV size and positive adaptations in insulin sensitivity. The relationship between EV cargo and the individual response to exercise is unknown. Our results highlight the need to distinguish EV subtypes to delineate their respective functional properties, and subsequent role in response to exercise training. Further study of EV size and cargo differences can have important implications for diagnostics of exercise intolerance, and tailoring exercises to youth to improve their health on the understanding of EVs and the physiological mechanisms that produce them.

Author Contributions

TMP and AM performed most of the experiments in the current study. TMP and AM analyzed data, created figures, and helped write the manuscript. AS designed the project, and helped synthesize data, create figures, helped write and edit the manuscript. POO, SS, BB, HIL provided technical support and assistance with experiments and theoretical expertise. ALE, KRB, JMM, MS conducted the original EXIT trial clinical study. All authors were involved in manuscript revisions. AS is the corresponding author and directly supervised the project. All authors have read, edited and agreed to the published version of the manuscript.

Ethics Approval and Consent to Participate

The study was approved by the University of Manitoba Biomedical Research Ethics Board (Ethics no. B2014:064) and performed according to the Declaration of Helsinki. All participants and parents gave written informed consent and assent prior to the start of the study.

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Conflict of Interest

K.R.B has a direct financial interest in nanotechnology innovation as a director and shareholder of NBBM Inc. All other authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; or in the writing of the manuscript.

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