SARCOPENIA, SKELETAL MUSCLE AND EXOSOMES

CIRCULATORY AND SKELETAL MUSCLE EXOSOME RESPONSE IN OLD PARTICIPANTS FOLLOWING A 12-WEEK RESISTANCE TRAINING PROGRAM

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LAY ABSTRACT

Aging is the slow and time-dependent process that our organs, down to the cellular level, deteriorate in function reducing the biological fitness of our bodies. Aging specific to skeletal muscle, or sarcopenia, is especially important because skeletal muscle makes up 40% of our weight, is essential for posture, balance, locomotion and breathing. Sarcopenic individuals have low muscle mass, strength, and function and as a result are associated with low independence in activities of daily living and increased risks of falls and fractures. Exercise, and in particular resistance training, has been shown to be beneficial and cost-effective in treating sarcopenia and delaying aging throughout the body. Part of the underlying mechanism regarding how exercise affects us in a multi-systemic manner is not well understood. We know that skeletal muscle releases a multitude of molecular factors during exercise. Amongst them, extracellular vesicles and specifically exosomes are worth investigating because they have been shown to function in intercellular communication by delivering molecular signals, called microRNAs, from origin cells to recipient cells throughout the body. In this thesis project, we investigate exosomes in circulation of older individuals before and after a 12-week resistance training program. We found that aging alters the exosome pool in circulation as well as their miRNA content. After resistance training, many of miRNAs altered with age, return to levels comparable to young. In addition, we showed that at the skeletal muscle level, aging and resistance training affect exosome biogenesis and miRNA expressions. In conclusion, we provide evidence that aging significantly alters circulatory exosomes and miRNA and show that resistance training normalizes the miRNA profile to levels seen in exosomes derived from young plasma. How exosomes and their molecular signals change with aging and how exercise affects them gives us an insight on how exercise elicits multi-systemic benefits against aging and sarcopenia.

ABSTRACT

Sarcopenia is the age-related progressive loss of skeletal muscle (SkM) mass, function, and strength. It has been well elucidated that resistance exercise can attenuate the development of sarcopenia. A population of extracellular vesicles, termed 'exosomes' (EXO), can contain microRNA and facilitates intercellular communication, including within SkM, though the response to prolonged training is not well understood. Given the potential role of SkM-derived exosomes in the response to exercise, we examined older adults (n = 30, OLD) before (PRE) and after a 12-week (POST), resistance training program. Healthy, young controls (n = 12, YNG) were used for comparison of baseline measures. Exosomes were isolated from platelet-free plasma using size exclusion chromatography in combination with ultracentrifugation (SEC-UC) and characterized via western blotting, nanoparticle tracking analysis and electron microscopy. To assess exosome biogenesis and miRNA synthesis in skeletal muscle, biopsies were taken from the *vastus lateralis.* Circulating EXO-enclosed and SkM miRNA expression was measured using RT-PCR. In SEC-UC isolates, EXO-markers CD81 and CD9 were significantly lower in PRE compared to YNG ($p<0.05$) but did not change with training. At baseline, ALIX, TSG101 and CD63 (markers of exosomes) were not altered with aging as compared to YNG; however, their expression significantly increased with training ($p<0.05$) Circulating EXO-derived mir-1, -133, -23 and -27a were significantly lower in expression of OLD participants as compared to YNG. Following resistance training, their expression significantly increased ($p<0.05$), returning to a YNG phenotype. Next, we aimed to investigate the contribution of skeletal muscle in the exosome responses. Our data indicate that a small fraction of circulatory exosomes may originate from skeletal muscle. In addition, in biopsy-derived SkM tissue, expression of proteins involved in EXO and miRNA biogenesis (Alix, XPO-5, DICER) were significantly higher in PRE compared to YNG ($p<0.05$), and further increased with resistance training (POST, $p<0.05$). Expression of Rab27a, a marker of exosome trafficking, was significantly higher in PRE ($p<0.05$) but did not respond to training. In conclusion, here we show alterations in circulating EXO content and cargo with age and resistance training partially restores the values to a younger phenotype.

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List of acronyms

AB – Apoptotic bodies

ALIX – ALG-2 interacting protein-X

ApoA1 – Apolipoprotein A1

CD9 – cluster of differentiation 9

CD81 – cluster of differentiation 81

CD63 – cluster of differentiation 63

CKD – chronic kidney disease

EV – Extracellular vesicles

ESCRT – Endosomal sorting complexes required for transport

EM – Electron microscopy

eWAT – Epididymal white adipose tissue

ILV – Intraluminal vesicles

MVB – Multivesicular bodies

mtDNA – mitochondrial DNA

mTORC1 – mammalian target of rapamycin complex 1

NTA – Nanoparticle tracking analysis

PI3K/Akt – Phosphatidylinositol-4,5-bisphosphate 3-kinase/Protein kinase B

SEC – size exclusion chromatography

SkM – skeletal muscle

TSG101 – Tumor susceptibility gene 101

UC – ultracentrifugation

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OVERVIEW

The world population continues to grow at an unprecedented rate. By 2050, ~25% of the population will comprise of individuals older than 65 years of age (Wortley et al., 2017). As a result, an increase in all age-related chronic diseases is predicted (Christensen et al., 2009). Sarcopenia is the age-related loss of muscle mass, function, and strength and the condition is strongly associated with trips and falls, frailty, and loss of independence (Bowen et al., 2015). These consequences lead to a decrease of quality of life and have major financial implications on the public health care system (Goates et al., 2019). Exercise, and specifically resistance training, has been well reported in the literature to be at the front-line of prevention and treatment against age-related declines in skeletal muscle (Morton et al., 2018). Exercise in the form of resistance training induces hypertrophy within aged skeletal muscle fibres, improving their contractile activity and force production generated by the muscle fibre (Bowen et al., 2015). This leads to functional and strength improvements necessary to mitigate sarcopenia. In part, the functional benefits are underlined by the molecular adaptations that occur with resistance training (Ziaaldini et al., 2017). Specifically, increased cross sectional area, reductions in fat infiltration, as well metabolic improvements of glucose handling and increased insulin sensitivity can lead to physiological benefits seen with resistance training (Bowen et al., 2015). Although the benefits of exercise are evident, the underlying mechanism of exercise and resistance training on a systemic level are not well understood. In the past two decades, it has been shown that skeletal muscle releases factors in circulation during exercise which are thought to play a beneficial role (Murphy et al., 2020). These factors include cytokines, proteins, and metabolites, altogether termed “myokines” which exert autocrine and paracrine functions on peripheral tissue and thereby hypothesized to elicit multisystemic benefits throughout the body (Murphy et al., 2020). Classic

myokines include IL-6, Irisin, FGF21 which act on brain, liver, and adipose tissue to improve cognition and memory, glucose metabolism and improve browning and increase energy expenditure (Bente K. Pedersen, 2013). Recently, among the secretome of skeletal muscle, extracellular vesicles (EVs) have also been shown to be released during exercise (Brahmer et al., 2019). Contrary to individual protein based myokines, EVs consist of small, nanosized membranous vesicles, produced from all cell types and contain molecular cargo (including myokines) to recipient cells where they induced functional changes and thereby mediate intercellular communication (Colombo et al., 2014). Part of the cargo that exosomes carry include nucleic acids, proteins, lipids, and metabolites and by delivering this cargo, they have shown to be involved in various aspects of cell biology ranging from regulation of immune response, waste management, cellular migration, and role in metabolic and cardiovascular disease (Kalluri & LeBleu, 2020). Given their role in intercellular communication, and the functional adaptations that are known to occur with exercise at the skeletal muscle level and throughout the body in different tissues, exosomes may potentially underly the mechanism behind the multi-systemic benefits of exercise. In addition, aging is known to alter intercellular communication and exosomes may provide insight how aging affects the exosome response and whether resistance training may normalize it. Currently, there are numerous gaps in the literature for optimal characterisation of circulating exosomes in young and old individuals (Rong et al., 2020). Understanding the cellular process that drive exercise-induced adaptations in old individuals is beneficial because it will increase our knowledge of sarcopenia and how the exercise-induced exosome response is affected in sarcopenia (Rong et al., 2020).

LITERATURE REVIEW

Biological maladaptations with aging

Biological aging is the time-dependent, slow, and progressive deterioration of cellular fitness that results in tissue and organ functional decline (Sarkar & Fisher, 2006). Although the process of aging is complex, there are several distinct characteristics that are attributed to the underlying cause. In a landmark review, Lopez-Otin and colleagues (2007) identify i) genomic instability, ii) telomere attrition, iii) epigenetic alterations, iv) loss of proteostasis, v) deregulated nutrient-sensing, vi) mitochondrial dysfunction, vii) cellular senescence, viii) stem cell exhaustion, and ix) altered intercellular communication as the classical hallmarks of cellular aging (López-Otín et al., 2013). Importantly, these dysregulated factors have been causally linked to aging in that they occur over the lifespan, they accelerate aging when upregulated; and lastly, when ameliorated, they are shown to delay aging and increase survival (López-Otín et al., 2013). The combination of all these cellular dysfunctions can be a driving force through the progression of age-related diseases and make aging the single most prevalent risk factor in all other major chronic diseases (Khosla et al., 2020). Due to the many different and independent factors that involve aging, it is important to find therapeutic strategies that are cost effective, and beneficial on a multisystemic level, by delaying and attenuating aging throughout the body (Breen & Phillips, 2011).

Sarcopenia: age-related muscle loss

Sarcopenia is the age-related loss of muscle mass, function, and strength (Cruz-Jentoft et al., 2019). Coined by Rosenberg and colleagues in 1989, the term sarcopenia originates its roots from Latin which literally translates to “the lack or deficiency of the flesh” (Rosenberg, 1989). After being formally recognized as a disease in 2016 (Cao & Morley, 2016), sarcopenia is now diagnosed based on criteria set forth by the European Working Group on Sarcopenia in Older People (Cruz-Jentoft et al., 2019). The identification of sarcopenic individuals is done through a number of

designated criteria including low muscle strength, low muscle mass quantity and/or quality and limited physical performance. Sarcopenia has been reported in upwards of ~30% of the population in community-dwelling older adults ranging 59 to 86 years (Cruz-Jentoft et al., 2019). Aging and the growing population present with a financial burden on the health care system. More than $60B is spent in treatment and management of age-related chronic diseases. In addition, the estimation that in 2050, ~25% of the population will be over 65 years of age will only worsen this financial burden. Specifically, the cost of disability and sarcopenia, driven by loss of skeletal muscle mass and function can not be ignored (Goates et al., 2019). Patients with sarcopenia when hospitalized have 2-fold increase in cost of treatment than non-sarcopenic individuals (Steffl et al., 2017). With the increasing age of the world population, models suggest a concomitant increase in cases of sarcopenia. Consequently, understanding the underlying causes of sarcopenia are of critical importance. Decreased skeletal muscle mass and function, overall frailty, increase risk of falls and fractures and along with an increased risk for all other age-related co- morbidities has major economic implications for health care systems worldwide when sarcopenia and age-related diseases are left untreated (Cruz-Jentoft et al., 2019).

Pathophysiology of sarcopenia

The loss of muscle mass and strength in aged individuals is attributed to the progressive decrease in myofiber cross-sectional area- or skeletal muscle atrophy and the loss of skeletal muscle fibres (Ryall et al., 2008). The reduction in muscle fibre area and number leads to a decreased production in force and strength impairments seen with aging. In addition, the quality of existing muscle fibres is reduced due to infiltration of fat within the muscle fibre reducing the contractility of the muscle fibre and its overall force production (Bowen et al., 2015). The underlying mechanism(s) of skeletal muscle atrophy with aging are complex and multifactorial. A loss in the anabolic stimulus

of dietary protein in aged muscle blunts the response to muscle protein synthesis and contributes to the increased rate of protein breakdown in aged muscle, called "anabolic resistance" (Breen & Phillips, 2011). An imbalance in protein breakdown to protein synthesis contributes to the decreased muscle fibre area and strength. Other factors including, a loss of neuromuscular junction stability in aged muscle leads to progressive decrease in muscle innervation which leads to decreased muscle use and subsequently loss of mass, function and strength (Gonzalez-Freire et al., 2014). Furthermore, mitochondrial dysfunction has been reported, including decreased mitochondrial content in fibres of aged skeletal muscle (Murgia et al., 2017). In addition, impairments in the regenerative capacity of aged muscle, particularly reduced satellite cell pool and capillarization, are thought to contribute to the accelerated loss of muscle mass seen with aging (Joanisse et al., 2017). Altogether, there are many different factors contribute to the etiology of sarcopenia. Therefore, it is important to study and find ways to delay and offset age-related muscle loss. A cost-effective form of treatment that combats sarcopenia, improves muscle mass function, and strength and also shown to target aging hallmarks and potentially confer anti-aging benefits across all tissues is exercise and increased physical activity (Rebelo-Marques et al., 2018).

Exercise against aging and sarcopenia

This past century has established the beneficial effects of life-long exercise. Initial, ground-breaking work by Morris and colleagues (1953) were the first to show that increased physical activity and exercise are beneficial for cardiovascular health and longevity, reducing the risk of all-cause mortality by 20%-50% (Morris et al., 1953; Paffenbarger et al., 1978). Exercise has shown to be beneficial against all forms of cancer, metabolic disease, and other age-related cognitive diseases (B. K. Pedersen & Saltin, 2015; Warburton et al., 2006). Specifically, resistance training has shown to be beneficial in preventing age-related skeletal muscle loss and improve

overall muscle quality and function (McKendry et al., 2020) as adults who participated in lifelong resistance training showed immense benefit in this regard compared to age-matched controls. The safety and efficacy of resistance exercise was revealed by Fiatarone and colleagues (1994), showing that resistance exercise can combat age-related skeletal muscle loss (Fiatarone et al., 1994). More recently, the literature suggests that resistance exercise training increases fat-free mass in older adults, fibre cross sectional area (CSA) and mid-femur CSA (Morton et al., 2018). In part, the molecular adaptations of resistance exercise that culminate with increased hypertrophy are as a result of the upregulation of the phosphatidylinositol 3-kinase (PI3-k)–Akt–mammalian target of rapamycin (mTOR) signaling cascade which monitors protein synthesis and therefore cell growth (Hawley, 2009). The mTOR complex is sensitive to environmental stimuli, such as exercise and protein ingestion, and activates ribosomal protein S6 kinase – which upregulates translation of myofibrillar proteins. Although this effect may be partially blunted in aged muscle (anabolic resistance), resistance training adaptations can improve signaling and elicit muscle hypertrophy and improve strength and function. In addition, metabolic improvements including glucose handling and insulin sensitivity are evident with resistance exercise in older adults leading to better fuel metabolism and overall health benefits (Consitt et al., 2019).

Despite the overwhelming evidence of exercise being beneficial against sarcopenia and other age-related disease, barriers affecting people from exercising and adhering to a training program prevail. Amongst these barriers, access to gyms in low socioeconomic areas may impact physical activity levels in older adults. Therefore, the reduction of the barriers to exercise, providing evidence that home-based resistance training programs can be beneficial, will also increase the levels of physical activity amongst elderly and help prevent biological complications, as well as offset financial costs (Merchant et al., 2021).

Skeletal muscle as an endocrine organ

Pioneering work in the discovery of skeletal muscle as a secretory organ have suggested that exercise release factors in circulation, which may underly the multisystemic benefits of exercise (Bente K. Pedersen, 2013). The idea that skeletal muscles possess a "humoral factor" that is released in circulation during contraction stems from work examining the production of glucose from the liver (Goldstein, 1961). In early 2000, it was discovered that skeletal muscle has secretory capacity, releasing IL-6 in circulation following an acute bout of endurance exercise (Steensberg et al., 2002). Since that discovery, over >300 proteins have been identified, part of the exercise-induced skeletal muscle secretome, collectively called "myokines" (Murphy et al., 2020). Part of this secretome, extracellular vesicles and exosomes have been receiving a lot of interest given that they function in intercellular communication, and they may potentially play a role in intercellular communication between skeletal muscle and other cell types and thereby facilitate the benefits of exercise in tissues across the body (Vechetti, Valentino, et al., 2021).

Many of these studies investigating the secretory capacity of skeletal muscle during exercise primarily utilized endurance exercise as the mode of stimulus. Given the benefits of resistance training on skeletal muscle, investigating the circulatory factors and how they change with training warrants further investigating. In addition, given that altered intercellular communication is a hallmark of aging, it is also of interest to identify alterations in circulating extracellular vesicles, as agents of intercellular communication and aim to see if exercise and resistance training, is able to correct these alterations. Being able to quantity and measure the response of resistance exercise on circulating exosomes may contribute to the mechanism that underlies the benefits of resistance exercise against age-related muscle loss. It may also open avenues for optimal exercise intensity.

Lastly, it may possibly open avenues in identifying specific exercise-induced exosomes with therapeutic potential.

Extracellular vesicle biology

Extracellular vesicles (EVs) are membranous vesicles that are found to be released in all biological fluids, by virtually all cell types (Colombo et al., 2014). They are thought to carry wide variety of cellular cargo from donor cells to recipient cells and thereby mediate intercellular communication (Colombo et al., 2014). EVs are largely separated into three major subpopulations based on their size and origin of production. Apoptotic bodies (ABs) pertain to the largest vesicles with a size diameter > 1000 nm (Battistelli & Falcieri, 2020). They are released from dying cells during the apoptotic process and carry cellular remnants and potentially intact organelles (Battistelli & Falcieri, 2020). Secondly, microvesicles are mid-sized vesicles which although don't generally have an upper size limit, they most vary between 400-1000 nm. Microvesicles, in line with other EVs, traffic and carry cargo to recipient cells. They are greatly implicated in tumour progression as it has been shown that tumour cells release and deliver oncogenic growth factor receptors to non-cancerous cells via the shedding of microvesicels (Al-Nedawi et al., 2008).

Exosomes are the smallest amongst EVs, ranging in size between 30-100 nm (Colombo et al., 2014). They are distinct from other EVs based on their size, as well as their origin of production (Kalluri & LeBleu, 2020). Unlike ABs and MVs, which both result from the shedding of the plasma membrane, exosomes originate from the endosomal pathway within the donor cell (Colombo et al., 2014). The earliest evidence that cells secrete vesicles in the extracellular milieu came from Pan and Johnson in 1983 (Pan & Johnstone, 1983). While aiming to explain the process by which erythrocytes lose the ability to produce hemoglobin during maturation – they stumbled upon cup shaped vesicles – secreted by reticulocytes carrying transferrin receptor – which binds

iron and internalized within the cell and used it to produce heme (Pan & Johnstone, 1983). This provided evidence of cup shaped vesicles- later termed ‘exosomes’- which cells can release and thought at the time, to discard cellular content.

Exosome biogenesis occurs via the double invagination of the plasma membrane- resulting in intraluminal vesicles (ILVs) within a multivesicular bodies (MVBs) (Colombo et al., 2014). The first invagination initiates the process and forms an early endosome. The early endosome is then matured into a late endosome or MVB containing many ILVs- the endosomal precursor to exosomes. The process is intricate requiring the fine orchestration of four protein complexes, endosomal sorting complex required for transport (ESCRT) and associated proteins, VPS4, VTA1, and ALIX (Vietri et al., 2020). Briefly, ESCRT-0 captures ubiquitinated cargoes, that are destined to be sorted and trafficked out of the cell. ESCRT-I and -II facilitates the invagination of the endosomal membrane and the formation of the ILV, and finally ESCRT-III drives the scission of the ILV membrane. TSG-101 is a crucial protein of ESCRT-I which facilitates recruitment of its complex, whereas ALIX is involved in the recruitment of ESCRT-III. Given their role in ILV and exosomes biogenesis, TSG-101 and ALIX are both used as exosome markers (Théry et al., 2018). In addition to ESCRT-dependent mechanism, studies of silenced ESCRT proteins can still secrete exosomes, suggesting of an ESCRT-independent mechanism of exosome biogenesis. Specifically, exosome membrane proteins called tetraspanins have been shown to independently sort proteins in ILVs and subsequent exosomes. In an ESCRT-independent fashion, tetraspanins have been shown to be involved in exosome biogenesis, as well as in cargo selection and uptake of exosomes in recipient cells. Lastly, given their location and function, tetraspanins, namely CD9, CD81 and CD63 are all frequently used exosome markers.

The ILV-containing MVBs are then transported to the plasma membrane where they fuse and releases the exosome populations to the extracellular space. The evidence of multiple mechanisms of biogenesis, undeniably adds to the heterogeneity of different subpopulations of exosomes. Within the same cell source, different types of exosomes may function to communicate differently (Kalluri & LeBleu, 2020). In addition, given that during exercise, metabolic demand at the muscle increases by 100-fold (Murphy et al., 2020), this acute metabolic stress stimulus may potentially affect exosome biogenesis, exosome cargo and release.

Exosomes and cargo: microRNAs

In 2007, in a landmark paper, Valadi and colleagues provided evidence to suggest that miRNA species are shuttled and packaged in exosomes (Valadi et al., 2007) . This breakthrough shifted the perspective on exosome and extracellular vesicles, as the prevailing idea at the time was that exosomes primarily functioned as means of discarding unwanted cellular content. However, by exchanging miRNA and mRNA- exosomes were then thought to be capable of modifying protein expression in recipient cells.

MicroRNAs are small single stranded, noncoding RNA species that modify the expression of proteins within a cell by targeting their respective mRNA (Bartel, 2009). They silence target mRNA through three mechanisms: 1) by cleaving target mRNAs, 2) destabilizing them by reducing the poly-A tails and thereby promoting degradation and, 3) interfering with ribosome machinery and reducing translation (Bartel, 2009). They are produced in specific hairpin regions of transcripts that loop back on themselves known as pri-miRNA (Winter et al., 2009). While in the nucleus, pri-miRNA are then cleaved by the microprocessor complex of Drosha and DGCR8 (Pasha) making pre-miRNA, which is then transported outside of the nucleus through Exportin-5 protein (Winter et al., 2009). In the cytoplasm, an RNA nuclease called Dicer, cleaves the pre-

miRNA into the mature miRNA (Winter et al., 2009). Once in its mature form, several RNA binding proteins, including ALIX, have been suggested to be involved in the sorting of miRNAs into exosomes (Fabbiano et al., 2020).

Depending on their function, specific miRNAs can be found at different expression levels across tissue. Several miRNAs have recently been found to be enriched in skeletal muscle, collectively called myomiRs. Their expression levels are directly related to their function, as they are all involved in the regulation of healthy skeletal muscle fibre homeostasis (Güller & Russell, 2010). Specifically, miR-133a is shown to be upregulated during proliferation, while miR-1 and -206 are both involved in differentiation of myoblasts to myotubes.

Acute exercise-induced exosomes

Over the past decade, there has been a rise in the interest of exosomes and exercise. Frühbeis and colleagues were the first to report that following a maximal exercise bout – the levels of exosome-related proteins increased in circulation (Frühbeis et al., 2015). More recently, Brahmer and colleagues (2019) performed a study with considerable improvements in their methodology of exosome isolation and subsequent verification (Brahmer et al., 2019). These methodological improvements included utilizing two different methods of exosome isolation, size exclusion chromatography which, filters the plasma and isolates its components based on size, and immunoaffinity-based method by isolating exosomes using antibodies against their surface markers (e.g., CD9, CD81, CD63). A wide panel of exosome protein markers all saw significant increases following an acute bout of exercise. In addition, they were also first to report that cell surface markers specifically corresponding to circulatory immune cells were found on exosomes, suggesting a contribution of circulating immune cells to the exercise-induced release of exosomes. Interestingly, the authors also reported minimal contribution from skeletal muscle to their

circulating exosome pool based on protein levels of α-sarcoglycan (SGCA) in their SEC-derived ELV isolates. SGCA is a component of the dystrophin-glycoprotein complex which mediates interactions between the muscle fibre and the extracellular matrix. Given its location in proximity to the membrane of the muscle cell, it is has been hypothesized that SGCA may be used as a marker for EVs derived from skeletal muscle (Guescini et al., 2015). Skeletal muscle is the largest organ in the body and given that it is under the most metabolic stress during exercise, it is unlikely that its contribution to the circulating pool of exosomes is minimal. What may be underlying this apparent lack of contribution of ELV from skeletal muscle may lie within the inherent limitation in SEC isolation. Indeed, an established limitation in SEC isolation as a means of exosome isolation is the low protein yield due to high specificity, and therefore it is possible that signal of SGCA is reduced in relation to exosome-specific proteins. Studies performed *in vitro* examining human skeletal muscle primaries suggests that myoblasts are capable of releasing exosomes and involved in intercellular communication (Le Gall et al., 2020; Mytidou et al., 2021). Furthermore, Guescini and colleagues also showed they were able to successfully isolate α-sarcoglycan^{+} -exosomes in circulation (Guescini et al., 2015). Upon characterization of their miRNA content, the authors found them to be enriched in skeletal muscle specific miRNAs, suggesting their origin of release. At the level of the skeletal muscle, Garner and colleagues investigated the effect of an acute bout of two different modes of exercise- cycling and resistance by taking biopsies before and after the acute bouts (Garner et al., 2020). They reported an upregulation in the gene expression of ALIX, EXPORTIN-5 and DICER- proteins involved in exosome biogenesis and miRNA processing (Garner et al., 2020). Other proteins involved in exosome biogenesis pathway, such as TSG-101, and ESCRT-III related protein, VPS4A showed no changes at the mRNA level. Despite the small number of studies, there is some evidence to suggest that exercise induces an exosome

response, not only in skeletal muscle, but also in other cell types. However, much work is needed in identifying and establishing a feature or linking functional benefits to exercise-induced exosomes.

Exosomes following chronic training

The number of studies investigating the effect of training on exosome release is lacking. To our knowledge, in human participants, there has only been a single study investing the effect of resistance training on circulating exosomes. In this training study, Estébanez and colleagues (2021) investigated the change in protein markers of plasma-derived exosomes with resistance training, and age, as compared to young controls (Estébanez et al., 2021). The authors saw decrease in CD63 protein markers with training of old participants and no significant differences of exosome protein markers compared to young at baseline (e.g., CD9, CD81, CD63, Flot-1). In addition, they showed no difference in plasma levels of miR-146a, a microRNA known to be a mediator of senescence-associated inflammation seen in old age (Olivieri et al., 2015). The authors claimed that the reduced expression of CD63 may suggest a decrease in the levels of low-chronic systemic inflammation that presents in old age. First, the authors have used ultracentrifugation as the means of exosome isolation and when dealing with blood as biological fluid, UC has been shown to co-isolate a significant amount of blood protein contaminants which may negatively affect the validity of the results (Patel et al., 2021).

Contrary, in mice studies of chronic exercise training, most have reported increases in exosome particles. Bei and colleagues (2017) found that 3-weeks of swimming training in mice increased the particle concentration of their serum-derived exosome isolates by 1.85-fold, as determined by nanoparticle tracking analysis (Bei et al., 2017). In addition, they saw increases in the CD63 protein marker, measured with immunoblotting of exosome isolates. Similarly, Bertoldi and

colleagues (2018) found that 2-weeks of exercise training on rats increased CD63 level proteins across all young and aged rats. In addition, they reported that aged-rats had significantly lower CD63 protein levels 1-hour post the last training session as compared to young rats. (Bertoldi et al., 2018). These results indicate an effect of exercise on exosome marker levels.

The challenge for an optimal characterization of the exosome response during exercise remains. Part of the challenge is due to the many isolation protocols that exist in the literature. Currently over 10 different methods of isolation have been used, with the most common being ultracentrifugation (Royo et al., 2020). Recently, the usage of size exclusion chromatography has increased significantly (Royo et al., 2020). Regardless of isolation method, future studies investigating the effect of both acute and chronic exercise on circulating exosomes should aim to follow proper guidelines in isolating and characterizing exosomes (Théry et al., 2018). The lack of proper exosome characterization in the literature has increased the inter-variability between studies and ultimately have led to inconsistent and variable findings. More standardized studies are warranted to better elucidate the function of exosomes and how they pertain to age-related skeletal muscle atrophy, and their involvement in exercise-induced multisystemic benefits.

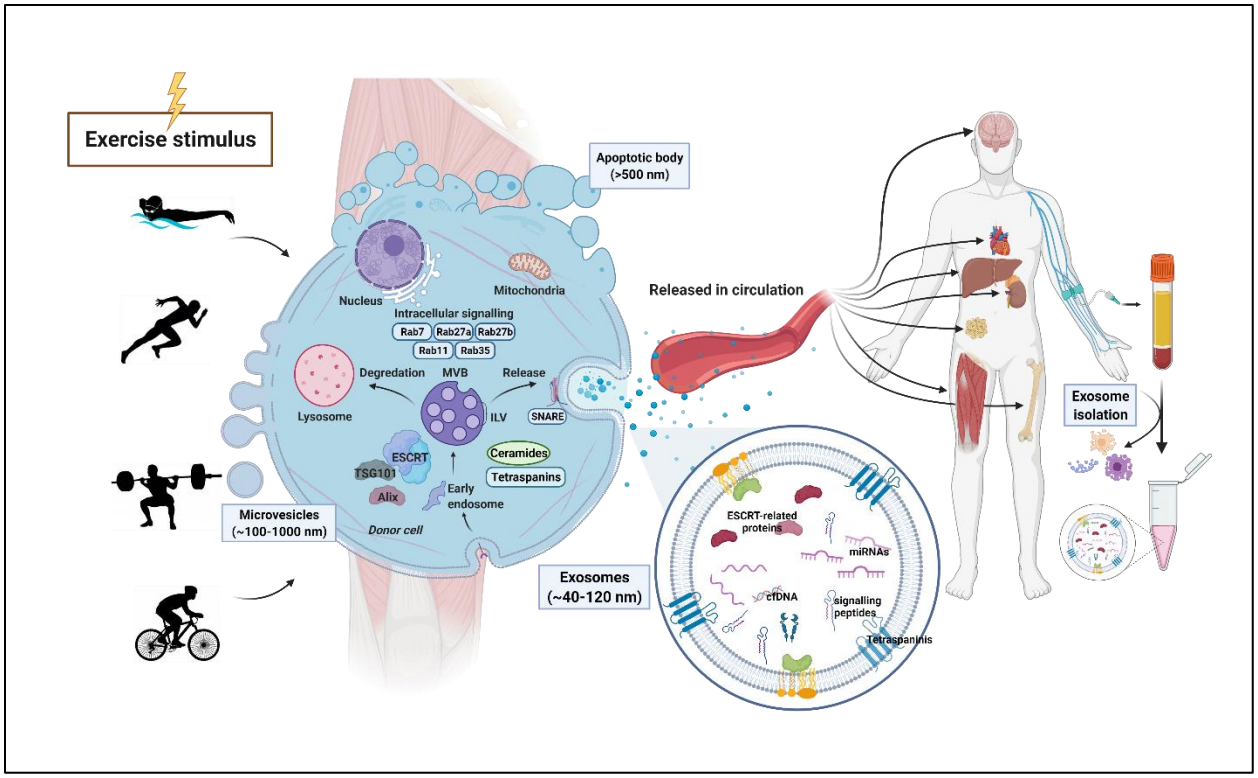

Figure 1. Proposed schematic mechanism of exercise-induced exosome biogenesis and release from skeletal muscle in circulation.

Exosome-encapsulated miRNAs and exercise

In many functional studies of exercise-induced exosomes, miRNAs which are found to be contained within exosomes, and facilitate intercellular communication. For example, Fry and colleagues (2017) (Fry et al., 2017) showed that during mechanical overload-induced hypertrophy, myogenic progenitor cells (MPCs) release exosomes and regulate the muscle extracellular matrix by inhibiting collagen deposition through miR-206. Although more work is needed, this study provided evidence to suggest that exosomes and miRNAs are involved in local cellular communication of ECM remodelling to facilitate muscle fibre growth. In addition, exosomes and miRNAs have been proposed to be involved in other cellular signaling during exercise-induced hypertrophy. Vechetti and colleagues showed that following a hypertrophic bout of exercise, miR-1 decreased in skeletal muscle and concurrently increased in serum EVs (Vechetti et al., 2021). In

addition, the authors found that serum EVs were preferentially taken up by epididymal white adipose tissue (eWAT). Within eWAT, they identified Tfap2α as a target of miR-1, inhibiting its translation and thereby inducing lipolysis (Vechetti et al., 2021). This study provides further evidence of how skeletal muscle communicates with other tissues via EVs and miRNAs. With respect to cardiac tissue, there has been some studies to suggest that long term exercise-derived EVs can be beneficial for cardioprotection. Specifically, Hou and colleagues subjected rats to swim training for 4-weeks and isolated EV from their plasma and performed miRNA sequencing (Hou et al., 2019). The authors found that miR-342-5p was differentially expressed in exercised rats, compared to control littermates. Additionally, EVs from exercised rats provided significant protection against myocardial ischemia/reperfusion injury. Interestingly, when inhibiting miR-342-5p, the protection was blunted. Together, these studies suggest that miRNAs during exercise facilitate inter-cellular communication and mediate exercise-induced benefits in different tissues.

miRNAs in skeletal muscle

In skeletal muscle, the effect on miRNA expressions with respect to exercise-induced hypertrophy or even dysregulation of skeletal muscle function that occur with sarcopenia are yet to be fully elucidated. Drummond and colleagues investigated the effects of a potent anabolic stimulus (resistance exercise + EAA ingestion) on skeletal muscle of young and old individuals (Drummond et al., 2008). At basal levels, there was no myomiR level differences between young and old. Following the acute bout, miR-1 levels decreased only in young individuals, while the other myomiRs (miR-133, -206) did not significantly change. Drosha and Exportin-5 however, significantly increased following exercise in old individuals suggesting an upregulation in the miR biogenesis pathway, although the myomiR level expressions reported do not increase despite the

upregulation in their biogenesis pathway. An explanation for this discrepancy could be the timepoints at which the biopsies are taken. 3h post could be too late to capture the response.

Some miRNAs have not been identified as myomiRs but undoubtably seem to affect skeletal muscle. Particularly, miR-23 and -27 have been shown to be implicated with skeletal muscle atrophy in CKD mice (Wang et al., 2017; Zhang et al., 2018). Studies where overexpressing miR-23 and -27 have shown to attenuate muscle loss and improve grip strength in CDK mice. Authors also saw a decrease in the expression of proteins associated with muscle atrophy, suggesting that miR-23 and -27 target atrophy related genes and inhibit their transcriptions (Wang et al., 2017). Other, non-myomiR related miRNAs include miR-155, which has been proposed to facilitate muscle regeneration through mediating pro- and anti-inflammatory macrophages (Nie et al., 2016). These studies suggested that there are many miRNAs that are implicated in skeletal muscle function. Studies investigating the miRNA profile of aged individuals, and the effect that resistance training has on them are further warranted in order to deduce and potentially discover further mechanisms that target sarcopenia-related atrophy and how we can prevent it.

Exosomes and miRNA: Altered with aging?

The potential therapeutic use of circulatory factors derived from healthy or young organisms have been hypothesised for many years (Loffredo et al., 2013). Parabiosis experiments of anatomically joining two mice and thereby connecting their circulatory systems have shown to improve age-related complications (Conboy et al., 2005), strengthening the evidence of potential therapeutic circulatory factors. Whether these “youthful” factors originate from skeletal muscle has yet to be fully elucidated.

In humans, there is a limited number of studies investigating the effect of age on exosomes. Specifically, Picca and colleagues showed that sarcopenic individuals had a proposed lower

amount of exosomes in circulation based on CD63 and CD9 protein markers (Picca et al., 2020). In addition, the authors reported that exosomes isolated from sarcopenic individuals, contained a lower content of mitochondrial proteins as compared to non-sarcopenic individuals. In addition, Lazzo and colleagues showed similar findings, reporting that in EVs isolated from individuals aged 30-64y, mtDNA levels decrease with age (Lazo et al., 2021). Furthermore, the authors found that aged-plasma derived EVs negatively affect oxygen respiration and decrease maximum mitochondrial respiration in HeLa cells (Lazo et al., 2021). It is important to note that in both studies, the use of ultracentrifugation to isolate plasma-derived exosomes may yield co-isolated contaminants, thereby affecting the validity of the results. Nevertheless, the characterisation of young and old- plasma derived exosomes is important and warrants further investigation with more rigorous methodology.

In conclusion, exosomes have shown to be a promising therapeutic strategy- due to their involvement in intercellular communication in both health and disease. Whether they are exercise-induced or derived from young plasma, there is some evidence to suggest that their delivery of cargo may provide some benefits.

Purpose, Objectives and Hypothesis

There are numerous gaps in the literature for optimal characterisation of circulating exosomes in young and old individuals. In addition, how these circulating exosomes and their cargo respond to resistance training are also not well understood. Exosomes represent an exciting new field of research whereby their investigation may provide us with insight on understanding underlying mechanism of the benefits of resistance training in an aged population. Understanding the cellular process that drive exercise-induced adaptations in old individuals is beneficial because it will increase our knowledge of sarcopenia and how the exercise-induced exosome response is affected in sarcopenia. Therefore, the purpose of this thesis project is to investigate the effect of 12-week resistance training program on the skeletal muscle and circulatory exosome response in old participants. The specific objectives are:

1. Characterize circulatory exosomes of aged participants, as compared to young controls, before and after 12-week resistance training program by isolating plasma-derived exosomes utilizing western blotting, nanoparticle tracking analysis and electron microscopy

2. Measure the miRNA profile expression through RT-PCR of isolated circulatory exosomes as potential indicators of exosome function.

3. Investigate the contribution of skeletal muscle as a potential source of miRNA-containing exosomes by taking muscle biopsies and through western blotting and RT-PCR and immune capture of skeletal muscle-specific exosome subpopulation

We hypothesize that aging alters the exosome release and cargo. Resistance training may normalize the altered exosome response and cargo in circulation and locally in the skeletal muscle based on functional adaptations known to occur with exercise.

MANUSCRIPT

Circulatory and skeletal muscle exosome response in old participants following a 12-week resistance training program

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Introduction

Sarcopenia is the age-related loss of muscle mass, function, and strength (Larsson et al., 2019) and is strongly associated with trips and falls, frailty, and loss of independence (Bowen et al., 2015) in elderly individuals. These consequences lead to a decrease of quality of life and have major financial implications for the public health care system (Goates et al., 2019). Exercise and specifically resistance training has been well reported in the literature to be at the front-line of prevention and treatment against age-related muscle loss (Morton et al., 2018). Exercise in the form of resistance training induces hypertrophy within aged skeletal muscle fibres, improving their contractile activity and force production generated by the muscle fibre (Bowen et al., 2015). This leads to functional and strength improvements, as well as overall health, which altogether are protective against sarcopenia and other age-related chronic diseases (Bowen et al., 2015; B. K. Pedersen & Saltin, 2015). Although the benefits of exercise are evident, the underlying mechanism of exercise and resistance training on a systemic level are not well understood. Exercise factors released from, termed "myokines", released in circulation during exercise and engaged in tissue cross talk have established the role of skeletal muscle as an endocrine organ (Murphy et al., 2020). In addition, the term "exerkines" includes all encompassing exercise factors that are released in circulation by any cell, not exclusive to skeletal muscle. Recently, among these factors, extracellular vesicles (EVs) have also been shown to be released during exercise (Brahmer et al., 2019). EVs are nanosized membranous vesicles, produced from all cell types containing molecular cargo to recipient cells and thereby mediating intercellular communication shown to be involved in various aspects of cell biology ranging from regulation of immune response, waste management, cellular migration, and role in metabolic and cardiovascular disease (Colombo et al., 2014; Kalluri & LeBleu, 2020). Part of the exosome cargo include small RNA species, called microRNAs that

inhibit translation of target proteins and thereby affecting protein expression and function in target tissues (Valadi et al., 2007). Given their role in intercellular communication, and the functional adaptations that are known to occur with exercise at the skeletal muscle level and throughout the body in different tissues, exosomes may potentially underly the mechanism behind the multisystemic benefits of exercise (Vechetti et al., 2021). In addition, aging is known to alter intercellular communication and exosomes may provide insight how aging affects the exosome response and whether resistance training may normalize it. Currently, there are numerous gaps in the literature for optimal characterisation of circulating exosomes in young and old individuals (Rong et al., 2020). Therefore, in our current study, we characterized circulatory exosomes isolated with size exclusion chromatography and ultracentrifugation and their miRNA expression in old participants as compared to young controls before and after a 12-week resistance training program. In addition, we explored the contribution of skeletal muscle as a potential source of exosomes in the circulatory pool. Our results indicate that aging and exercise training alter different exosome subpopulations and miRNA cargo. Lastly, our results suggest that skeletal muscle may be involved in aging and exercise-induced changes of exosome response.

Methods

Ethical approval, study participants and training program. The study was conducted as part of a larger project with published primary outcomes (Nilsson et al., 2020). All methods and procedures utilized within the project were approved by the Hamilton Integrated Research Ethics Board (2018-4656-GRA). Study participants included exclusively males and were recruited within the Greater Hamilton Area through newspapers and poster advertisements aimed at targeting sedentary free living elderly individuals. For the current thesis project, only participants who completed the study were included in the analysis. In addition, in order to investigate age differences, young individuals were recruited for the study but did not undergo the training program.

The training program consisted of 3X week of progressive, whole body resistance exercises using elastic bands, in the setting of their homes. The participants were given detailed instructional videos on the exercise, sets and reps that they were to perform. Briefly, the participants were instructed to perform each exercise with correct form for 3 sets X 10-15 repetitions for each exercise. Resistance force of elastic bands was progressively increased to ensure that adaptations occur (starting with yellow, 1.32 kg; red, 1.77 kg; green, 2.27 kg; blue, 3.22 kg; and black, 4.40 kg). Compliance to the training program was checked using email and phone calls to the participants, bi-weekly, as well as they were instructed to keep an exercise log to make sure they adhered to the training program.

Blood draws and exosome isolation. Participants arrived at the laboratory following an overnight fast, approximately one week after their last training session. Blood draws were taken from the antecubital vein into blue-citrated plasma (Lacroix et al., 2013) following an overnight fast for young and old participants (PRE) and following training (POST). The blood was immediately spun for 15 minutes at a speed of 2000*g* to obtain platelet-free plasma (PFP). PFP is then subjected to a

centrifugation spin of 20,000*g* to remove large EV, such as apoptotic bodies and microvesicles. The supernatant containing small EVs is then loaded onto size exclusion chromatography (SEC) columns (qEV Izon). Prior to loading the samples, the SEC columns are first washed thoroughly with 20mL of PBS as they are calibrated and reach room temperature. Upon calibration, 500 µL of PFP supernatant is added and EV-rich fractions, consisting of fractions #7-9, are captured and pooled. Following that, the samples are concentrated down with UC, normalized to volume, and then stored in -80ºC, to be used later in protein quantification and RNA extraction. The columns were washed thoroughly between each subject and their usage was standardized to minimize variability between samples.

Skeletal Muscle biopsies. Skeletal muscle biopsies were taken following an overnight fast for all subjects, before and after training intervention as previously described (Tarnopolsky et al., 2011). Local anesthesia (2% xylocaine) was applied to the vastus lateralis, taking care not to infiltrate the skeletal muscle and with the use of a 5-mm Bergstrom needle and manual modified for suction, approximately 150 mg of muscle biopsy was obtained. The samples were inspected for any visible fat tissues, snap frozen and stored at -80ºC to be used for later analysis.

Western blotting. Controlling for equal volume between subjects and timepoints, the SEC samples were concentrated with UC overnight and the liquid pellet is resuspended in equal volumes of RIPA buffer supplemented with phosphatase/protease inhibitors. Additionally, 6X Laemmli buffer is added to the samples, which are subsequently heated at 95ºC for 5 minutes, to fully denature the proteins. Upon denaturing, the samples are then loaded onto 2-20% SDS-PAGE and all EV proteins are separated by size. They are then transferred onto 0.2 µm Nitrocellulose membranes and incubated overnight at 4 ºC with antibodies against specific proteins (Table 1). Following overnight incubation, conjugated secondary antibodies are added for 1 hour at RT and detection

of protein expressions is done the use ECL substrate reagents and ChemiDoc Imaging system (BioRad).

Nanoparticle tracking analysis. Pooled EV fractions were diluted in 0.05% DPBS/ultrapure water (1:200) and analyzed for size and concentration by ZetaView PMX 110 operating with software version 8.04.02 (Particle Metrix, Meerbusch, Germany). As detailed previously (Helwa et al., 2017), but with minor modifications, an automated quality control, including a cell status check, instrument alignment, and focus optimization, was performed prior to running any samples. Accuracy and consistency were confirmed by applying nanobead standards (Applied Microspheres) to the cell unit prior to every sample run. Two mL of the diluted EVs was injected into the cell, followed by assessment of particle size/concentration at 11 different positions, with three cycles of readings per position. Pre-acquisition parameters were set a temperature of 23ºC, camera sensitivity of 85, shutter time of 120, and a frame rate of 30 frames per second. Post-acquisition parameters were set at a minimum brightness of 30, minimum size of 5 pixels, and maximum size of 200 pixels. Acquisition parameters remained the same and temperature, conductivity and pH were kept constant between subjects and timepoints. All EV fractions were run in duplicates to ensure accurate concentration and size/diameter readings of particles.

Transmission electron microscopy (TEM). For transmission electron microscopy (TEM), 1.5 mL of pooled EV fractions were concentrated via ultracentrifugation at 120,000 g for 24 hours at 4ºC. The supernatant was discarded and the fluid pellet (50 µL) containing the EVs was stored at -80ºC until analyzed by TEM. The EVs were then thawed, diluted 1:5 in nuclease-free water (UltraPure; Invitrogen, Life Technologies), and 5 µL was added to a Cu/Pd grid (200 mesh with Formvar). Samples were allowed to settle for 10 min and excess fluid removed by ‘blot-drying’ with filter paper (KimWipes), followed by staining in 3 µL of 1% uranyl acetate in ultrapure water for 1 min.

Excess stain was removed and slides were visualized under a JEOL JEM 1200 EX TEMSCAN transmission electron microscope (JEOL, Peabody, MA, USA) operating at an accelerating voltage of 80 kV. The presence of EVs was verified by obtaining images at 50,000 x to 150,000 x with a 4-megapixel side-mounted digital camera (Advanced Microscopy Technique, Woburn, MA). The current TEM method is similar to 'Protocol A' as described by Rikkert et al. (2019) which yields high-quality images with a robust number of EVs, and is particularly suitable after protein removal by SEC (Rikkert et al., 2019).

miRNA extraction and RT-PCR. We used the MagMAX™ *mirVana*™ *Total RNA* Isolation Kit (cat.# A27828; Thermo Scientific) as per the manufacturer's instructions. Briefly, 100 µL of EV isolates were plated onto extraction plates where magnetic beads aid in the isolation of 50 µL of total RNA. Prior to the extraction, *cel-miR-54*-3p (custom oligo; Thermo Fischer Scientific) spike-in was added to each sample at a concentration of 1×10^{-9} M. 2 µL of eluted RNA was then used as input to synthesize cDNA using TaqMan™ Advanced miRNA cDNA Synthesis Kit (cat.# A28007; Thermo Scientific). Expression of the miRNAs of interest were then measured using TaqMan™ Fast Advanced Master Mix (cat.# 4444558; Thermo Fisher Scientific) and the CFX Connect Real-Time PCR Detection System (Bio-Rad, California, United States). Each sample was loaded in duplicates or triplicates and Ct scores >37 with 1 Ct scores between duplicates were excluded from the analysis. *cel-miR-54-3p* was used as a reference gene in normalization and expressions were calculated using the $2^{-\Delta C_T}$ method (Schmittgen & Livak, 2008).Values were then expressed as fold changes compared to YNG expression levels.

In skeletal muscle, approximately 10 mg was used for total RNA extraction using the TRiZOL method, as previously described (Nederveen et al., 2019). Following RNA extraction, 10 ng of RNA is used as input to synthesize cDNA using TaqMan™ Advanced miRNA cDNA Synthesis

Kit (cat.# A28007; Thermo Scientific), as per manufacturer's instructions. Expression of the miRNAs of interest were then measured using TaqMan™ Fast Advanced Master Mix (cat.# 4444558; Thermo Fisher Scientific) and the CFX Connect Real-Time PCR Detection System (Bio-Rad, California, United States) and performed in duplicates. For normalization, the geometric mean of three housekeeper genes was used: an exogenous control, *cel-miR-54-3p* along with two endogenous control RNA species, *snRNAU6* and *hsa-miR-320d*. Values are then calculated using the $2^{-\Delta C_T}$ method (Schmittgen & Livak, 2008) and expressed as fold changes compared to YNG expression levels.

Immune capture of α-sarcoglycan$^+$ exosomes. Prior to immunocapture, an α-sarcoglycan antibody (Abcam) was biotinylated with Pierce™ Antibody Biotinylation Kit (REF. 90407, Thermo Scientific) as per manufacturer's instructions. Once biotinylated, the α-sarcoglycan antibody, along with Dynabeads™ Biotin binder (REF 11047, Invitrogen)- magnetic beads are added to the SEC-isolated exosome samples and incubated overnight at 4ºC with constant rotation. The Dynabeads™ are magnetic beads coated with streptavidin which has a high affinity for biotin conjugated on the α-sarcoglycan antibody. The amount of antibody protein and volume of beads are standardized between samples and added per volume of exosome samples. A negative control containing PBS was included in the experimental design. Following overnight incubation, the samples were placed in a DynaMag™ Magnet for 5 minutes. Without disturbing the magnetic pellet – the flow through is retained. The pull down was washed thoroughly and then resuspended in RIPA buffer supplemented with phosphatase/protease inhibitors. The samples were then loaded onto gel electrophoresis and controlled per volume of flow-through and pull down.

Statistical analysis. Statistical analysis was performed using GraphPad Prism 8.4.3 software. One-way analysis of variance (ANOVA) and Student's t-tests were employed to compare means between age at baseline or with exercise intervention, as appropriate. Significance was accepted at $p < 0.05$. Data are presented as means ± SEM.

Table 1. Antibody information

Primary antibodies			
Antibody	**Species**	**Cat. Number**	**Dilution**
Alix (100kDa)	Rabbit	ab76608	1:2000 in 5% BSA
Tsg101 (47 kDa)	Mouse	ab83	1:2000 in 5% BSA
CD9 (28 kDa)	Rabbit	EXOAB-CD9A-1	1:1000 in 5% BSA
CD81(26 kDa)	Rabbit	EXOAB-CD81A-1	1:1000 in 5% BSA
CD63 (30-70kDa)	Rabbit	EXOAB-CD63A-1	1:1000 in 5% BSA
Albumin (69 kDa)	Rabbit	ab207327	1:5000 in 5% BSA
ApoA1 (31 kDa)	Rabbit	ab52945	1:2000 in 5% BSA
Calreticullin (46 kDa)	Rabbit	12238S	1:2000 in 5% BSA
Exportin-5 (136 kDa)	Rabbit	12565S	1:2000 in 5% BSA
Dicer (220 kDa)	Rabbit	3363S	1:2000 in 5% BSA
Rab27 (25 kDa)	Rabbit	ab55667	1:2000 in 5% BSA

Results

Participants and training program

The home-based resistance training program successfully elicited hypertrophy in participants and has been previously published (Nilsson et al., 2020). Type IIa and type IIx fibre cross sectional area significantly ($p<0.05$) increased by ~21% in both fibre types following 12-weeks of resistance training. Type I non-significantly ($p>0.05$) increased by 14% (Supplementary Table 1). In addition, previously reported increases in total lean mass (TLM) and appendicular skeletal mass (ASM) were observed in response to skeletal muscle adaptations following training (Nilsson et al., 2020). Lastly, strength and functional testing measures significantly improved with exercise, namely leg press (1RM), max grip strength and 5-Times sit to stand (s) test.

Isolation and characterization of circulating exosomes

We utilized size exclusion chromatography in combination with UC as our isolation protocol (SEC-UC). SEC has been previously shown to efficiently isolate exosomes from platelet free plasma (Böing et al., 2014). Consistently, exosome protein markers were present in SEC-UC samples (Figure 1A). In addition, SEC samples are devoid of any plasma contaminant such as Albumin and endoplasmic reticulum contaminant, Calreticulin, suggesting the high purity of the isolation method (Figure 1A). However, SEC samples express lipoprotein contamination marker, ApoA1, a previously reported limitation of the isolation protocol (Brahmer et al., 2019).

Following isolation, the exosome samples were then characterized in accordance to the guidelines set forth by the International Society of Extracellular Vesicles (ISEV) (Théry et al., 2018). Specifically, the detection of wide panel of exosome protein markers (Figure 1A) along with particles characterization (Figure 1B) and visual confirmation with electron microscopy (Figure

1C). ESCRT-related proteins (ALIX, TSG101) and tetraspanins (CD9, CD81, CD63) were all present in SEC samples. The particles isolated also fall within the size range of exosomes, 40-140 nm and visually, they depict the cup-shape of exosomes.

Circulating exosome markers are differently expressed with age and training.

Following characterization of SEC-UC derived samples, we sought to investigate how age and exercise affect the expression of exosome protein markers. Total particle concentration measured using NTA in SEC-isolated samples was not different amongst YNG and OLD participants (Figure 2A). In addition, the size diameter between YNG and OLD was not significantly different (Figure 2B). It is important to note that NTA depicts measurements of all particles within SEC-UC samples, including lipoproteins, and therefore its measurements are not suitable to determine the accurate effect of age and exercise on plasma EVs (Brahmer et al., 2019).

Using immunoblotting, different exosome marker expressions reveal different levels of expression. At baseline, protein levels of ALIX, TSG-101 and CD63 were not different than those seen in YNG. However, following exercise training, ALIX and CD63 increased by 1.2-fold while TSG101 increased by 1.4-fold ($p<0.05$). In contrast, CD81 and CD9 levels were not significantly altered with training; however, at baseline, their levels were significantly lower in OLD compared to YNG (Figure 3). Approximately, 0.6-fold lower levels in CD81 and ~0.7-fold lower in CD9 ($p<0.05$). Taken together, our data suggest that different exosome protein markers are expressed differently with age and resistance training.

SEC- isolated exosomes carry miRNAs that are dysregulated by age and respond to exercise training.

To investigate the miRNA cargo of isolated exosomes, we extracted total RNA and measured the expression of miRNAs previously shown to be involved in aging, skeletal muscle atrophy or exercise. At baseline we saw significant lower expression levels of skeletal-muscle specific miRNAs (miR-1, -133a) and miRNA cluster 23a/27a which have previously been implicated in reducing skeletal muscle atrophy (Wang et al., 2017; Zhang et al., 2018). Specifically, miRNA-23a and -27a were ~0.2- and 0.3-fold lower, respectively. In addition, miRNA-1 and -133a were ~0.5- and 0.2-fold lower in OLD participants, respectively ($p<0.05$). With exception of miR-1, all other three miRNAs significantly increased with resistance training, returning to expression levels comparable to YNG. Specifically, miRNA-23a and -27a increased ~3.0- and 2.5-fold respectively ($p<0.05$) while miRNA-133a increased ~2.0-fold following resistance training. Other miRNAs, shown to be altered with aging, such as miRNA-92a, significantly increased with resistance training. Lastly, miRNA-146a also significantly increased with exercise training by ~2.0-fold. It is important to note that the miRNA expression measured in SEC-samples represents the total expression measured in all different exosome subpopulations. In addition, there were several miRNAs that were not detected in SEC samples (Table 1). In particular, skeletal muscle-specific miRNA-206 expression was not detected, contrary to previous literature where it has been reported to be expressed in exosomes (Fry et al., 2017).

$SGCA^{+}$ exosomes represent a small fraction of total circulatory pool

To assess the contribution of SkM as a possible origin of circulatory exosomes, we aimed to isolate a subpopulation of vesicles which contain the skeletal muscle specific membrane protein, SGCA.

Using immunocapture of tissue-specific skeletal muscle subpopulation, our data indicate that ALIX protein expression can be detected in samples isolated with immunocapture of $SGCA^+$ pulldown samples (Figure 4C) . Compared to the expression of ALIX in the flowthrough, our data suggest that $SGCA^+$ exosomes represent a small fraction of total circulatory pool.

Muscle- specific exosome and miRNA biogenesis is altered with age and exercise

With alterations seen in plasma-derived exosomes and miRNA expression and the presence of α-$SGCA^+$ ALIX expression in circulatory exosomes, we sought to investigate the contribution and involvement of skeletal muscle as it pertains to exosome biogenesis and miRNA expressions. We hypothesized that with age-related atrophy, proteins involved in exosome and miRNA biogenesis would be altered. Similarly, we hypothesized that miRNA expressions in skeletal muscle would also be different, given the structural and functional alterations in aged muscle. We saw significantly higher protein expression of DICER, XPO-5, ALIX and RAB27 in aged muscle, as compared to young controls ($p<0.05$). Specifically, DICER and XPO-5 are both proteins involved in the miRNA biogenesis pathway, which were seen to be elevated in aged muscle by ~1.5 and ~2-fold respectively. Interestingly, following resistance training, both DICER and XPO-5 increased in expression levels, with only XPO-5 increase being significant. Similarly, ALIX was observed to be ~2-fold higher in aged muscle, which continued to increase following training to levels of 3-fold higher than YNG ($p<0.05$). RAB27, a protein involved in exosome release was also shown to be higher expression in aged skeletal muscle which was not significantly altered by exercise. Other proteins of exosome biogenesis (TSG101, CD63, CD9, CD81) were shown to not be significantly altered by age or exercise.

Muscle- specific miRNA expression is altered with age and exercise

With regards to miRNA expression within SkM, a few significant changes were observed. Specifically, myomiR expression of miR-1 and -206 were significantly lower in aged skeletal muscle by ~0.6- and ~0.4-fold respectively (Figure 7). miR-133 was not significantly altered with age nor exercise. In addition, within the miR-23a/23b/27a cluster, miR-23b expression is ~0.5-fold lower in aged muscle ($p<0.05$) while miR-23a/27a were not significantly altered with age at baseline. However, miR-27a significantly increased with resistance training by ~1.3-fold (Figure 7). Further, miR-181a was also observed to be significantly lower expression in aged skeletal muscle by ~0.6-fold. No significant changes occurred with resistance training. Lastly, several miRNAs were observed to be unaffected by age nor exercise (miR-199, -422, -146a, -130a, -92a).

Figures

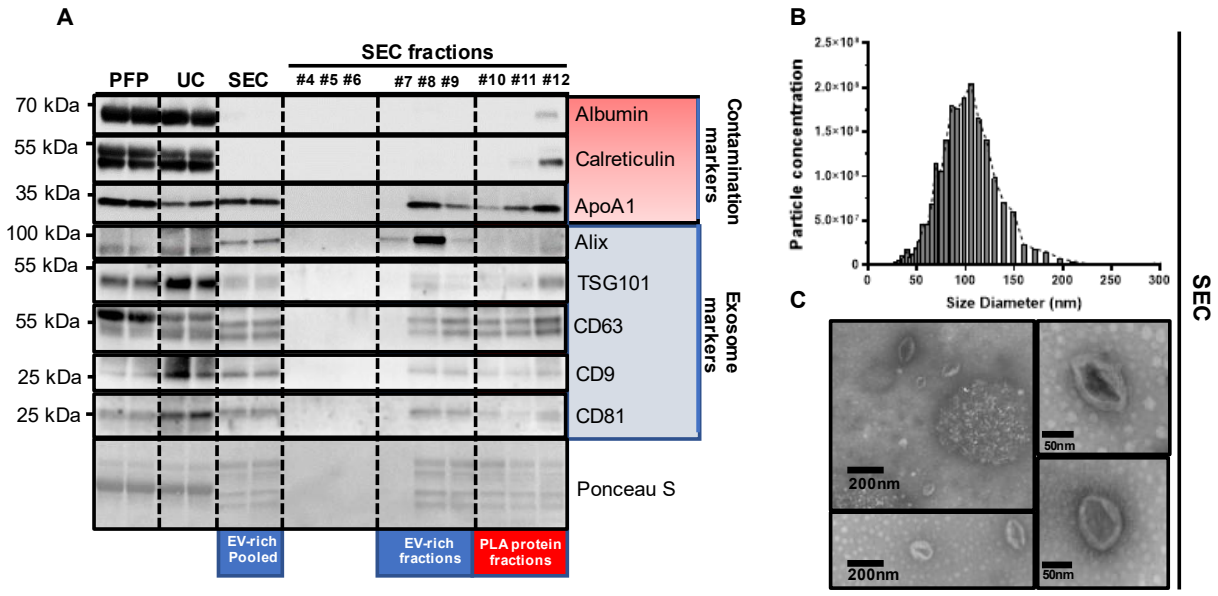


Figure 1. Characterization of plasma exosomes isolated with size exclusion chromatography. **A)** Immunoblotting of exosome-specific markers (ALIX, TSG101, CD63, CD9, CD81) and plasma contaminants (Albumin, ApoA1) and endoplasmic reticulum contaminant (Calreticulin) in platelet-free plasma (PFP), extracellular vesicles isolated through ultracentrifugation (UC) and all SEC fractions. **B)** Representative size distribution and particle concentration profile of SEC-exosome (SEC-EXOs) particles, as measured through nanoparticles tracking analysis. **C)** Transmission electron microscopy image of SEC-exosomes. Scale bar refers to 200 nm, 50 nm as indicated.

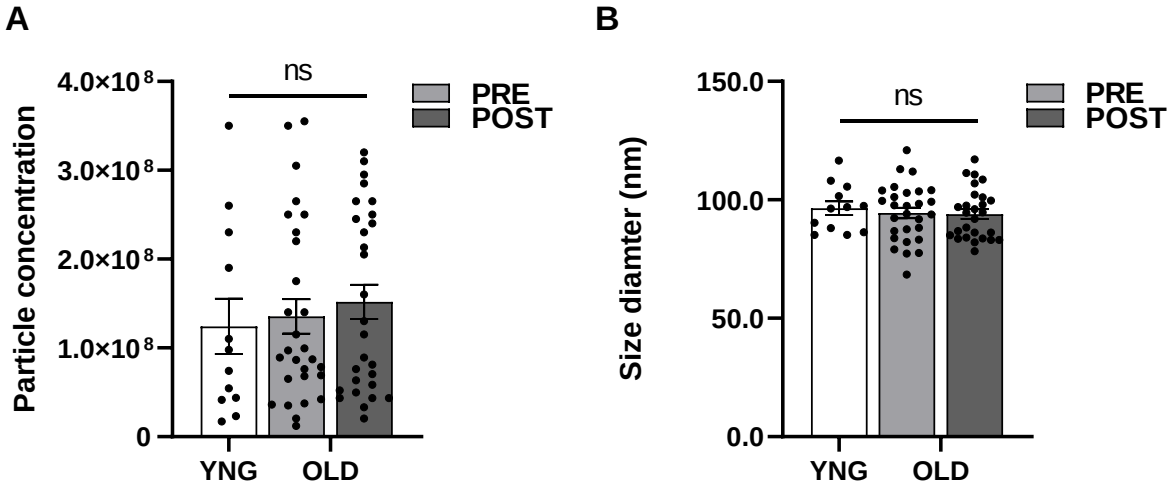


Figure 2. Particle characterization of SEC-EXOs using nanoparticle tracking analysis. Mean **A)** particle concentration and **B)** size diameter of SEC-EXOs isolated from YNG and OLD plasma and following training. Data expressed as mean ± SEM. One-way ANOVA with Fisher's LSD. ns = no significant difference between groups, *n* = 12 for YNG, *n* = 28 for OLD.

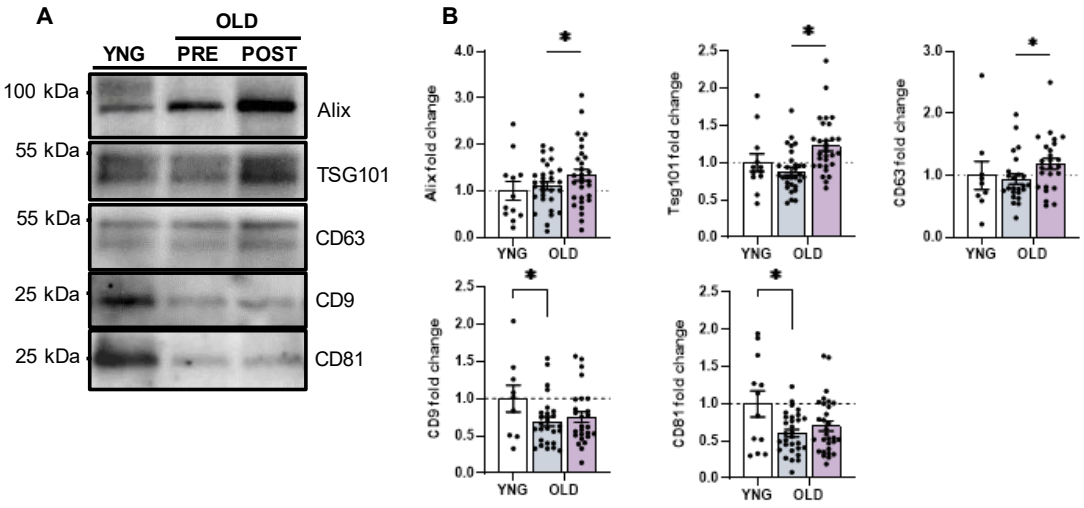


Figure 3. Circulating exosome markers are differently expressed with age and training. **A)** Representative western blot (WB) images of exosome-specific markers in SEC-EXOs derived from young (YNG) and old plasma, before (PRE) and after 12-weeks of home-based resistance training (POST). **B)** Densitometric quantification of western blot signals. Individual (circle) and mean ± SEM (bar) fold changes of protein markers. PRE and POST values expressed in relation to YNG. One-way ANOVA with Fisher's LSD, n = 12 for YNG, n = 26-30 for OLD, * = $p < 0.05$.

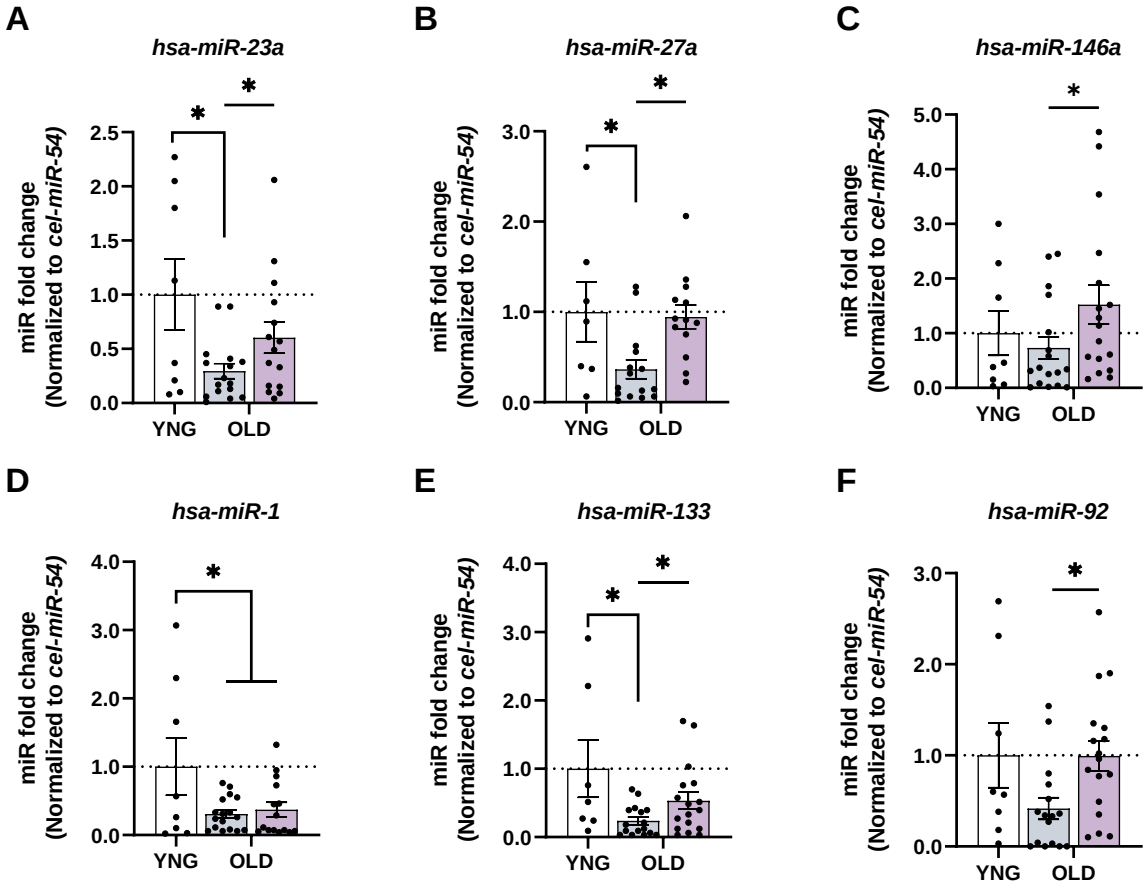


Figure 4. SEC- isolated exosomes carry miRNAs that are dysregulated with age and respond to exercise training. miRNA gene abundance of **A)** *hsa-miR-23a*, **B)** *hsa-miR-27a*, **C)** *hsa-miR-146a*, **D)** *hsa-miR-1*, **E)** *hsa-miR-133a*, **F)** *hsa-miR-146a*. miRNAs normalized to exogenous miR, *cel-miR-54* added prior to RNA isolation at a concentration of 1 pM. Individual (circle) and mean ± SEM (bar) fold changes of miR expression. PRE and POST values expressed in relation to YNG. One-way ANOVA with Fisher's LSD, n = 8 for YNG, n = 18-20 for OLD, * = $p < 0.05$.

Table 1. List of miRNAs not detected in SEC-isolated exosomes samples

miRNAs not detected in SEC-samples
hsa-miR-206
hsa-miR-141
hsa-miR-27b
has-miR-130
hsa-miR-181
hsa-miR-199

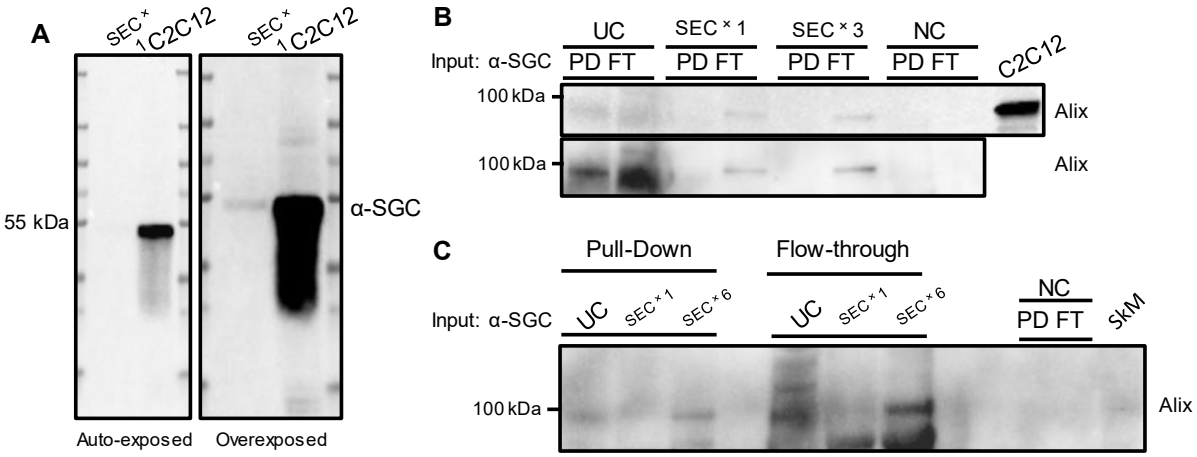


Figure 5. Exploratory experiments of isolating circulating α-sarcoglycan^{+} exosomes. **A)** α-SGCA expression in SEC-isolated samples as compared to highly expressed C2C12 murine myoblast cell line. Detection presented through autoexposure and overexposure to reveal small protein expression of SGCA protein in SEC-isolated exosomes. Principle of immunoprecipitation of α-SGCA in plasma-derived EXOs isolated using UC and **B)** SEC (×1 and ×3 concentrated) and **C)** SEC (×1 and ×6 concentrated) and representative Western blot respectively of the pull-down (PD) and flow-through (FT) of the immunoprecipitation, with quantification of the relative ALIX and α-SGC expression. UC = ultracentrifugation, SEC = size exclusion chromatography, NC = negative control, IgG only with no EXO sample.

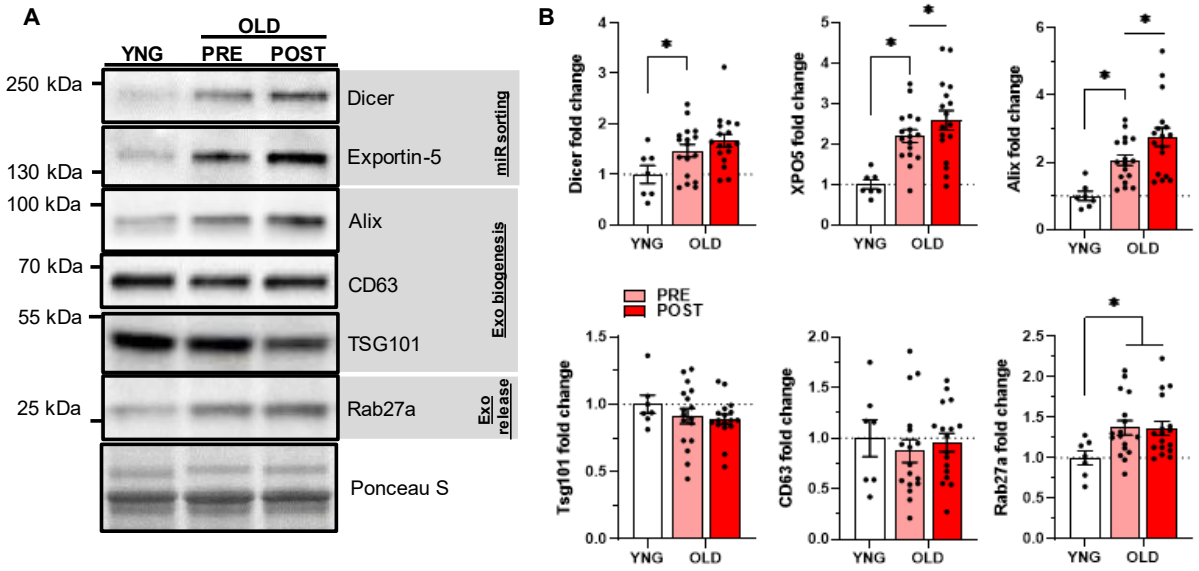
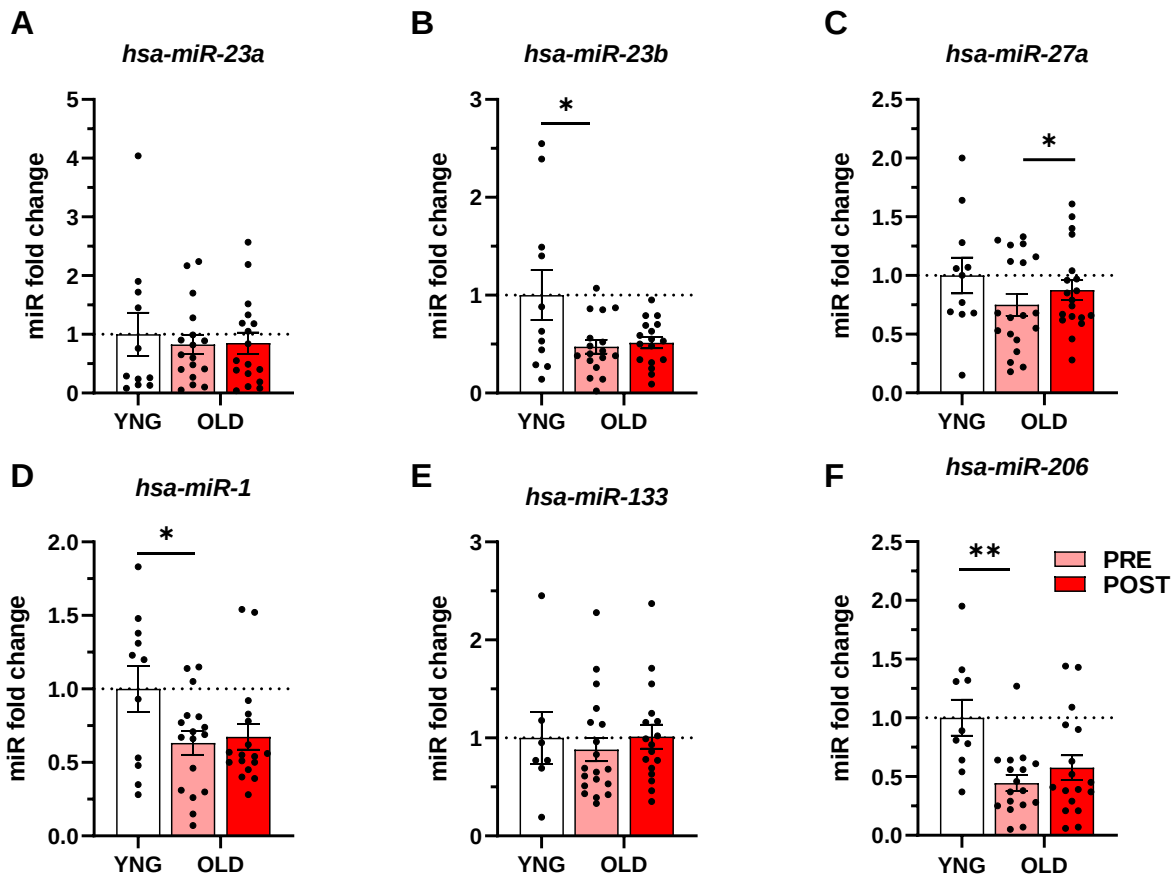


Figure 6. Aged skeletal muscle upregulates proteins involved in miR and exosome biogenesis. **A)** Representative western blot (WB) images of exosome-specific markers in skeletal muscle of young (YNG) and old, before (PRE) and after 12-weeks of home-based resistance training (POST). **B)** Densitometric quantification of western blot signals. Individual (circle) and mean ± SEM (bar) fold changes of protein markers. PRE and POST values expressed in relation to YNG. One-way ANOVA with Fisher's LSD, n = 12 for YNG, n = 26-30 for OLD, * = $p < 0.05$.


A
hsa-miR-23a
miR fold change
YNG
OLD
B
hsa-miR-23b
*
C
hsa-miR-27a
*
D
hsa-miR-1
*
E
hsa-miR-133
F
hsa-miR-206
**
PRE
POST

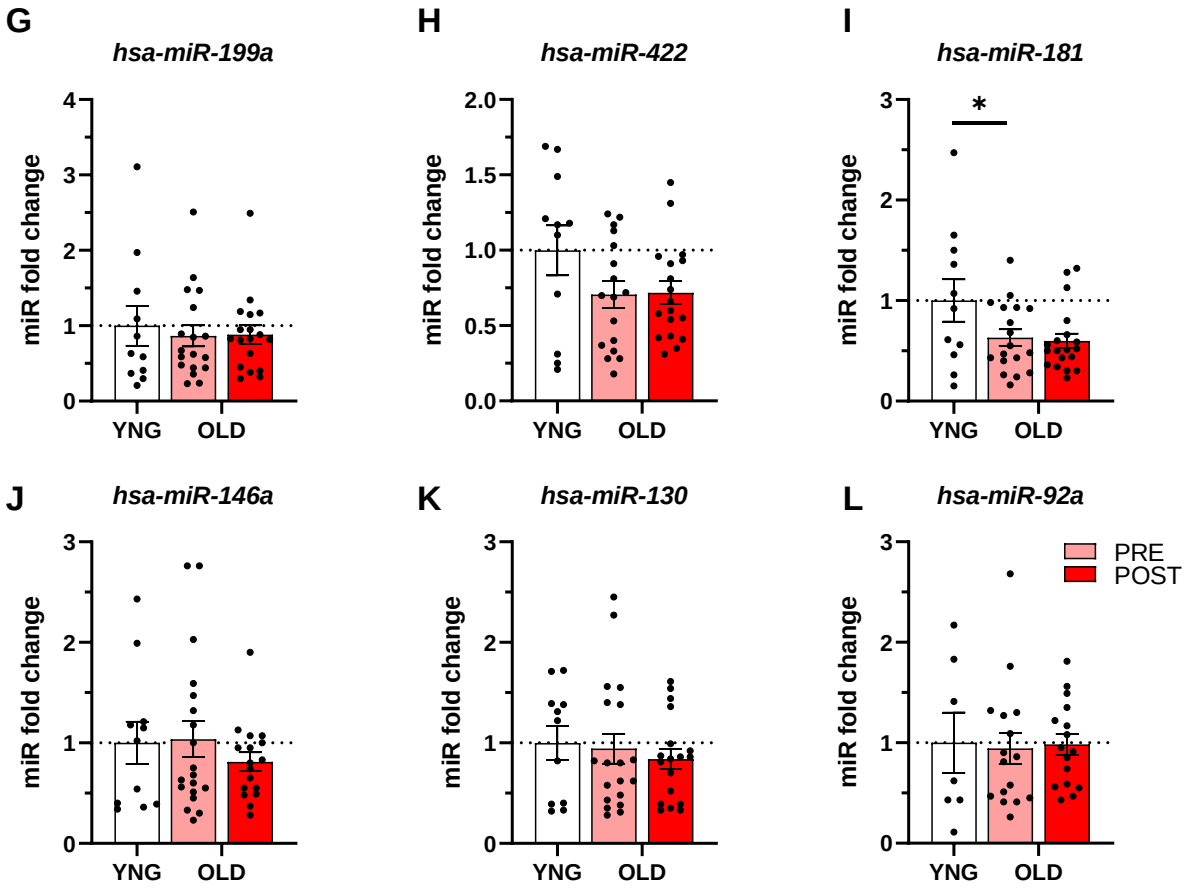


Figure 7. miRNA gene expression in skeletal muscle of YNG and OLD participants, following training. **A)** *hsa-miR-23a*, **B)** *hsa-miR-23b*, **C)** *hsa-miR-27a*, **D)** *hsa-miR-1*, **E)** *hsa-miR-133a*, **F)** *hsa-miR-206a*. **G)** *hsa-miR-199a*, **H)** *hsa-miR-422*, **I)** *hsa-miR-181*, **J)** *hsa-miR-146a*, **K)** *hsa-miR-130a*, **L)** *hsa-miR-92a*.miRNAs normalized to exogenous miR, *cel-miR-54* added prior to RNA isolation at a concentration of 1 pM., as well as two endogenous controls, *snRNA U6* and *hsa-miR-320d.* Individual (circle) and mean ± SEM (bar) fold changes of miR expression. PRE and POST values expressed in relation to YNG. One-way ANOVA with Fisher's LSD, n = 10-12 for YNG, n = 18-20 for OLD, * = $p < 0.05$.

Discussion

The aim of this project was to investigate the circulatory and skeletal muscle exosome response in old participants, as compared to young controls, and the subsequent effect of a 12-week resistance training program. Our study is the first to characterize circulatory exosomes in old participants, using size exclusion chromatography before and after resistance training program. In addition, we investigated miRNA cargo of isolated exosomes and how they are affected by age and resistance training. Lastly, we examined skeletal muscle tissue, as a potential source of exosome pool in circulation. Indeed, our data suggest that different exosome subpopulations are affected by age and exercise. In addition, miRNA expressions within exosome samples are altered with age and some increase with exercise training, returning to levels comparable to YNG. Within skeletal muscle, we see significant differences with age and training in miRNA biogenesis related proteins and different miRNA expressions. Future studies should aim to investigate further on the significance of these results and determine function.

Exosome isolation and characterization with aging and exercise

We used size exclusion chromatography in combination with ultracentrifugation to isolate exosomes from platelet-free plasma. To date, more than 10 different isolation methods have been used in the literature with the most common method of isolation being differential centrifugation (DC) with ultracentrifugation (UC) (Royo et al., 2020). Indeed, many of the foundational studies in exosome literature utilized DC+UC as their means of exosome isolation (Valadi et al., 2007); however, it is important to note that these studies utilized cell supernatant as the source origin of exosome isolation. Plasma and serum are very different and much more complex biological fluids which may require further filtration, such as size exclusion chromatography. We compared the purity of the different isolation techniques, DC/UC, and SEC/UC by blotting for different

contamination markers (Figure 1A) and SEC is significantly more deplete of albumin and calreticulin suggesting that SEC yields the "cleaner" isolates. We also characterized our isolated exosomes, in accordance with the guidelines set-forth in the ISEV statement paper (Théry et al., 2018), blotting for a wide panel of canonical exosome markers, particle characterization analysis and electron microscopy. All exosome markers are present in the isolated samples and the size distribution of particles falls within the range of exosomes. Visually, within the SEC samples, cup-shaped vesicles can be seen, evident of exosome samples. Collectively, we confirm successful isolation of plasma-derived exosome samples using size exclusion chromatography.

When assessing how age and exercise affects particle concentration, we found non-significant differences between young and old participants at baseline, nor did we find any changes with resistance training. Particle characterization with nanoparticles tracking analysis uses lasers to bounce the light off particles which is then captured by cameras and read-outs on concentration and size distribution are computed. NTA does not discriminate on the particles that are present within the sample and given that a known limitation of SEC is its co-isolation of lipoprotein particles, the contamination of other non-exosome related particles may affect the reading of the instrument and therefore the validity of the results (Mørk et al., 2017). This is consistent with other studies in which they investigate the effect of acute exercise in SEC-isolated samples and saw similar, non-significant results (Brahmer et al., 2019). In that study authors concluded that NTA is therefore not a reliable measure to assess exosome release under a given intervention (Brahmer et al., 2019).

In contrast, measuring the expression of the protein markers and how those are affected by age and exercise training reveals interesting results. Our findings show that different exosome subpopulations respond differently with age, as well as with resistance training. Older adults

showed significantly lower expression of CD9 and CD81 but were not significantly altered with exercise. Contrary, ALIX, TSG-101 and CD63 showed no significant differences with age, but all three protein markers expressions significantly increased with resistance training. In part, our findings confirm the previously known effect of exercise on exosome markers. Although during an acute bout, exercise has been shown to increase exosome markers in circulation (Brahmer et al., 2019; Frühbeis et al., 2015). Estébanez and colleagues reported lower expression of CD63 protein in old participants compared to young (Estébanez et al., 2021). In addition, they showed no differences following resistance training, which is contrary to what we see in our current study. The discrepancy in the results reported by Estébanez and colleagues may be attributed to the cruder isolation protocol that they utilized within their study (UC). In addition, the lack of ISEV recommended exosome characterization also questions the validity of the results.

In our current study, the novel finding of heterogeneity within exosomes as to how they may respond to exercise and how may be affected by age is in line with recent studies discriminating between different exosome subpopulations. Specifically, Mathieu and colleagues utilized KO cell line models and intricate methodology of live intercellular tracking of different EV markers from their subcellular origin to release and found that $CD63^+$ EVs may represent a distinct subpopulation from $CD9^+$ EV (Mathieu et al., 2021). CD63 marker localized more in the MVB while CD9 localized more in the plasma membrane. Based on marker localization within cell compartments from origin to release, the authors suggested that $CD63^+$ EVs represent exosome-like EVs based on origin, while $CD9^+$ EVs represent ectosomes, a distinct EV subpopulation which is smaller in size than exosomes which are also derived from the plasma-membrane (Mathieu et al., 2021). In addition, the authors also suggested that $CD81^+$ EVs behave similarly to $CD9^+$ EVs which may represent a similar subpopulation of ectosomes. Given the findings reported by

Mathieu and colleagues, as they pertain to our data, we suggest that CD63 and other endosomal-EV origin-based markers (such as ALIX, TSG101), are affected by exercise training. In addition, CD9 and CD81, seeing that those may be present primarily in ectosomes, we suggest that aging has a significant effect on ectosomes. Future studies should aim to validate these findings and investigate further the effect of exercise and age on the different EV subpopulations.

SEC-isolated exosome miRNA cargo

Within our SEC-isolated exosome samples we aimed to investigate the miRNA cargo expression with the purpose of providing insight on the functional roles of circulating exosomes and how they are affected by age and exercise training. The selected miRNAs of interest were chosen based on previous studies showing their involvement in skeletal muscle atrophy, exercise, and aging (Hudson et al., 2014; Silva et al., 2017; Zhang et al., 2018). In our study, we saw significantly lower miRNA expressions at baseline of old participants (miR-23a, -27a, -1, -133). Interestingly, following resistance training, miR-23a, -27a, -133 significantly increased, returning to levels comparable to young participants. In the literature, the effect of aging and resistance exercise on miRNA-23a/27a has not been well elucidated. However, their role in atrophy has been more studied. miR-23a/27a have been shown to target atrophy related genes and inhibit their translation (Wang et al., 2017; Zhang et al., 2018). Zhang and colleagues reported that miR-23a/27a expressed in UC-isolated exosomes can attenuate atrophy by reducing the expression of TRIM63/MuRF1 and FBXO32/atrogin-1 in skeletal muscle, two atrophy-inducing E3 ubiquitin ligases. In our results, we show significant increases in miR-23a/27a following resistance exercise suggesting that following skeletal muscle uptake of circulating exosomes, the expression of miR-23a/27a may attenuate atrophy. However, further detailed studies are warranted in order to appropriately elucidate function. In addition, the authors reported an attenuation in kidney fibrosis when

overexpressing miR-23a/27a in skeletal muscle suggesting intercellular crosstalk between muscle and kidney whereby exosomes containing miR-23a/27a and originating from skeletal muscle are taken up by the kidneys and possibly eliciting beneficial effects in renal recipient cells by inhibiting TGFβ/SMAD cascade pathway responsible for the fibrotic process. This is particularly important because fibrosis and upregulated TGFβ/SMAD have been shown to be prevalent in aging (Biernacka & Frangogiannis, 2011; Harvey et al., 2016). The increased expression miR-23a/27a in our SEC-isolated samples seen following resistance exercise may provide some evidence of the multisystemic benefits of resistance training. However, further studies are warranted to determine conclusive results.

Moreover, we saw significantly lower expression of skeletal muscle specific miR-1/133 in circulating exosomes from older participants. In addition, miR-133 significantly increased following training. In the literature, the effect of age and exercise on circulating levels of myomiRs in exosomes are not well defined. miR-1/133 have been shown to be enriched in skeletal muscle and involved in skeletal muscle homeostasis (Ultimo et al., 2018). In mouse models; however, miRNA-133 has been previously shown to increase in circulating exosomes following high intensity interval training (Castaño et al., 2020). In addition, the authors identified transcription factor forkhead box O1 (FoxO1) in the liver as a target of miR-133 and proposed that miR-133 levels in exosomes released in circulation following exercise, travel to the liver and subsequently induce gene expression changes that improve glucose metabolism and insulin sensitivity. The authors concluded that the benefits of HIIT training on glucose handling, may be, in part due to exosome-mediated intercellular communication between skeletal muscle and liver (Castaño et al., 2020). Given that sarcopenic individuals present with metabolic complications and insulin resistance (Consitt et al., 2019), increasing circulatory levels of miR-133 through resistance

training, may partially underly the mechanism behind resistance training-induced benefits on glucose metabolism reported in older adults (Consitt et al., 2019). However, further studies are warranted, particularly in humans to determine conclusive results on function of myomiRs in circulating exosomes and the effect of age and exercise.

Circulatory exosomes originating from skeletal muscle

Using immunocapture-based methodology, we aimed to isolate a specific skeletal muscle-origin subpopulation of α-sarcoglycan^{+} exosomes in our SEC isolated samples. Initially, we were unsuccessful in detecting ALIX protein within our pull-down samples most likely due to the limited protein yield of SEC-isolated samples. However, when increasing the amount of starting plasma volume, indeed a small expression of ALIX can be detected in the pull-down samples. Compared to the expression of ALIX in the flow through, our data suggest that α-sarcoglycan^{+} exosomes expressing ALIX represent a small portion of the total circulatory exosome pool. In the literature, Brahmer and colleagues utilized similar exosome isolation methodology to our current study and were unsuccessful at detecting SGCA in their SEC samples (Brahmer et al., 2019). However, that may be partially explained due to the low protein yield of SEC samples, which we struggled with initially. Indeed, two other studies have used SGCA as a marker of muscle-specific exosomes. Guescini and colleagues were the first to isolate α-SGCA^{+} exosomes using density gradient isolation. In addition, Rigamonti and colleagues also measured the α-SGCA^{+} exosomes in plasma following and acute bout of exercise where they showed a significant increase in α-SGCA^{+} exosomes in plasma with exercise (Rigamonti et al., 2020). Despite successful measurements α-SGCA^{+} exosomes, the previous studies lacked the rigorous methodology in EV isolation. Future studies should aim to investigate the miRNA cargo of α- SGCA^{+} exosomes

isolated with SEC-UC to better validate their isolation protocol and aim to investigate the effect of age and exercise on skeletal muscle-specific exosome subpopulation.

Exosome biogenesis and miRNA expression in skeletal muscle

A secondary aim for the study was to investigate the contribution of skeletal muscle as a source of EVs. Given that with sarcopenia, skeletal muscle undergoes structural and functional changes, we hypothesized that proteins involved in exosome and miRNA biogenesis would be altered with aging. Indeed, we saw significantly higher levels with ALIX in old muscle at baseline compared to young, which continued to significantly increase with training. Apart from exosome biogenesis, ALIX has been shown to be involved in cytoskeleton remodeling within skeletal muscle. Knockdown of ALIX in C2C12 cell lines showed impaired formation of sarcolemmal projections indicating defective cell motility (Bongiovanni et al., 2012). In addition, KO ALIX cell lines also represented a reduced release of EVs (Romancino et al., 2013). In the literature, the effect of age and exercise on skeletal muscle ALIX function has not been previously shown before. However, given its important function in exosome biogenesis, we suggest that an upregulation of ALIX can be seen in response to the molecular perturbations of skeletal muscle aging, and impairments in cytoskeleton of the muscle fibres- thereby upregulating ALIX in an attempt to restore homeostasis with regards to cytoskeleton remodelling.

In addition, we observed significantly higher protein expression with age of miRNA biogenesis-related proteins (XPO-5, DICER). XPO-5 was further increased following resistance training. Previously, the gene expression of XPO-5 has been reported to not be altered between young and old muscle and increased with an acute bout of resistance exercise (Drummond et al., 2011). The higher protein expression of XPO-5 and DICER with aging is seen in parallel with the increase in

expression levels of miRNAs in skeletal muscle, and likely increases in miRNA expression in circulating exosomes.

Furthermore, we measured the expression levels of miRNAs within skeletal muscle and investigated the effect of age and exercise training. In miR-23b we saw significantly lower expression with age. In contrast no age differences were seen in miR-23a/27a. With respect to exercise training, miR-27a significantly increased but no significant differences reported in miR-23a/23b. Previously in the literature, the cluster of miR-23/27 has been reported to be downregulated in skeletal muscle of muscle wasting conditions (Hudson et al., 2014; Silva et al., 2017; Wang et al., 2017). Given that sarcopenia is a condition of muscle loss, downregulations we see in miR-23b confirm previous findings. Similarly, increases in miR-27a following resistance training have been previously reported (Wang et al., 2017). Specifically, in mice model of hypertrophy, following mechanical overload, miR-27a increased and was shown to be implicated in reducing atrophy (Wang et al., 2017; Zhang et al., 2018).

Lastly, we found significantly lower expressions with age in skeletal muscle specific miR-1 and -206. Lower expression of miR-1 with aging has been previously shown before (Rivas et al., 2014), confirming our results. In contrary, miR-206 has been previously reported in the literature to not be significantly altered with aging of human skeletal muscle (Drummond et al., 2008). Knocking out of miR-206 has been reported to significantly impair and delay myotube differentiation, providing evidence for the important role of miR-206 in myogenesis (Salant et al., 2020). Furthermore, genetic deletion of miR-206 in mice significantly altered skeletal muscle regeneration induced by cardiotoxin (Liu et al., 2012). Given that with aging, skeletal muscle regeneration is impaired (Joanisse et al., 2017), it is expected that we see significantly lower expression of miR-206 in aged muscle. In addition, it also important to note, that the expression

of different miRNAs in skeletal muscle varies on the different types of cells present. In particular, miR-206 has been shown to be released by satellite cells (Fry et al., 2017), which also decrease in abundance with age, suggesting that it is possible that the decrease in satellite cell pool (Joanisse et al., 2017) means less production of miR-206, explaining the results that we see in our study. Following resistance training, miR-206 levels increased in most participants, although these changes were not statistically significant. Our findings suggest that aging significantly alters miRNA expression in skeletal muscle and the age-related defects in skeletal muscle function may be partially due to the altered miRNA expression.

Nevertheless, limitations for the study remain. As previously discussed, size exclusion chromatography co-isolates lipoproteins in addition to exosomes. Given that miRNA species have recently been identified to be transported in lipoproteins as well, understandably, when isolating for miRNAs in SEC-samples, the addition of lipoprotein-derived miRNA would indeed affect their expression and add variability to the effect of age and exercise on exosome-transported miRNA (Michell & Vickers, 2016). In addition, another limitation in the study design may be the timing of the last training session. If the timing between the last bout of acute exercise and the collection of POST data is not standardized, the acute effect of exercise may add variability to the exosome release and other results. Future studies should aim to standardize the timing between the last training session and the collection of blood and/or other tissues.

Conclusion

Our study is the first to characterize circulating exosomes in old participants using size exclusion chromatography and investigate the effect of age as compared to young controls and exercise training. Our findings suggest that aging significantly alters miRNA cargo of circulating exosomes which are partially restored to young levels following 12 weeks of resistance training program. In addition, our findings suggest that aging and exercise alter different exosome subpopulations in circulation, whereby age affects CD9/CD81^{+} exosomes; whereas, ALIX, TSG101 and CD63-expressing exosomes are not significantly altered with age; however, they significantly respond to exercise. In addition, we show alterations with age in proteins involved with exosome and miRNA biogenesis and report that these expression levels also respond to resistance training. We show altered expression levels of miRNAs in aged muscle and potential restoration with resistance training. Altogether our findings suggest that age dysregulates exosome markers, cargo, and skeletal muscle specific contributions. With exercise training, we see some of those levels restored to a young phenotype. However, future studies are warranted in linking function of exosomes to age-related muscle atrophy and complications, as well as further studies warranted in elucidating that the benefits of resistance training in older individuals are mediated through exosomes.

Supplementary Table1: Participant characteristics

	YNG	OLD	
		PRE	POST
Age (years)	21.4 (3.5)#	77.4 (5.8)	
Height (cm)	176.8 (7.5)	173.1 (4.7)	
Weight (kg)	77.9 (14.6)#	85.4 (8.4)	86.3 (8.9)
BMI (kg/m^2)	24.89 (4.1)#	28.2 (3.6)	28.5 (3.1)
Fat %	24.795 (8.4)#	32.42 (7.3)	32.2 (7.7)
ASM (kg)	26.5 (4.5)#	23. 9 (3.3)	24.7 (3.6)
Total lean mass (kg)	55.4 (9.1)	54.4 (8.8)	55.7 (8.2)
CSA Type I (μm^2)	5527.3 (919.5)	5209.5 (1072.0)	5922.7 (1946.4)*
CSA Type IIA (μm^2)	6056.9 (826.4)#	4788.3 (932.0)	5878.6 (2032.4)*

Statistically significant between YNG vs PRE ($p < 0.05$), * Statistically significant between PRE vs POST ($p<0.05$)

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