The Effects of Frailty on Extracellular Vesicles (EVs) and the Ability of EVs to Rescue Age-Associated Cellular Dysfunction

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Abstract

In Canada and across the world, the global population of older adults is rising. Within the next 15 years, approximately 25% of Canadians will be 65 years of age or older. This shift in population demographics will be a stressor for healthcare systems due to a concomitant loss of functional independence with age. While strategies exist to promote healthy living, there is also growing interest in research focused on attenuating the biological hallmarks of aging. Previous parabiosis experiments have shown that factors in the circulatory system may be key to reversing the cellular aging process. We propose that these youthful circulatory factors are encapsulated by extracellular vesicles (EVs), nanoparticles released by all cell types that are critical in cellular communication. To test our hypothesis, we obtained samples from the WARM Hearts Study (Clinical Trial #NCT02863211). In this study, we: 1) isolated and biochemically characterized EVs from women who were classified as robust, pre-frail or frail, and 2) co-cultured robust/young EVs, and frail/old EVs with chronologically young and old primary human skeletal muscle cells. Our results indicate that EVs isolated from frail subjects yielded 22% more protein than EVs isolated from robust subjects (*p=0.01, N=23) and 48.5% more protein than EVs isolated from pre-frail subjects (*p<0.001, N=12-23). Moreover, frail EVs had 119% lower ApoA1, a non-EV marker, than robust EVs (*p<0.01, N=8). Next, robust and frail plasma samples were stratified for epigenetic age (biological age) and EVs were isolated for co-culture experiments with young (19 year old) and old (92 year old) human skeletal muscle cells. Young and old cells were co-cultured with robust/biologically young and frail/biologically old plasma and EVs. We observed no difference in cell count or mitochondrial staining in any treatment groups. Treating old cells with EVs isolated from robust/biologically young subjects resulted in a 48% reduction in senescence as measured by beta-galactosidase staining (*p=0.02, N=7), and a 24% increase in cell viability (*p=0.02, N=6). Treating young cells with plasma isolated from frail/biologically old subjects resulted in a 16% decrease in cell viability (p=0.05, N=6). Treating young cells with EVs isolated from frail/biologically old subjects increased senescence by 73% (*p=0.007, N=7). The data show that EVs from frail/biologically old subjects have more protein, contain less ApoA1, and induced senescence in young cells, whereas EVs from robust/biologically young subjects rescued senescence in old cells.

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Dedication

I would like to dedicate this thesis to my parents Rob and June, grandparents Bob and Sylvia, the rest of the Bydak family, and Koko.

List of Abbreviations

AT-MSC = adipose tissue-derived mesenchymal stem cells

CpG sites = cytosine-phosphate-guanine site

CAT = catalase

DCF = dihydrodichloroflourescein

DNMT = DNA methyltransferase

eAgeAccel = Epigenetic age acceleration

eNAMPT = extracellular nicotinamide phosphoribosyltransferase

ESCRT = endosomal sorting complex required for transport

EVs = extracellular vesicles

GSH = glutathione

GST = glutathione s transferase

GM-CSF = granulocyte-macrophage colony-stimulating factor

HDPSC = human dental pulp stem cells

HUVEC = human umbilical vein endothelial cells

IL = interleukin

Lin^- = lineage negative bone marrow cells

MDA = malondialdehyde

miR = microRNA

miRNA = microRNA

mtDNA = mitochondrial DNA

MVB = multivesicular body

NAD+ = nicotinamide adenosine dinucleotide

PEG = poly-ethylene glycol

PARP = poly (adenosine diphosphate- ribose) polymerase

PGC-1α = peroxisome proliferator-activated receptor gamma coactivator 1-alpha

ROS = reactive oxygen species

Rb = retinoblastoma

SASP = senescence-associated secretory phenotype

SA-β-Gal = senescence-associated beta galactosidase

SEC = size exclusion chromatography

sEVs = small extracellular vesicles

SOD = superoxide dismutase

UV = ultraviolet

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Chapter 1: Literature Review

1.1. Epidemiology of aging

By the year 2036, roughly 25% of the Canadian population will be 65 years of age or older[1]. Aging is generally defined as the progressive deterioration of physiological processes in the body over time[2]. This functional decline over time occurs in tandem with increased onset of diseases affecting the cardiovascular, nervous, musculoskeletal, and endocrine systems, as well as increased incidence of cancer[3]. As a result of this increased onset of chronic diseases, the aging population requires significant healthcare interventions to maintain a healthy lifestyle and ensure independence with age[4]. Facilitating healthy aging free of age-related physiological dysfunction can alleviate healthcare costs, foster improved quality of life for older adults, and support independence in old age. A number of nutritional and physical activity strategies to promote a healthy lifestyle exist, as well as strategies to combat the appearance of aging with the anti-aging consumer market generating over $190 billion USD in 2019[5]. However, attenuating the aging process at the cellular and organismal levels has only been consistently achieved in *C. elegans* and rodent models, and to a lesser extent in human cells in culture[6,7]. To address rejuvenation of aging at the cellular level, it is important to first understand how cellular modifications drive the process of aging.

1.2. Cellular aging

At the cellular level, aging is characterized by the presence of nine hallmarks of aging which include genomic instability, telomere attrition, epigenetic alterations, loss of proteostasis, deregulated nutrient-sensing, mitochondrial dysfunction, stem cell exhaustion, altered intercellular communication, and cellular senescence[89]. Cellular senescence, first described in 196[10], is the process of arrest of cell proliferation, either due to a cell being fully differentiated or because the cell is no longer undergoing the cell cycle[11,12]. Senescence can lead to morphological changes in the nucleus or mitochondria of the cell, and result in visible changes in cellular hypertrophy and irregular shape[13]. Senescent cells show increased beta-galactosidase (SA-β-Gal) activity, an enzyme responsible for cleaving lactose that is established as an identifiable marker of cellular senescence[14,15], as well as higher reactive oxygen species (ROS) production[16], and expression of senescent-associated markers such as p16 and p21[17,18]. With age, senescent cells accumulate in most tissues throughout the body[19] due to an accumulation of DNA damage within the cell, the

subsequent DNA damage response, and telomere shortening[20], leading to sustained accumulated damage over time[21].

1.2.1. Epigenetic changes with age

DNA damage that occurs with age as a consequence of UV or oxidative damage[22,23] includes epigenetic modifications as well as mutations or changes in the sequence of the genetic material. The DNA damage theory of aging postulates that DNA damage, through exogenous or endogenous pathways, promotes aging. Over time, an accumulation of DNA mutations and damage leads to senescence, atrophy, or inflammation[24]. These changes can affect not only the damaged cell but also neighbouring cells, through altered intercellular communication in senescent and aged cells[24]. Epigenetic alterations involve changes in expression of genes that are inherited without changing the actual underlying DNA sequence[25]. Epigenetic regulation is mediated by modifications in DNA acetylation or methylation, and modifications of histone and other chromatin-associated proteins[26]. During aging, epigenetic alterations can include changes in chromatin structure, reduced histone protein synthesis resulting in general loss of histones, changes in DNA methylation, chromatin relaxation, and nucleosome remodelling[25,27]. DNA methylation (DNAm) is of particular importance because of its relevance to the epigenetic or biological clock. While chronological age refers to the age in terms of time that has passed from birth, biological or physiological age describes how old the cells are. Biological age is controlled by genetics as well as nutritional and lifestyle factors[28,29]. Individuals with younger biological age than chronological age are at lower risk of chronic diseases[30,31]. Interestingly, while chronological age is irreversible, biological age can be reversed[32]. Changes in DNAm at CpG sites, first characterised as a biological aging clock in 2013 by Dr. Steve Horvath[33], have been shown to be consistent measures of aging[34–36], and as such DNAm is used as a viable measure of biological age[37].

1.2.2. Changes in metabolism with age

Loss of metabolic function is an established marker of aging. Impaired mitochondrial respiration, enhanced ROS production, and a gradual decline in ROS scavengers with age can lead to oxidative stress and subsequent damage to DNA, proteins, and lipids[38–41]. Accumulation of mutations in the mitochondrial DNA (mtDNA) can in turn lead to an increase in ROS production, which in a feed-forward cycle can further damage mtDNA. Loss in mitochondrial mass, function and activity, independent of mtDNA mutations, can also accelerate the aging process[42]. Critical regulators of

mitochondrial function and biogenesis such as nicotinamide adenine dinucleotide (NAD+), peroxisome-proliferator-activated receptor-γ coactivator-1 (PGC-1) alpha among others decrease with age, which contributes to age-associated mitochondrial dysfunction[43]. NAD+ is important for sirtuin proteins (SIRT 1-7) to function as coenzymes, with mitochondrial sirtuins being essential in maintaining optimal metabolism[38,44]. PGC-1alpha is the 'master' regulator of mitochondrial biogenesis, and increasing PGC-1alpha expression in skeletal muscle by exercise or genetic manipulation rescues age-related decline in skeletal muscle mitochondrial function[45,46]. The decrease in mitochondrial content with age often occurs in tandem with sarcopenia, the gradual loss of skeletal muscle mass and strength with age. Sarcopenia is highly prevalent in older adults, ranging from 11-50% in older adults > 80 years of age[47]. Sarcopenia is a risk factor for onset of other age-related chronic diseases and predictor of overall mortality[48,49]. It is also accompanied by another age-associated condition called frailty.

1.3. Frailty

Frailty is a condition whereby an individual shows impeded ability to return to their baseline level of health after experiencing a physical injury or stressor[50]. First defined by Fried *et al.* in 2001, frailty is characterized by unintentional weight loss, self-reported exhaustion, poor grip strength, slow walking speed, and low physical activity[51]. Physical frailty scores are generally based on these criteria. In addition to the Fried frailty score, a different approach to assess frailty is the frailty index method, which incorporates physiological, behavioural, and social factors into a frailty score by accounting for deficits experienced by an individual[52–54]. Currently, 25% of Canadians over the age of 65 live with frailty[55]. While frailty can occur at any age, frailty incidence increases with age, and women generally experience frailty at a higher rate than men[56,57].

1.4. Senescence-associated secretory phenotype

As cells undergo senescence, they can communicate in a paracrine manner with neighbouring cells through the senescence-associated secretory phenotype (SASP). The SASP involves the secretion of various proteins including cytokines, chemokines, growth factors, and proteases to neighbouring cells[58]. SASP can have positive effects inducing cellular plasticity and cellular regeneration[59], however senescent cells can also release inflammatory cytokines, that are pro-tumorigenic, and impair insulin sensitivity[58,60,61]. Although the SASP can vary according to cell type, general pro-inflammatory factors found in the SASP include pro-inflammatory cytokines

such as interleukin-6 (IL-6), interleukin-8 (Il-8), and monocyte chemoattractant protein-1 (MCP1)[20]. These cytokines are involved in cellular pathways such as inflammation, the immune response, and cancer, and their dysregulation as a part of the SASP can affect neighbouring cells[20,62–64].While the SASP can be released as soluble circulating factors, it can also be released via small, membranous nanoparticles known as extracellular vesicles (EVs)[65,66], which are known to contribute to the pro-inflammatory effects associated with SASP[65,67].

1.5. Extracellular vesicles (EVs)

EVs are nanoparticles that can be secreted from all living cells, and play a vital role in intercellular communication. EVs can carry a plethora of molecular cargo, such as proteins, nucleic acids, and lipids, throughout the body[68]. Classically, there are three main types of EVs: apoptotic bodies, microvesicles, and exosomes. They differ in size, biogenesis, and cargo. Despite these differences, all EVs share a lipid bilayer membrane that protects their cargo from the extracellular environment[68]. More recently, the Minimal information for studies of extracellular vesicles (MISEV) 2018 guidelines recommend classifying EVs as either small (sEVs, <200 nm) or medium/large (m/lEVs, >200 nm) in size[69], until purity of endosomal origin is confirmed quantitatively by expression of marker proteins, and imaging by transmission or scanning electron microscopy.

1.5.1. Types of EVs

Apoptotic bodies, the largest type of EV, range from 500-5000 nm in size[68]. They are released by the outward blebbing of the plasma membrane of cells undergoing apoptosis, and as a result largely contain the remnants of these cells[68]. Specifically, apoptotic bodies protrude outward from apoptotic cells by stacking on top of each other in a bead-like formation and are regulated by actin-myosin interactions, pannexin-1, and plexin-B2[70,71].

Microvesicles are about 100-1000 nm in size, and also released by an outward blebbing of the plasma membrane whereby the membrane pinches in on itself to release the vesicle[72,73]. Due to their unique biogenesis, microvesicles can be identified via surface markers such as ADP-ribosylation factor 6 (ARF6) and matrix metallopeptidase 2 (MMP2)[74]. ARF6 is necessary for microvesicle formation in tumour cells, regulating actomyosin-based release of outward blebbings from the plasma membrane[75]. MMP2 has been consistently found to be incorporated into the

membranes of microvesicles, although the exact mechanism by which this occurs remains unclear[76,77]. While EV research generally focuses on exosomal content, microvesicles have been shown to harbour a wide variety of content, both within the vesicle and on its membrane. Tumour-derived microvesicles with exposed Fas ligand have been shown to induce apoptosis in lymphocytes[78,79]. Furthermore, microvesicles can contain organelles (mitochondria), nucleic acids and proteins as intraluminal cargo[80].

Exosomes, or sEVs are on average 30-150 nm in size[72,81]. Exosome biogenesis involves the formation of intra-luminal vesicles inside of a multivesicular body (MVB). MVB cargo can either be recruited in an endosomal sorting complex required for transport (ESCRT)-dependent or independent manner. ESCRT proteins 0, I, and II recruit ubiquitinated cargo to the MVB membrane, where it is shuttled along the membrane by ESCRT II[82–84]. ESCRT-independent MVB cargo recruitment is achieved through various proteins, such as tetraspanins CD9, CD63, and CD81[85,86].Once cargo has arrived at the MVB membrane, ESCRT III is responsible for cutting off the membrane invagination away from the cytoplasm, thus forming an intraluminal vesicle[83]. MVBs can either travel to the lysosome to deposit their contents, or fuse with the plasma membrane by way of SNARE proteins to release their contents as exosomes[82,83] The purity of exosomal preparations is ascertained by Western blotting for specific marker proteins such as CD9, HSP70, Flotillin-1, and TSG101[68], biophysical characteristics (size, density), as well as by electron microscopy (transmission, scanning, or cryo) imaging. An important caveat to note is that not all exosomes express each exosome-specific protein, but are enriched in the expression of one or more of them. It is common to identify exosomes by the markers they express e.g. $CD9^+/CD81^+$ exosomes.

1.5.2. EV isolation methods

There are a plethora of validated protocols that can be used for isolating EVs. Standard methods include differential ultracentrifugation (dUC), size exclusion chromatography (SEC), poly-ethylene glycol (PEG) precipitation, and ultrafiltration among others[81]. Choosing a particular method of isolation is often dependent on the medium from which EVs are being collected. This is because EV isolation methods are governed by two important factors: yield and specificity[69]. For example, using an isolation method such as PEG will result in a high protein yield from the sample. Unfortunately, this high yield comes at the cost of a relative lack of specificity as PEG

will co-precipitate non-EV proteins as well. Conversely, isolating EVs using SEC will result in high specificity for EVs at the cost of overall protein yield. As a result, some methods are more feasible than others depending on the experimental design. For isolation of sEVs from cell culture media, dUC is recommended[87]. For isolation of sEVs from human plasma, SEC has emerged as an ideal EV isolation method due to the rich abundance of EVs in plasma[81,87,88]. Often a combination of one or more techniques is needed to obtain the optimal yield and purity of sEVs or exosomes. This means using dUC or SEC in combination with ultrafiltration, or immune-affinity capture methods[81].

1.6. EVs and aging

While EVs are known to be involved in the SASP[89–91], the relationship between EVs and aging is still being explored. A number of studies have evaluated the biochemical characteristics, concentration, and cargo content of EVs with age using *in vivo*, *in vitro* and *ex vivo* methods in different species as described below. Interestingly, EVs derived from young and old mice have also been used successfully in longevity experiments in mice, and in co-culture experiments using human cells[92,93]. The remarkable ability of EVs to transmit cellular changes seems very promising in the quest to generate anti-aging strategies for the burgeoning aging population worldwide.

Reference	Donor species/tissue	Sample Size	Population age	EV size	EV concentration	EV protein yield	EV protein markers
Alberro *et al*., 2016[94]	Human / *in vivo*	37	21-92 y/o	N/A	No difference	N/A	N/A
Eitan *et al*., 2017[95]	Human / *in vivo*	74	32-61 y/o	No difference	↓	N/A	N/A
Davis *et al.*, 2017[96]	Mouse / *in vivo*	16	2-28 m/o	No difference	No difference	N/A	N/A
Lee *et al.*, 2018[97]	Rat / *in vivo*	6	7-22 m/o	No difference	N/A	N/A	No difference
Terlecki-Zaniewicz *et al.*, 2018[98]	Human / *in vitro* human dermal fibroblasts	3		No difference	↑	N/A	N/A
Bertoldi *et al.*, 2018[99]	Rat / *in vivo*	4-6	3-26 m/o	N/A	↓	N/A	N/A
Su *et al.*, 2019[100]	Mouse / *in vivo*	6-7	3-18 m/o	No difference	No difference	N/A	N/A
Huang *et al.*, 2019[101]	Human / MSC *in vitro*	2	25-72 y/o	N/A	No difference	N/A	No difference
Alberro *et al.*, 2019[102]	Human / *in vivo*	51	20-104 y/o	N/A	N/A	N/A	No difference in

							senescent markers
Zhang *et al.*, 2020[103]	Human / immune cells *in vivo*	28	40-68 y/o	N/A	↓	N/A	N/A
Willis *et al.*, 2020[104]	Mouse / *in vitro*	7-11	1-3 m/o	No difference	No difference	N/A	N/A
Alibhai *et al.*, 2020[105]	Mouse / *in vivo*	8	3-21 m/o	↓	↓	N/A	↑ TSG101, CD63, CD81 ↓ APOA1
Grenier-Pleau *et al.*, 2020[106]	Humans / *in vivo*	35	20-85 y/o	No difference	No difference	No difference	N/A
Tsukamoto *et al.*, 2020[107]	Mouse / *in vivo*	12	2-18 m/o	No difference	No difference	N/A	N/A
Khanh *et al.*, 2020[108]	Human / *in vitro* Adipose-tissue MSCs	10	1-80 y/o	No difference	N/A	N/A	No difference
Mensa *et al.*, 2020[109]	Human / *in vitro* Human umbilical vein cells (HUVEC)	1		No difference	↑	N/A	N/A

Table 1: EV characteristics comparing young and aged samples. Summary of recent findings documenting changes in EV biochemical characteristics with age *vs*. young. Studies examining senescent cells are highlighted in blue. N/A = measurement was not made in the study. No difference = no difference between age groups. *M/O = months old; Y/O = years old.*

1.6.1. Biochemical characteristics of aged EVs

A summary of literature comparing the biophysical characteristics of EVs in young and old subjects is outlined in **Table 1.** Alberro *et al.* (2016) provide the first direct comparison of EVs between chronologically young and old humans[94]. While studies prior to this compared microparticles from young and old participants[110,111], a lack of methodological details and absence of standardization experiments for EV characterization as mandated by MISEV 2018 guidelines[69,112] made it difficult to extrapolate and interpret data from these studies.

The data on changes in EV biochemical characteristics such as size, concentration, protein yield and expression of specific markers is variable. Alberro and colleagues compared three groups of young participants (21-30 year old; 31-40 year old; 41-50 year old) to older adult participants (72-92 year old), who were further divided into robust, frail, or non-autonomous groups[94]. The authors reported that EV concentration did not change between age groups[94]. Several other studies as detailed in **table 1** reported lack of any differences in EV concentration with age in

humans[101,104,106] or rodents[96,100,107]. Conversely, others have reported that EV concentration decreases with age. Eitan *et al.* (2017) compared EVs from participants 30 *vs.* 60 years of age and found EV concentration was reduced with age[95]. This finding was also reported in other studies in humans[103], mice[105], and rats[113]. In opposition to this, studies examining senescent cell-derived EVs demonstrated that senescent cells show an increased concentration of EVs released from the cells[89,109]. The variability in results could be a result of differences in EV isolation and characterization, as well as due to differences in cohort with respect to sex, gender, and race/ethnicity. Taken together, these results illustrate that the relationship between EV concentration, biogenesis, release and age has yet to be fully elucidated, and requires the use of standardized, validated EV methodologies.

While the literature on the relationship between EV concentration and age is not unanimous, there appears to be more of a consensus on the relationship between EV size and aging. Eitan *et al.* (2017) were the first to directly compare and report that EV size distribution did not change with age[95], with others demonstrating similar findings in humans[89,104,106,108,109], mice[100,107,114], and rats[97]. Using rodent models in aging literature is generally performed by using the average lifespan of the rodent to make an approximation to human age. The average lifespan of mice is considered to be 2 years[115]. While laboratory rats are established to have an average lifespan of 3 years, Sprague Dawley rats are known to incur increased susceptibility to pneumonia and tumour incidence as early as 12 months of age[116,117]. In opposition to this consensus, Alibhai *et al.* (2020) found mean EV size to be reduced with age when comparing 3-month old to 18-month old mice[105]. Finally, when examining the relationship between EV sub-type protein marker expression (mostly exosomal proteins) and age, Lee *et al.* (2018) compared EVs from 7-month and 22-month old Sprague Dawley rats and found no change in expression of exosome-markers CD63 and Alix[97], with several studies in humans reporting the same[101,102,108]. In contrast, Alibhai *et al.* (2020) found that 18-month old mice expressed greater levels of exosome-specific markers such as TSG101, CD63, and CD81 than their 3-month old counterparts, as well as less APOA1, a marker of lipoprotein contamination. Only one study has compared EV protein yield in young *vs.* old to date and it found no differences in participants aged 20-39, 40-59, and 60-85 years[106].

1.6.2. EV cargo content with age

Literature on EV content and age is largely focused on miRNA content of EVs due to the potential of miRNAs in EVs to serve as biomarkers and potential therapeutics[118]. A full list of EV cargo content changes with age is listed in **Table 2**. Interestingly, of all miRNAs found to increase or decrease in EVs with age, no two studies found the same miRNA to be related to age[89,95,97,99,100,105,107,109,119–124], except for miR-21which was found to be increased with age in 3 studies[105,107,109] and miR-145 increased in two papers[103,105]. miR-21 is a highly conserved, and ubiquitously expressed microRNA that has been used a biomarker for ~29 diseases[125] and miR-145 is thought to function as a tumor suppressor[126]. This indicates that further research is needed, and that EV miRNA changes in response to age may be transcript-, species- and/or tissue-specific. Notably, proteins (specifically those not used for EV sub-type determination) found to decrease with age in EVs included proteins related to apoptosis[95], scavengers of free radicals[113], and those linked with cellular metabolism[120]. Since EV content changes with age, along with its cargo, it can modulate cellular function of recipient cells. Comparing the treatment effects of administering young *vs*. aged EVs on cellular hallmarks of aging can facilitate a better understanding of the role of EVs in maintaining homeostatic balance with age.

Reference	Species/tissue	Increased in Aged EVs	Decreased in Aged EVs
Weilner *et al.*, 2016[123]	Human / *in vivo*	N/A	Galectin-3
Eitan *et al.*, 2017[127]	Human / *in vivo*	CD151 MUCIN16	p53 Cleaved PARP Cleaved Caspase-3
Lee *et al.*, 2018[124]	Mouse / *in vivo* plasma	miR-184-3p miR-200b-5p miR-708-5p	miR-126b-5p miR-466c-5p
Kulkarni *et al.*, 2018[119]	Mouse / *in vitro* MSC	miR-17 miR-34a	N/A
Lee *et al.*, 2018[97]	Rat / *in vivo*	miR-500-3p miR-770-3p miR-6324 miR-455-3p miR-487b-3p miR-26b-3p miR-127-3p miR-148-3p	miR-450a-3p miR-196c-3p miR-34b-3p miR-10a-3p
Bertoldi *et al.*, 2018[99]	Rat / *in vivo*	DCF	SOD
Terlecki-Zaniewicz *et al.*, 2018[98]	Human / *in vitro* human dermal fibroblasts	miR-23a-5p miR-137	N/A
Yoshida *et al.*, 2019[120]	Humans / *in vivo* plasma Mouse / *in vivo* plasma		eNAMPT eNAMPT
Su *et al.*, 2019[100]	Mice / *in vivo*	miR-29b-3p	N/A
Khayrullin *et al.*, 2019[128]	Human / *in vivo*	C24:1 ceramide	N/A
Zhang *et al.*, 2020[103]	Rat / *in vivo*	miR-150-3p miR-378-3p miR-199a-5p miR-145-5p miR-598-3p miR-122-5p miR-194-5p miR-203a-3p miR-202-3p miR-145-5miR-532-5p	miR-181-5p miR-133-5p
Alibhai *et al.*, 2020[105]	Mouse / *in vivo*	miR-145a miR-21 miR-22 miR-223 miR-145 let-7a	N/A
Tsukamoto *et al.*, 2020[107]	Mouse / *in vivo*	miR-19b miR-322 miR-192 miR-21 miR-181	N/A
Mensa *et al.*, 2020[109]	Human / *in vitro* HUVEC	miR-21-5p miR-217	N/A

Table 2: Changes in EV cargo content with age. Summary of literature on the changes in EV cargo content with age. Data are stratified by cargo that 1) increased in EVs isolated from aged

subjects, 2) decreased with age, or 3) did not change with aging. Literature focusing on senescent cells is highlighted in blue. N/A = measurement was not made in the study.
MSC: mesenchymal stem cells, miR: micro RNA; PARP: poly ADP-ribose polymerase; SOD: superoxide dismutase; eNAMPT: extracellular nicotinamide phosphoribosyltransferase; HUVEC: human umbilical vein cells; DCF: oxidized 2',7'- dichloroflourescein diacetate; MUCIN16: cancer antigen 125.

1.7. Frailty and EVs

Literature comparing the relationship between frailty and EVs is scant. Alberro *et al.* (2016) compared EVs isolated from plasma of 72-92 year-olds of robust and frail status and found no change in EV concentration with frailty[94]. Ipson *et al.* (2018) identified several miRNAs increased in EVs with frailty[129] (**Table 3**). Of the miRNAs found to increase with frailty status, mir-92a-3p can regulate cartilage development in primary human osteoarthritic chondrocytes by targeting wingless-related integration site 5A (WNT5A), which is known to be integral to chondrogenesis, the process by which cartilage is formed[130,131]. Additionally, exosomal miR-326 can help relieve inflammatory bowel disease[132], and exosomal miR-532-5p has been shown to reduce intervertebral disc degeneration[133]. Finally, Picca and colleagues (2020) found that frail individuals produced higher amounts of sEVs than their robust counterparts, and these sEVs contained less CD9 or CD63, hallmark exosomal marker proteins[134].

Reference	Species/tissue	Increased with Frailty	Decreased with Frailty	No change with frailty
Alberro *et al.*, 2016[94]	Human / *in vivo*	N/A	N/A	EV concentration
Ipson *et al.*, 2018[129]	Human / *in vivo*	miR-10a-3p miR-92a-3p miR-185-3p miR-194-5p miR-326 miR-532-5p miR-576-5p miR-760	N/A	N/A
Picca *et al.*, 2020[134]	Human / *in vivo*	Concentration of sEVs	EVs positive for CD9 or CD 63	N/A

Table 3: Changes in EV cargo content from robust *vs.* frail EVs. Summary of literature comparing cargo content in EVs from robust and frail participants. Data are stratified by cargo that 1) increased in EVs isolated from frail subjects, 2) decreased with frailty, or 3) did not change with frailty status. N/A = measurement was not made in the study.

1.8. EV treatment using EVs isolated from young and old samples

A summary of studies that have utilized EVs from young/old samples to reverse cellular dysfunction with age is presented in **Table 4**. In 2016, Weilner *et al.* treated adipose-tissue (AT) derived mesenchymal stem cells (MSCs) with EVs from young or aged human plasma and found that young EVs induced significantly more osteogenic differentiation in treated cells compared to aged[123]. Given that young embryonic stem (ES) cells reduce SA-β-GAL staining and increase the presence of cellular antioxidants in treated human vascular cells[135], the effect of young human ES cell derived-EVs in reducing senescence and increasing antioxidant levels seems intuitive[8,108]. Treating senescent human dental pulp stem cells (HDPSC) with EVs from young HDPSC, also reduced SA-β-Gal activity in treated cells[136]. Wang *et al.* (2018) examined the effects of murine serum EVs from young and aged donors and found that young EVs reduced serum and splenic levels of inflammatory cytokines, and increased thymus size and thymocyte count in aged mice[137]. Treatment with young EVs also reduced the expression of proteins involved in cell turnover, and increased telomerase in the lungs and liver of treated mice, tissues where EVs preferentially localize[124]. Khayrullin *et al.* (2019) demonstrated that aged human and non-human primate EVs showed an increase in C24:1 ceramide levels compared to young EVs. Using a rodent model, Khayrullin *et al.* loaded serum EVs with C24:1 ceramide and treated primary bone marrow stem cells with these C24:1-enriched EVs. The authors noted that ceramide-enriched EVs induced senescence in the treated bone marrow stem cells that were derived from a young mouse[121]. Young murine EVs can reduce cell proliferation and senescence, increase antioxidant levels, and reduce levels of circulating inflammatory cytokines[8,119,135]. Interestingly, Yoshida *et al.* (2019) reported that injecting EVs isolated from young mice into 26-month old mice increased lifespan of treated mice by 2 months[120], demonstrating the potency of young EVs to attenuate cellular and organismal aging.

While young human EVs have been shown to induce positive benefits, the effects of aged human EVs on cellular function are less clear. Aged human EVs are known to induce calcification in human aortic smooth muscle cells, increase bone resorption in treated osteoclasts, and reduce differentiation in oligodendrocytes[104,138,139]. Furthermore, human senescent-cell derived EVs can reduce apoptosis in fibroblasts, and yet increase early and late apoptosis in human umbilical vein endothelial cells (HUVEC)[89,109]. Treatment of HUVEC with senescent EVs also reduced cellular

SIRT1, an established regulator of metabolism[140] and longevity[109,141]. Others have reported that treating C2C12 and 3T3-L1 cells with aged mice EVs inhibited glucose-stimulated insulin uptake, and treating young insulin-sensitive mice with aged mice EVs increased fasting blood glucose and insulin levels, and inhibited insulin signaling in several tissues[100]. Treating macrophages with aged mouse plasma EVs both reduced IL-6 expression and increased phagocytic activity[104,105] Collectively, this suggests an age- and tissue-dependent disparity in cellular effects with EV treatment.

While great strides have been made in deciphering the link between aging and EVs, information on the effect of frailty on EVs is thoroughly lacking. Further, the effect of biological age on the ability of EVs to reverse cellular signs of aging has not been investigated to date. Lastly, despite skeletal muscle representing 40% of body mass, no investigation into the effects of biologically young EVs on primary human skeletal muscle cells has been performed. Exploring the effects of biologically young and old EVs in tandem with frailty assessments on skeletal muscle cells can help develop a greater understanding of how EVs impact the musculoskeletal system with age.

Reference	Species/tissue	Sample size	Age of isolated EVs	Age of treated group	Young EV treatment	Aged EV treatment
Weilner *et al.*, 2016[123]	Human / AT-MSC	4	25-55 y/o	23-47 y/o	Increased osteogenic differentiation	N/A
Alique *et al.*, 2017[138]	Human / endothelial cells *in vitro*				N/A	Induced calcification in human aortic smooth muscle cells
	Human / plasma	26	23-78 y/o		N/A	Induced calcification in human aortic smooth muscle cells
Wang *et al.*, 2018[137]	Mouse / *in vivo* serum	4-8	2-20 m/o	18-20 m/o C57BL6	Reduced serum IL-6, serum IL-1b, splenic TNF-α, $CD3^+$ T-cell blood-derived macrophages Increased thymus size and thymocyte count	N/A
Lee *et al.*, 2018[124]	Mouse / *in vivo* plasma	3	3-24 m/o	15 m/o C57BL6	Localized in lungs and liver Reduced IGF1R, P16, Men1, and Mre11a in lungs and liver	N/A
Kulkarni *et al.*, 2018[119]	Mouse / *in vitro* Lin^- treated with MSC EVs	3	2-24 m/o	18-24 m/o	Reduced cell proliferation	N/A
Xie *et al.*, 2018[139]	Human / osteoclasts *in vitro*	62	21-70 y/o		N/A	Increased bone resorption in osteoclasts
Terlecki-Zaniewicz *et al.*, 2018[89]	Human / dermal fibroblasts	3			N/A	Reduced apoptotic activity in fibroblasts
Yoshida *et al.*, 2019[120]	Mouse / *in vivo*	4	4-26 m/o	20 m/o C57BL6	Extended lifespan	N/A
Chen *et al.*, 2019[135]	Mouse / *in vivo* ESC EV treatment	6		1.5-2 m/o C57BL6	Accelerated wound healing and angiogenesis at wound site	N/A
	Human / *in vitro* HUVEC treated with ESC EV	3			Reduced SA-β-Gal and MDA, increased SOD, GSH, CAT, and cellular miR-200a	
Khayrullin *et al.*, 2019[121]	Human / mouse BMSC *in vitro*	5	25-90 y/o		EVs loaded with C24:1 ceramide induced senescence	N/A
Su *et al.*, 2019[100]	Mouse / 3T3-L1 and C2C12 *in vitro*	6-7	18 m/o		N/A	Inhibited insulin-stimulated glucose uptake, increased cellular miR-29b-3p
	Mouse / *in vivo*	6-7	3-18 m/o	2 m/o C57BL6		Increased fasting blood glucose and fasting serum insulin, inhibited insulin signaling in epididymal white adipose tissue, skeletal muscle, and hepatocytes
Khanh *et al.*, 2020[108]	Human / *in vitro* Adipose tissue MSCs	10	1-80 y/o		Reduced SA-β-Gal and cellular ROS Upregulated SOD1 and SOD3 expression	Had no effect on SA-β-Gal or cellular ROS in treated aged cells

Fafian-Labora *et al.*, 2020[8]	Human / *in vitro* donor fibroblasts	4	1-3 y/o	67-81 y/o	Reduced senescence, cellular ROS levels, DNA damage Increased cell growth, GST activity, cellular GSH levels	N/A
	Mouse / *in vivo*	3-5	2.5-25 m/o	22-25 m/o C57BL6	Reduced SA-β-Gal staining in the liver, lungs and brown adipose tissue, reduced circulating IL-6 and GM-CSF, reduced serum ROS levels	
Tsukamoto *et al.*, 2020[107]	Mouse / plasma	16	2-18 m/o	2-18 m/o C57BL6/ 6JrSlc	N/A	Reduced IL-6 expression in macrophages
Alibhai *et al.*, 2020[105]	Mouse / *in vitro*	8	3-21 m/o		N/A	Aged EVs increased phagocytic activity in macrophages
Willis *et al.*, 2020[104]	Human / astrocytes	4			N/A	Reduced oligodendrocyte support and differentiation
Grenier-Pleau *et al.*, 2020[106]	Human / HSC *in vitro*	35	20-85 y/o		N/A	Increased colony forming capacity of HSCs
Mas-Bargues *et al.*, 2020[136]	Human / *in vitro* Dental pulp cells	3			Reduced SA-β-Gal	N/A
Mensa *et al.*, 2020[109]	Human / *in vitro* HUVEC	1			N/A	Increased cellular miR-21-5p and miR-217, cellular apoptosis Reduced cellular SIRT1, DNMT1

Table 4: EV treatment with age. Summary of literature comparing the treatment of young and aged EVs in mice and humans. Literature focusing on senescence is highlighted in blue. N/A = measurement was not made in the study.
IGF1R: insulin-like growth factor 1 receptor; Men1: menin 1; MSC: mesenchymal stem cells.

Chapter 2: Study Design and Methods

2.1. Statement of Problem

EVs are involved in cell-to-cell communication and can potentiate anti-aging effects. EV sub-type and cargo is plastic and can change according to the cellular milieu. Characterizing EVs from people along the spectrum of frailty (robust, pre-frail and frail), in tandem with biological age deceleration/acceleration will give insight into how EV-based cell-to-cell communication differs with biological age and frailty status. If EVs from biologically young, non-frail subjects carry protective cargo that is beneficial for rescuing age-associated decline in physiological and biochemical function, then it follows that treating old cells with young EVs will mediate positive adaptations and rescue cellular deficits associated with aging such as metabolic dysfunction and senescence. The corollary is that treating old cells with biologically old/frail EVs would exacerbate metabolic deficits and markers of senescence already inherent in the old cells.

2.2. Objectives

The objectives of this proposal are to:

1. Characterize changes in EV content and biophysical characteristics in frail, pre-frail, and robust participants from the WARM Hearts Study.
2. Examine the effects of EVs stratified by frailty and biological age, on modifying the cellular hallmarks of aging (metabolism, senescence) in young and old muscle cells *in vitro*.

2.3. Hypotheses

Our hypotheses are:

1. EV biochemical characteristics (size, stability, protein yield, expression of proteins related to EV sub-types) will differ according to frailty status.
2. Treating chronologically old skeletal muscle cells with robust, biologically young EVs will alleviate the cellular hallmarks of aging present in the treated cells.
3. Conversely, treating chronologically young skeletal muscle cells with frail, biologically old EVs will impair the cellular hallmarks of aging in the treated cells.

2.4. Research Design

We used a case control design to assess EVs in WARM Hearts participants as a function of frailty status. Furthermore, an *ex vivo* cell culture model was utilized to examine the effects of frail *vs*. robust EV treatment on the cellular hallmarks of aging e.g. metabolism, and senescence in young and old human skeletal muscle cells. EVs were isolated, characterized and used in line with the minimal information for studies of extracellular vesicles (MISEV) guidelines[69]. We characterized EVs by size, zeta potential, transmission electron microscopy (TEM), and analyzed the expression of EV sub-type markers and non-EV co-isolate proteins.

Study participants. Plasma samples were obtained from the WARM Hearts study (Clinical Trials #NCT02863211) led by Dr. Todd Duhamel at the University of Manitoba. Whole blood from Caucasian postmenopausal women (64±5.97 years) participating in the WARM Hearts study was collected for secondary EV analysis in our laboratory as detailed below. Research was conducted with institutional ethics approval from the Research Ethics Board at the University of Manitoba (REBH2019:063), and in accordance with the Declaration of Helsinki and STROBE guidelines[142]. Briefly, blood was collected in tubes containing EDTA and immediately centrifuged at 2000x *g* for 10 min at 4 °C. Plasma was carefully collected, aliquoted and frozen at -80 ºC. Members from Dr. Duhamel's laboratory stratified samples (N=330) by frailty status (23 frail, and 23 robust) using the Frailty Index method[143] and participants were matched by age, gender, ethnicity, smoking status, and socioeconomic status. A further subset of pre-frail participants (N=12-14) was also included. All plasma samples were used for EV isolation and biophysical characterization as described below in our laboratory (see study schematic, **Fig.1A)**. Frail and robust samples were further stratified by biological age as determined by epigenetic age (eAge) measured by DNAm characterization (Illumina EPIC microarray) by collaborator Dr. Meaghan Jones at the University of Manitoba. Briefly, Dr. Jones' group used the PhenoAge clock, a multifactorial epigenetic clock that predicts for outcomes such as all-cause mortality, health span, and physical function[144,145]. After samples were sorted by biological age, the top 10 frail and biologically oldest, and the 10 robust and biologically youngest samples were used for EV co-culture experiments.

Sample storage and preparation. Plasma samples were collected as described above and stored at -80 °C. Samples were thawed on ice for up to 90 min prior to EV isolation. Thawed plasma samples were filtered in the following manner: 0.22 µm filters (Millipore, SLGP033RB) were pre-

wet with 500 µl of 1X PBS. 500 µl plasma samples were then loaded into the filter using a 1 ml syringe. After filtration, 150 µl of plasma sample was immediately used for EV isolation as described below. Leftover filtered plasma was stored at -80 °C.

Small EV isolation. EVs were isolated using size exclusion chromatography (SEC) according to manufacturer's guidelines. qEVsingle columns (35nm, Izon) were thawed at room temperature for 30 min. Columns were then drained of void volume and flushed with 3.5 ml of 1X PBS. ***For EV characterization***: 150 µl of pre-filtered plasma was loaded into the column and flushed with 1.0 ml of PBS. Fractions 7 through 10 were collected by flushing columns with 800 µl of PBS as previously described[146]. After EV isolation was complete, 20 µl of EVs were immediately analysed for size distribution and zeta potential using a NanoBrook ZetaPALS instrument as described below. Remaining EVs were concentrated and stored at -80 °C for Western blotting analysis. ***For EV co-culture experiments***: 100 µl of pre-filtered plasma was diluted with 50 µl of PBS before being loaded into the column and flushed with 1.0 ml of 1X PBS. To improve small EV purity isolated from plasma, fractions 7 through 9 were collected by flushing columns with 600 µl of PBS[87]. Freshly isolated EVs were used for co-culture experiments as detailed below.

EV characterization. EV zeta potential and size were measured by phase analysis light scattering using a NanoBrook ZetaPALS instrument (Brookhaven) and analyzed using ZetaPlus software as we described before[146]. Freshly isolated EVs (20 µl) were diluted 50 times and loaded into 1.5 ml Fisherbrand Disposable Cuvettes (Fisher Scientific). The sample underwent 5 runs in the NanoBrook ZetaPALS and multimodal size distribution was used to determine the amount of EVs per binned size group. Zeta potential analysis was performed using a Solvent-Resistant Electrode (NanoBrook) and BI-SCGO 4.5 ml cuvettes (NanoBrook). Smoluchowski formula was used to calculate zeta potential and final results were averaged irrespective of number sign.

EV concentration, protein extraction and yield. Samples isolated with SEC were loaded onto Ultra-4 Centrifugal Filter columns (Amicon), and centrifuged at 14,000x *g* for 60 min at 4 °C, until approximately 50 µl of concentrated sample remained. Concentrated EV fractions were used for protein extraction as described before[146]. Briefly, RIPA solution (ThermoFisher) containing EASYPack Protease Inhibitor Cocktail (Roche) was added at a 1:1 ratio to the EV fractions, vortexed, sonicated 3 x 3 seconds at 50% amplitude, and then centrifuged at 16,000x *g* for 15 min at 4 °C. Supernate was collected and used for analysis of protein concentration using the

MicroBCA (ThermoFisher) assay as previously described[146]. Concentration of EVs (µg/µl) was multiplied by volume of EV yield (µl) to generate EV protein yield (µg).

Western blotting. For immunoblotting, 20 µl of β-mercaptoethanol were added to 180 µl of laemmli buffer to make a 5% solution of β-mercaptoethanol. Then, the solution was mixed with EV protein lysates and incubated at 95 °C for 5 min. Following protein denaturation, 5 µg of total protein were loaded onto 12% SDS-PAGE gels, and electrophoresed for 30 min at 90 volts, followed by 90 min at 120 volts. Proteins were transferred to nitrocellulose (NCL) membranes using a Trans-Blot® Turbo™ (Bio-Rad) for 9 min. Membrane were stained with Ponceau S to ascertain loading and transfer, and gels stained with Coomassie Blue to ascertain equal loading and transfer. Next, the membranes were washed with 1X Tris-buffered saline (TBS) for 10 min and blocked with 5% skim milk in TBS-Tween20 (TBST) solution for 1 hour at room temperature. Membranes were incubated with the primary antibodies in 1% skim milk overnight at 4 °C. The following day, membranes were washed 3 x 5 min with TBST and incubated with the appropriate secondary (anti-mouse/anti-rabbit) IgG horseradish peroxidase secondary antibody for 1 hour at room temperature. Membranes were then visualized by enhanced chemiluminescence (ECL) detection reagent (Bio-Rad). Membranes were imaged and scanned using trans-illuminator ChemiDoc^{TM} System (Bio-Rad). Band densities were normalized to the respective Coomassie Blue Gel as a loading control. EVs were analyzed for expression of ***proteins associated with exosomes***: TSG101 (Sigma-Aldrich, #T5701-200UL), CD81 (Santa Cruz, #sc-166029), CD9 (Sigma-Aldrich, #CBL162), Alix (Bio-Rad, #MCA2493), and Flotillin-1 (Sigma-Alrich, #F1180-200UL); ***for proteins enriched in microvesicles***: MMP2 (Santa Cruz, #sc-13595 AC), and ARF6 (Santa Cruz, #sc-7971); and ***non-EV marker proteins:*** Apo-A1 (Bio-Rad, #0650-0050) to determine purity of isolation.

Epigenetic age. DNAm characterization performed using the Illumina microarray was used to determine eAge, by collaborator Dr. Meaghan Jones' laboratory[145]. Epigenetic age acceleration (eAgeAccel) was then calculated by DNAm PhenoAge clock regressed on chronological age. Briefly, 23 robust and frail samples used for EV characterization were assessed for eAgeAccel. Robust EV samples were stratified as biologically young age if subjects presented with eAge deceleration, and frail samples were stratified as biologically old if they demonstrated eAge

acceleration. The 10 biologically youngest and robust samples, and 10 biologically oldest and frail samples were then used for co-culture treatment as described below.

Cell culture. Human donor skeletal muscle cells were purchased from Cook Myosite. Young (cat # PO1358-19F.2) and old (cat # PO1520-92F.1) donor cells were purchased from a 19- (F19) and 92-year-old (F92) female donor, respectively. Young and old cells were matched for gender and muscle tissue (vastus lateralis), as well as negative smoking and diabetes status. Cells were grown in Myotonic Basal Medium (Cook Myosite, #MB-2222), supplemented with 3.5 ml Myotonic Growth Supplement (Cook Myosite, #MS-3333), 10% fetal bovine serum (FBS, Life Technologies, #12483020) and 1 % penicillin-streptomycin (Fisher Scientific, #MT30002CI) per manufacturer's instructions.

Co-culture procedure. Young and old cells were plated into separate 6-well plates. Cells were seeded in 6-well dishes at 16,000 cells/well. The top row (3 wells) from both plates were co-cultured with 1) 5% plasma, 2) 100 µl small EVs (F7-9, isolated from 100 µl plasma) from robust and biologically young subjects and 3) 100 µl PBS (negative control). The bottom rows (3 wells) on both plates were treated with the same conditions (5% plasma, 100 µl EVs and 100 µl PBS condition) from frail and biologically old subjects (see study schematic, **Fig. 1B)**. Cells were treated on day 1 and day 3 and harvested on day 5 for measurement of all outcome variables. EVs were isolated fresh on each day of treatment (day 1, 3) as described above from the same starting volume of plasma (100 µl) from both groups to provide a physiological dose of EVs. After EV isolation was performed as described above, EVs were volumed up to 100 µl using PBS. Thus, each well was treated with the same volume (i.e. 100 µl) of either plasma, EVs, or PBS to standardize the additional volume added per well.

Cell harvest and cell count: Cells were washed twice with 1X PBS and trypsinized for 2 min in a 37 °C incubator. Growth media was added to neutralize the trypsin, and total media volume divided into two. Both halves were spun (1,000x *g* for 5 min at 22 °C). One half was frozen down as pellets to be used for DNAm analysis by Dr. Meaghan Jones' group. The other half was centrifuged at 1000x *g* for 5 min at room temperature. The supernate was discarded, cells resuspended in 1 ml of growth media, and counted using a BrightLine Hemacytometer (Sigma, #Z359629). Briefly, the cytometer was loaded with a 1:1 ratio of cells to 0.4% Trypan Blue (Sigma-Aldrich, #T8154-100ml) and cells counted by noting the total amount of cells in four

quadrants. Both halves of the hemacytometer were counted and the counts were averaged to derive cell count according to manufacturer's instructions. Treated cells were then divided into three: one-third were frozen to be used for immunoblot analysis of mitochondrial-related proteins. The other two-thirds were divided into two separate 24-well plates and seeded at 3,000-5,000 cells per well from each condition. Cells were allowed to adhere for 24 hours before analysis of: 1) mitochondrial mass (by MitoTracker Red CMXRos staining), and 2) senescence (SA-β-Gal) measurement.

MitoTracker staining. To prepare for mitochondrial staining, MitoTracker Red CMXRos (Invitrogen, #9082) was first prepared to a concentration of 0.1 M in 1X PBS. 60 µl of 0.1 M of MitoTracker Red CMXRos was mixed with 12 ml of aforementioned growth media to prepare staining solution. Cells were then washed twice with 1X PBS, stained with 1 ml of staining solution, and incubated for 30 min at 37 °C. Following incubation, cells were washed twice with 1X PBS and subsequently covered with 200 µl of growth media before imaging in a Zeiss Axiovert 200 Inverted Microscope. Images were taken in a single-blind fashion with 3 images at 10x intensity and 1 image at 40x intensity per condition and subsequently analysed using ImageJ.

Senescence measurement. Prior to co-culture, young and old cells were assessed for baseline senescence levels between age groups using a Senescence Detection Kit (Abcam, # ab65631). Senescence was then measured in treated cells harvested after 5-day treatment with EVs. First, cells were fixed using 20% formaldehyde. After 10 min, cells were washed with 1X PBS and stained with the staining solution to detect SA-β-Gal activity, a gold-standard marker of senescence[147]. After overnight incubation with the staining solution, representative images were taken at 4X and 10X magnification at 3 images per magnification using fluorescence microscopy (Cytation 5 Cell Imaging Multi-Mode Reader, in collaboration with Dr. Adrian West's group at CHRIM) and quantified using ImageJ.

Statistical analysis. Statistical analysis was performed using GraphPad Prism 9. EV biophysical characteristics between robust, pre-frail, and frail participants were measured using a one-way ANOVA with Tukey's post-hoc multiple comparisons test. Exosome and microvesicle markers were measured using Student's unpaired one-tailed t-test between robust and frail groups. Two-way ANOVA with Tukey's post-hoc multiple comparisons test was used to analyse readouts from co-culture treatment experiments, and EV size distribution. Effects of treatment within treated cells

were measured with paired Student's t-test between groups. Statistical significance was set at $p<0.05$. Exact p values are noted where applicable.

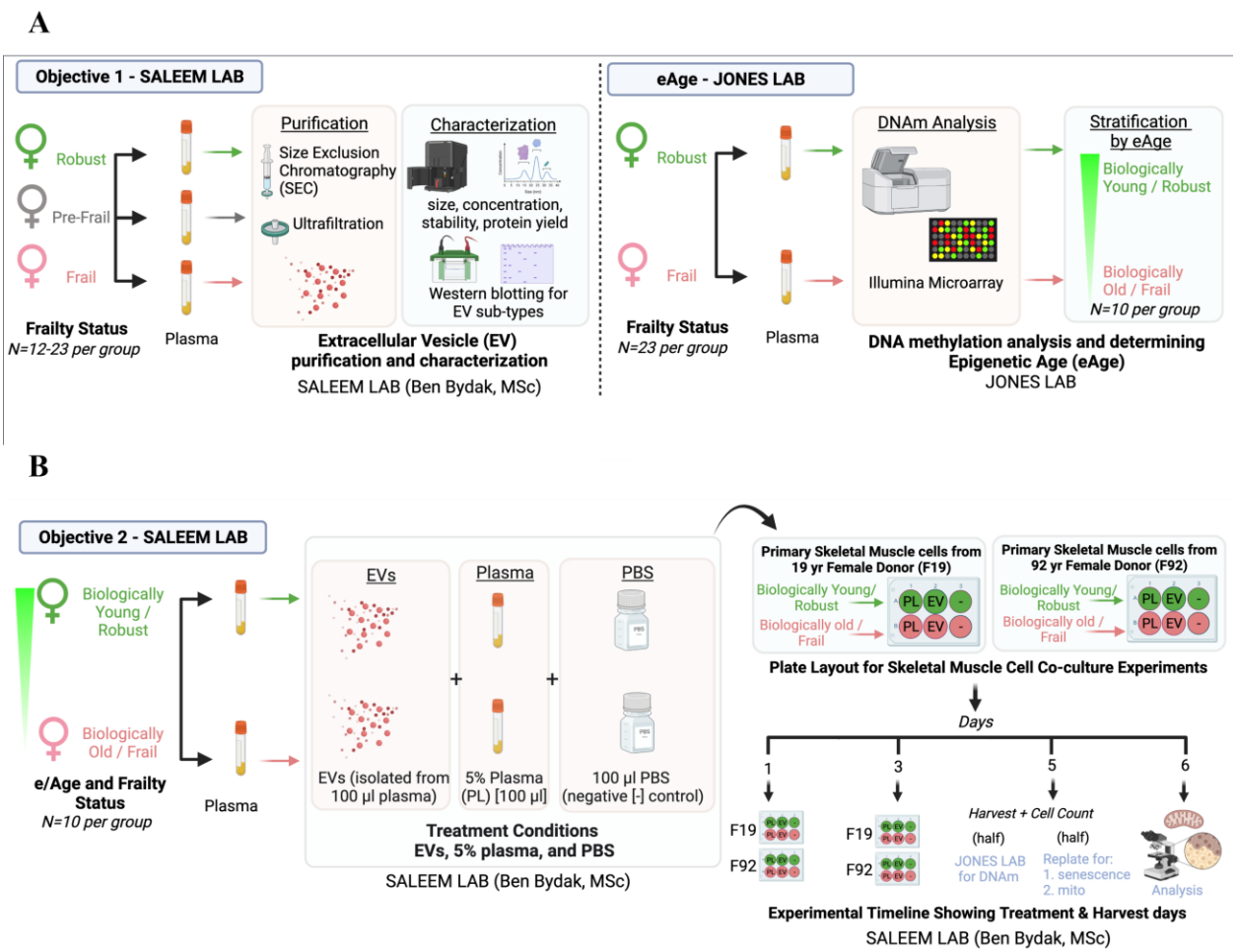

Figure 1. Schematic of study design - from EV isolation to co-culture treatment. (A) EVs were isolated from plasma samples using size exclusion chromatography. Isolated EVs were then characterized for size, zeta potential, protein yield, and protein markers. Epigenetic age was determined by the Jones lab and samples were stratified accordingly. **(B**) Young (F19) and Old (F92) donor primary human skeletal muscle cells were treated with 100 ul plasma or EVs (isolated from 100 ul plasma) from robust, biologically young or frail, biologically old participants. Treatment was performed on day 1 and 3 of co-culture. On day 5, media was saved for lactate analysis, cells collected and one half frozen to be used for DNAm analysis by Dr. Meaghan Jones lab. The other half was counted, and divided into thirds: one-third was used for immunoblot analysis, and the remaining plated into 24-well plates for mitochondrial and senescence assessment the following day.

Chapter 3: Results

EV biophysical characteristics with frailty status.

To ascertain EV biophysical characteristics in accordance with MISEV 2018[69], we first characterized EVs across frailty status for size, zeta potential, and protein yield. EVs from robust, pre-frail, and frail participants did not differ in average size (**Fig. 2A**), zeta potential (**Fig. 2B**), or size distribution (N=23, **Fig. 2D**). Frail EVs had 22% (*p=0.01) and 48.5% (*p=0.001) more protein than robust and pre-frail EVs, respectively (N=23, **Fig. 2C**). We next characterized robust and frail EVs by measuring the expression of protein markers enriched in EV subtypes (exosomes, microvesicles, non-EV protein co-isolates) according to MISEV guidelines[69]. Exosome markers CD9, TSG101, Flotillin-1, and HSP70 showed no significant difference in expression between robust *vs.* frail EVs (N=7-8, **Fig. 3A and 3B**). Microvesicle-specific marker ARF6 showed no significant difference in content between groups (N=6-7, **Fig. 3A and 3B**). ApoA1, a lipoprotein and non-exosome co-isolate, was 119% lower in frail EVs when compared to robust EVs (*p=0.01, N=8, **Fig. 3A and 3B**).

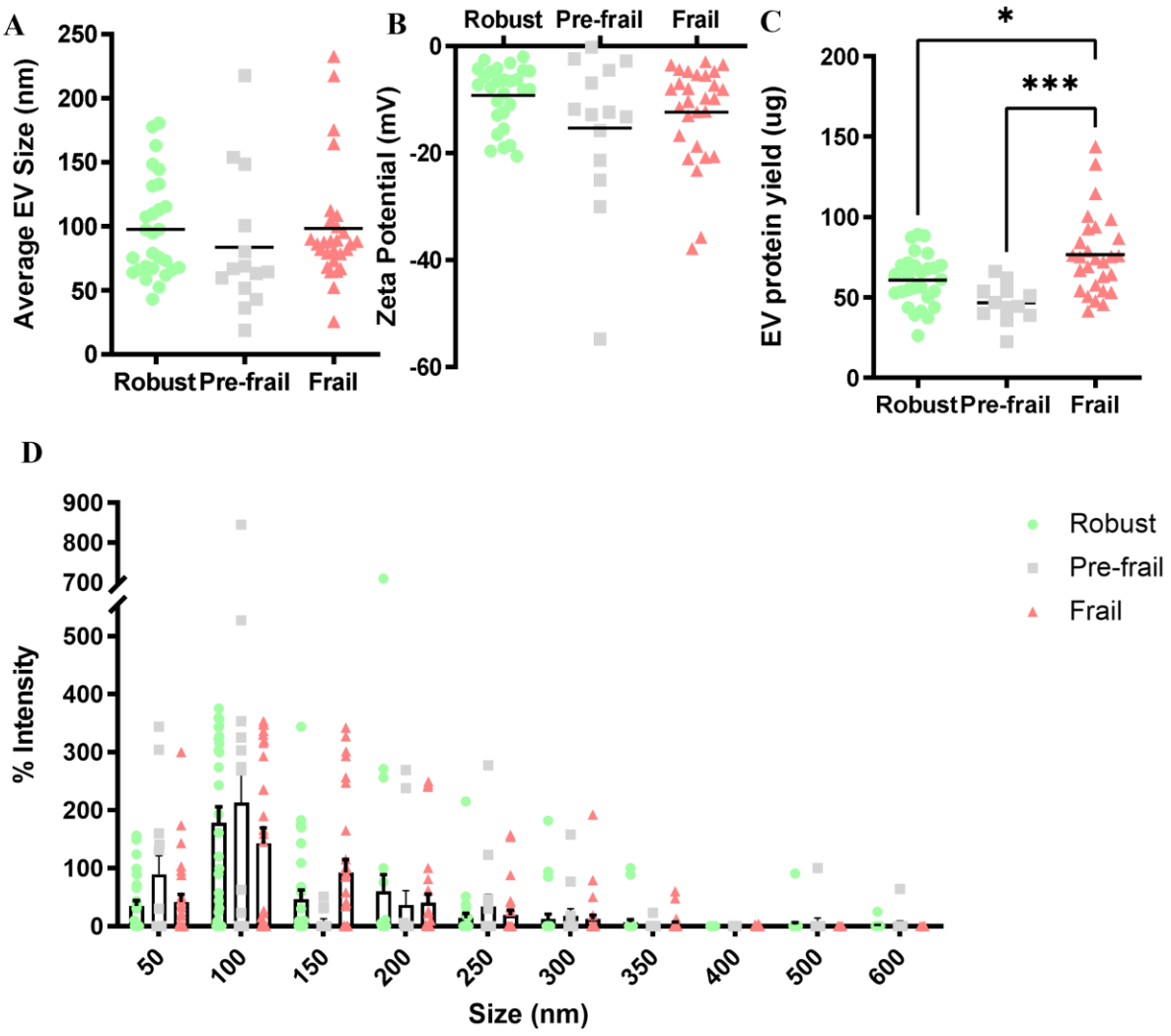

Figure 2. Changes in EV biophysical characteristics with frailty status. EVs from robust, pre-frail, and frail participants show no difference in **(A)** average EV size or **(B)** zeta potential. **(C)** EVs from frail participants yield 22% more protein when compared to EVs from robust participants, and 48.5% more protein when compared to EVs from pre-frail participants. Robust and pre-frail participants did not differ in EV protein yield. **(D)** All groups primarily expressed small EVs, between 50-100 nm. In the pre-frail group, there was a higher presence of 0-50 nm and 50-100 nm sized EVs, whereas frail participants displayed higher EV distribution of EVs in the 100-150 nm bin vs. the other two groups. Data were analyzed using multiple Student's t-tests, except in Fig. 1D where a 2-way ANOVA was used. Data are expressed as mean ± standard error, n=12-23, *p=0.01, ***p=0.001.

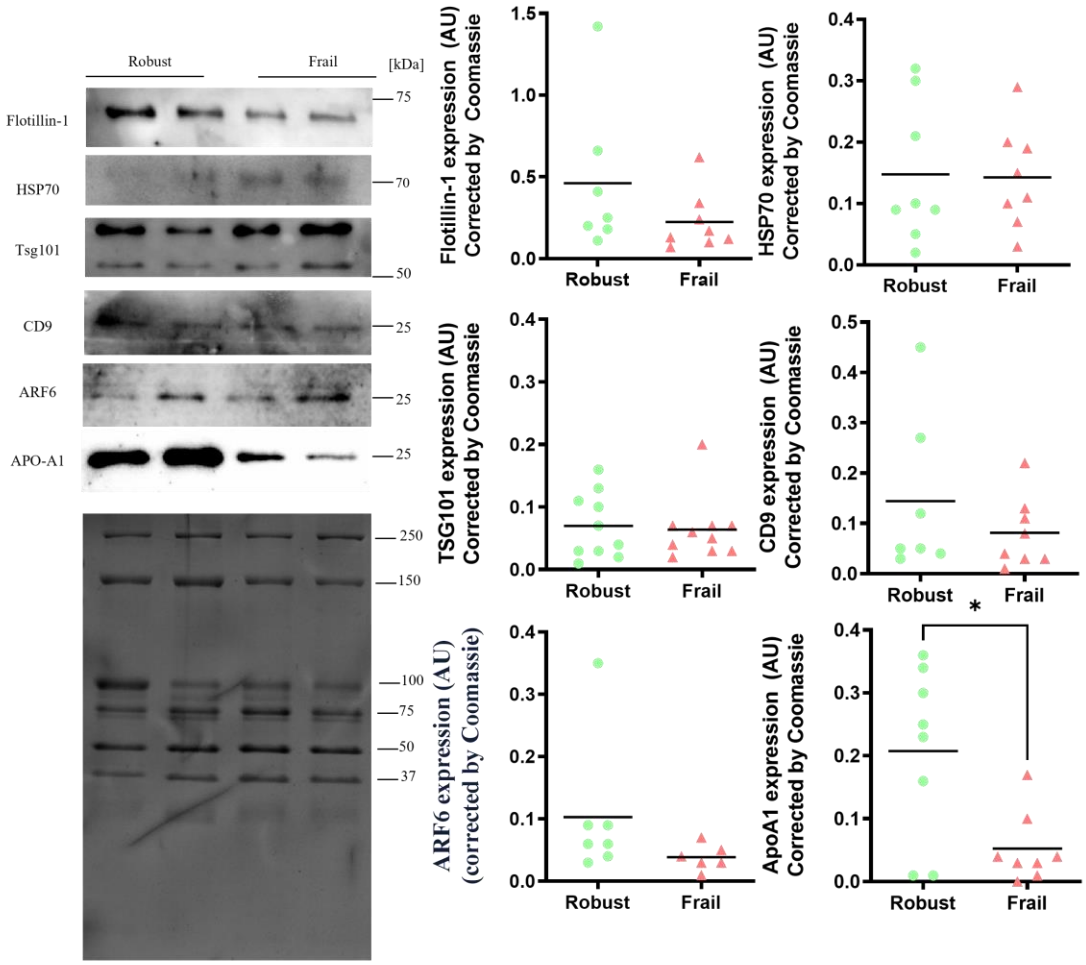

Figure 3. Effects of frailty status on the expression of EV subtype proteins. Equal amounts (5 µg) of EVs from robust and frail participants were subjected to 12% SDS-PAGE for Western blot analysis. Coomassie blue gel staining was used as a loading control. **(A)** Representative images and **(B)** the corresponding quantification of immunoblots for Flotillin-1, HSP70, TSG101, CD9, ARF6, and ApoA1 are shown. EVs from frail group had 1.2-fold lower expression of non-exosome marker ApoA1 when compared to EVs from robust group. Exosome markers flotillin-1, TSG101, CD9, and HSP70, and microvesicle marker ARF6 showed no significant difference between groups. Data were analyzed using a Student's t-test, are expressed as mean ± standard error, N = 7-8, *p=0.01.

EV co-culture experiments: effect on cell count.

To examine the effects of EVs from robust, biologically young and frail, biologically old participants, we performed co-culture with young (F19) and old (F92) primary skeletal muscle cells (**Fig. 1B**). Cells were plated at equal densities and treated over the course of 5 days. F19 cells treated with plasma or EVs from frail/biologically old subjects showed no significant difference in cell count when compared to plasma or EV treatment from robust/biologically young participants (N=5-6, **Fig. 4A**) as measured by Trypan Blue staining. Similarly, F92 cells showed no significant difference in cell count with any treatment condition (N=5-6, **Fig. 4B**).

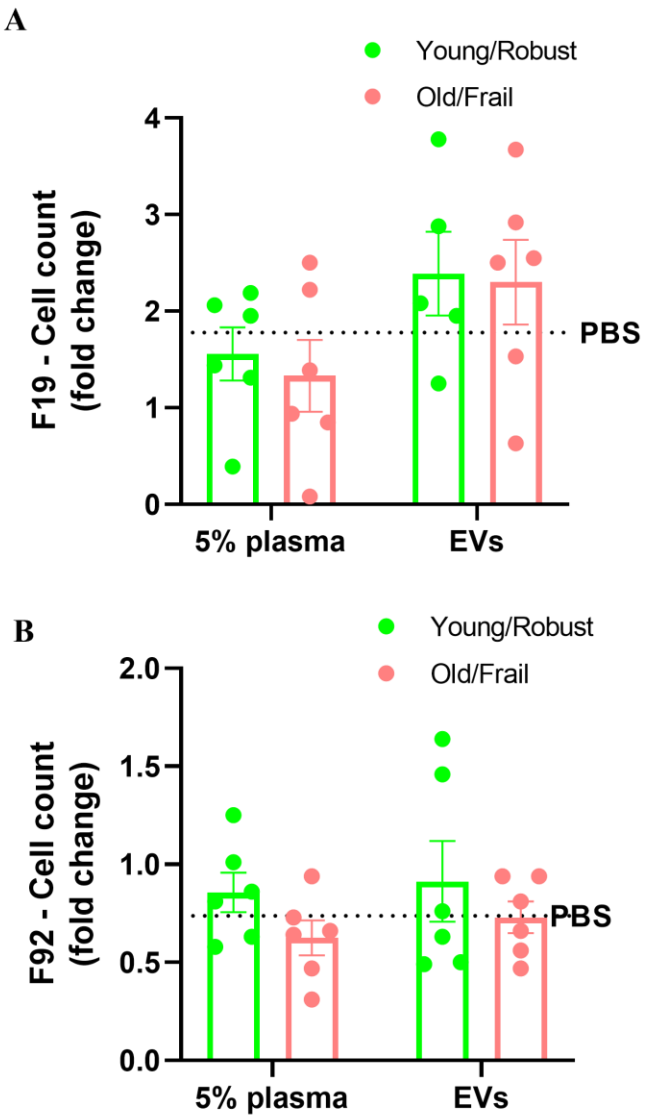

Figure 4. Effects of young/robust and old/frail EV and plasma treatment on cell counts. Treatment with plasma, EV, and PBS in **(A)** F19 (young) and **(B)** F92 (old) human donor primary skeletal muscle cells had no significant effect on total cell count. Data were analyzed using 2-way ANOVA. Data are expressed as mean ± standard error, N = 5-6.

EV co-culture experiments: effect on cell viability.

After counting cells, we analysed cell viability by recording the ratio of live/dead cells measured by Trypan Blue. As would be expected in chronologically young *vs.* old cells, F19 cells had higher viability than F92 cells overall (**Fig. 5**). F19 cells treated with plasma from frail/biologically old subjects showed a 16% decrease in cell viability when compared to plasma treatment from

robust/biologically young participants (N=6, p=0.05, **Fig. 5A**). F19 cells treated with EVs from either group showed no significant difference in cell viability (N=6, **Fig. 5A**). F92 cells treated with EVs from robust/biologically young subjects showed a 24% increase in cell viability when compared to treatment with EVs from frail/biologically old subjects (N=6, *p=0.02, **Fig. 5B**). F92 cells treated with plasma from either group showed no significant difference in cell viability (N=6, **Fig. 5B**).

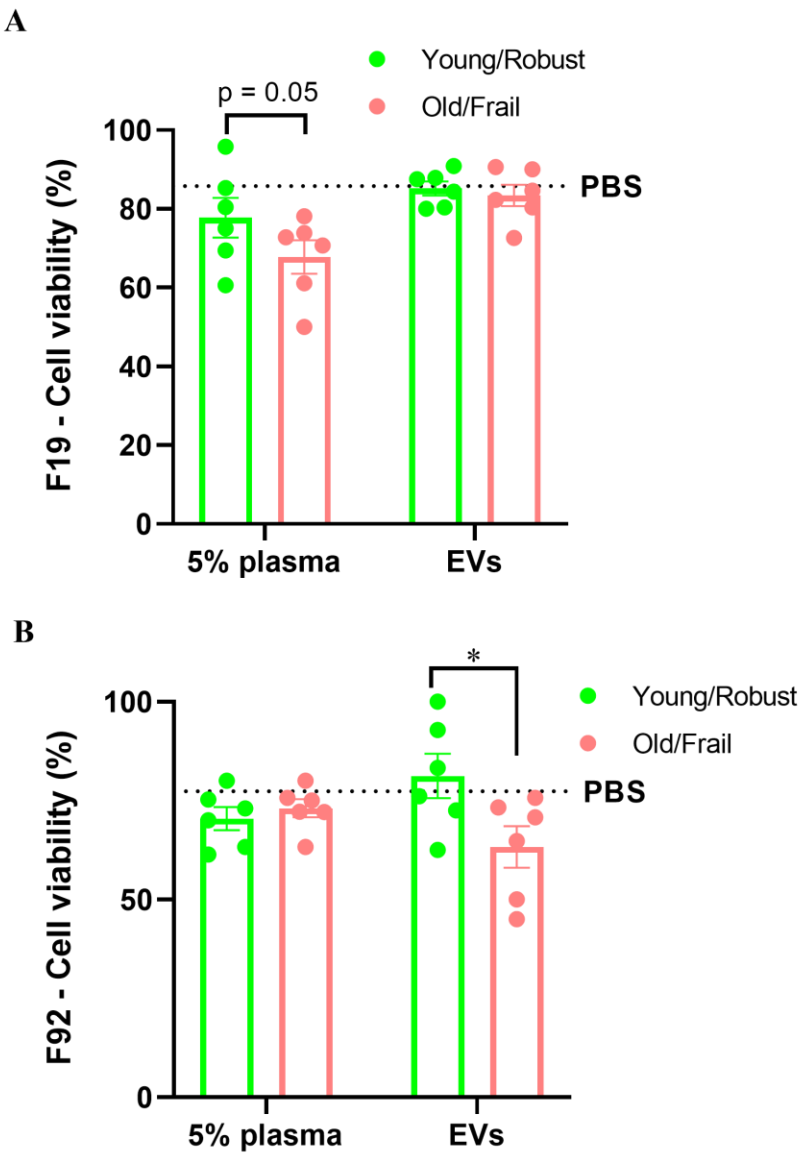

Figure 5. Effects of young/robust and old/frail EV and plasma treatment on cell viability. **(A)** Treating F19 (young) cells with frail/biologically old plasma showed a 16% decrease in cell viability when compared to treatment with robust/biologically young plasma (p=0.05, N=6). **(B)** Treating F92 (old) cells with robust/biologically young EVs resulted in a 24% increase in cell viability when compared to treatment with frail/biologically old EVs (*p=0.02, N=6). Data were analyzed using 2-way ANOVA, and plasma and EV treatment comparisons were performed using Student's t-test. Data are expressed as mean ± standard error, N = 6.

EV co-culture experiments: effect on mitochondrial content.

Next, we studied the effects of EV co-culture on two hallmarks of cellular aging; mitochondrial content and cellular senescence[9]. Treated cells were stained with Mitotracker Red CMX Ros to

assess mitochondrial mass, as a marker for overall mitochondrial content. Three images per condition were taken and MitoTracker values were averaged for each condition. Neither F19 (**Fig. 6A-C)** nor F92 cells (**Fig. 6D-F**) treated with plasma or EVs from frail/biologically old samples or robust/biologically young samples showed any changes in Mitotracker staining (N=7, **Fig. 6**).

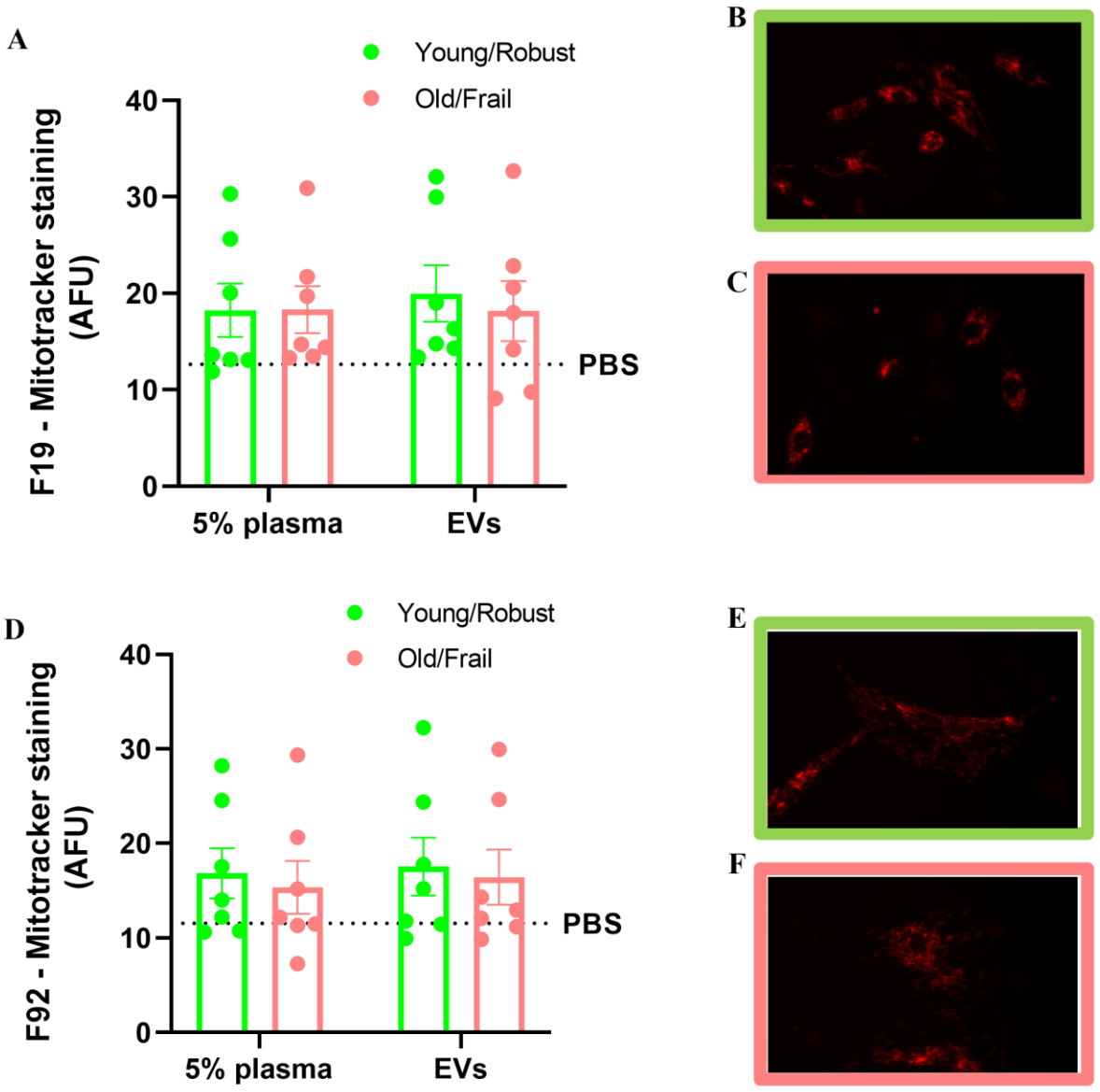

Figure 6. Effects of young/robust and old/frail EV and plasma treatment on mitochondrial content. **(A)** Treating F19 (young) and **(D)** F92 (old) cells with plasma and EVs from robust/biologically young or frail/biologically old subjects showed no significant effect on mitochondrial mass as measured by mitotracker staining. Data were analyzed using 2-way ANOVA. Data are expressed as mean ± standard error, N = 7. Representative images of F19 treatment of **(B)** robust/young EVs and **(C)** frail/old EVs, and F92 treatment of **(E)** robust/young EVs and **(F)** frail/old EVs.

EV co-culture experiments: effect on cellular senescence.

Finally, we measured SA-β-gal in treated cells, a hallmark measurement of senescence. Cells were stained overnight and imaged with fluorescent microscopy the following day. Similar to mitochondrial staining, three images were taken per condition and SA-β-gal values were averaged. In line with expectations, SA-β-gal levels were higher in F92 *vs*. F19 cells (**Fig. 7A and 7B**). F19 cells treated with EVs from frail/biologically old subjects showed a 73% increase in senescence as measured by ß-galactosidase staining when compared to treatment with EVs from robust/biologically young subjects (*p=0.007, N=6-7, **Fig. 7A**). F92 cells treated with EVs from robust/biologically young subjects showed a 48% reduction in senescence as measured by ß-galactosidase staining when compared to treatment with EVs from frail/biologically old subjects (*p=0.02, N=7, **Fig. 7B).** No effect of plasma from either group on SA- β-gal levels were observed in either young or old cells.

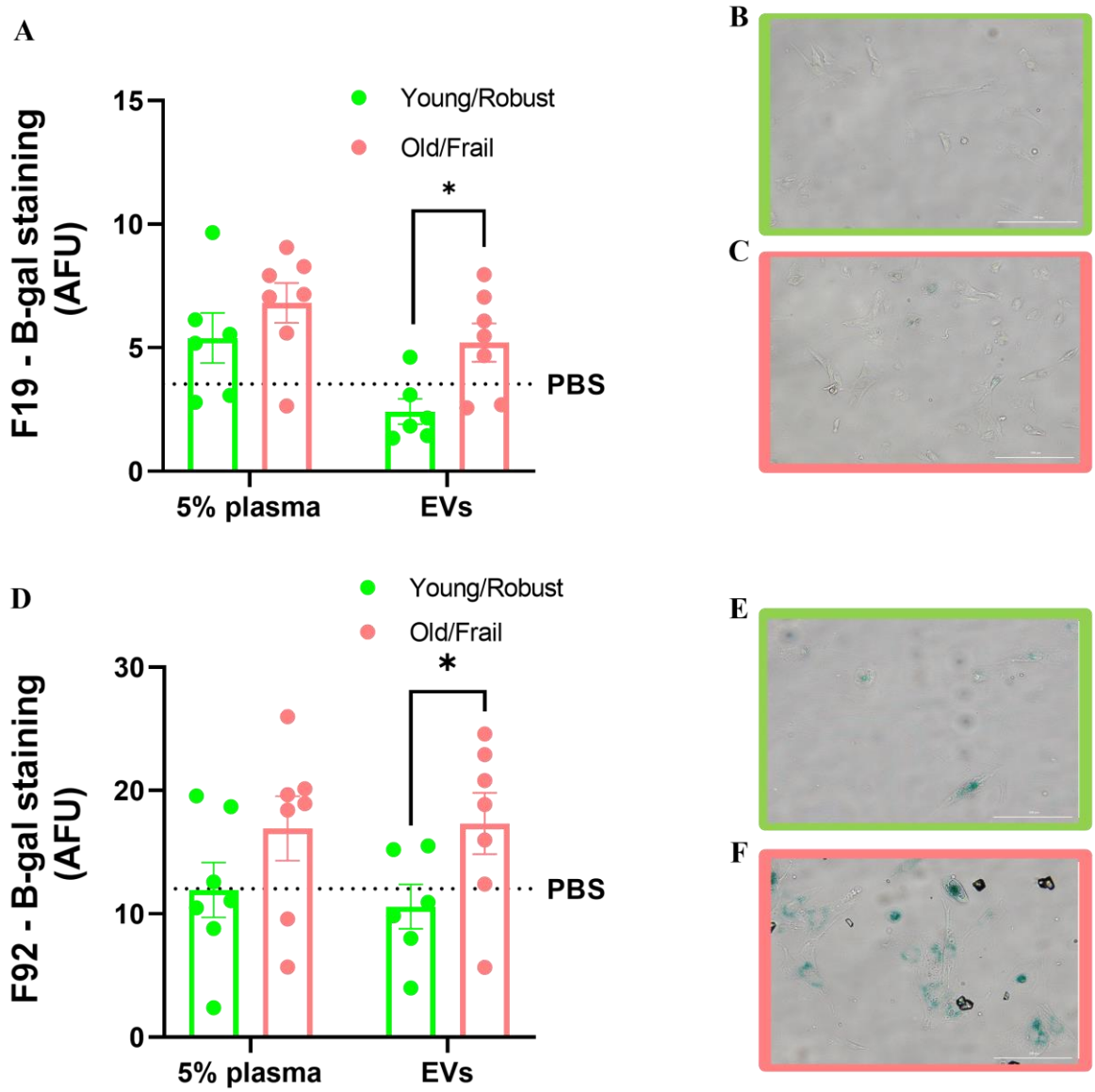

Figure 7. Effects of young/robust and old/frail EV and plasma treatment on cellular senescence. **(A)** Treating F19 (young) cells with **(C)** frail/biologically old Evs resulted in a 73% increase in β-galactosidase (B-gal) staining when compared to **(B)** robust/biologically young EVs treatment (*p=0.007, N=6-7). **(D)** Treating F92 (old) cells with **(E)** robust/biologically young EVs resulted in a 48% decrease in β-galactosidase staining when compared to **(F)** frail/biologically old EV treatment (*p<0.05, N=6-7). Data were analyzed using 2-way ANOVA, and EV treatment comparisons were performed using Student's t-test. Data are expressed as mean ± standard error, N = 6-7, *p=0.02. Scale bar = 200 μm.

Chapter 4: Discussion

Our results indicate that some EV biophysical characteristics differ according to frailty status. Specifically, EVs isolated from individuals with frailty have significantly more protein yield and less ApoA1 expression than EVs isolated from robust participants. Changes in protein yield could be indicative of increased EV concentration, or more proteins packed within each vesicle or both. While previous literature has not examined EV protein yield with frailty status, research has shown EV concentration (defined as EV particles per ml of blood) can either increase or remain unchanged with frailty status[94,134]. Alberro et al. (2016) stratified participants aged 79-92 years by frailty status according to the Barthel and Tilburg frailty indexes, used differential ultracentrifugation for EV isolation, and found that frailty status did not affect EV concentration[94]. On the other hand, Picca et al. (2020) assessed frailty based on a variety of physical performance measures, used differential ultracentrifugation for EV isolation, and found that EV concentration was significantly higher in EVs isolated from frail individuals when compared to non-frail participants. Based on these results, our work showing a significant increase in protein yield with frailty status could be indicative of higher EV concentration or more proteins within luminal cargo or both. To address this, further analysis is needed to measure particle count in isolated EV samples. Future work to characterize EV concentration from the cohort analysed in this thesis could help elucidate the connection between protein yield and EV concentration.

We are the first to examine EV protein markers in robust and frail participants according to MISEV guidelines[69]. Our work shows that exosome- and microvesicle-enriched protein markers were unaffected by frailty status. HSP70, a marker of exosomes, is released from cells independent of traditional protein trafficking mechanisms orchestrated through the Golgi complex[148], and its secretion from cells is confirmed to be exosome-dependent[149,150]. Due to its presence on the cell surface, HSP70 has been hypothesized to be recruited as intraluminal vesicle cargo via endocytosis and later released within exosomes[150]. Intracellular HSP70 functions as a chaperone protein involved with facilitating proteostasis and managing misfolded protein degradation[151,152]. Cell surface HSP70 in tumour cells can account for ~15-20% of total cellular HSP70, although this effect has not been shown in non-tumour cells. Thus, there may be cell-specific accumulation of cell surface HSP70 protein, and this in turn can potentially affect its recruitment into early endosomes[153]. Moreover, HSP70 translocates to the plasma membrane during stress and is

subsequently released from cells[154]. Subsequently we expected HSP70 expression in EVs would change with frailty, given the known effects of frailty on cellular stress and the lack of proteostasis in aged cells. Surprisingly, HSP70 expression in isolated EVs was unchanged with frailty status in our study. This may indicate that the EV-associated HSP70 protein content is derived mainly from the cell surface pool of the HSP70, and is largely dissociated from its intercellular functions.

TSG101 is a subunit of ESCRT I, which is essential to ESCRT-dependent exosome formation by cargo recruitment to the multivesicular body (MVB) membrane[155,156]. Interestingly, depleting cells of TSG101, decreases $CD63^+$ EVs[157], implying a dynamic and co-related relationship between ESCRT-dependent and -independent intraluminal vesicle (ILV) formation. Like HSP70, we did not measure any changes in TSG101 expression in the EV preparations. While we were unable to probe for CD63 in our EV preparations, the results showed no difference in tetraspanin CD9 expression between robust and frail EVs either. Although the exact mechanism by which CD9 recruits cargo to the MVB membrane is unclear, it is commonly used as an exosome marker, and cellular CD9 can be used as an anti-inflammatory marker in immune cells[82,158]. Interestingly, recent evidence suggests that $CD9^+/CD81^+$ EVs, that are also $CD63^-$, may be synthesized primarily via plasma membrane blebbing as microvesicles. Conversely, EVs enriched with $CD63^+$ but not $CD9^-$ may indicate the preferential release of exosomes synthesized via the endosomal/MVB pathway[159]. This demonstrates both the intricacies and importance of comprehensive EV characterization to decipher the cellular biogenesis pathway, and also indicates that CD9 may serve as both a microvesicle and exosome marker. CD9 is also closely associated with flotillin-1, another exosome enriched marker protein. Knocking out CD9 in mice generates exosomes with lower levels of flotillin-1 expression[86]. Flotillin-1 resides on the plasma membrane in lipid rafts and is involved in clathrin-independent endocytosis[160,161]. Interestingly, flotillin-1 has also been implicated in cargo sorting into MVBs[162]. We did not observe any changes in flotillin-1 expression with frailty status either. Given the lack of any observable alterations in EV size as measured by DLS, or EV protein markers associated with small EVs or exosomes, it was therefore not surprising to note there were no differences in the expression of microvesicle marker ARF6 between robust and frail EVs either. Together, our data suggest that robust and frail participants may share common mechanisms of exosome cargo recruitment and microvesicle release. We expect that EV protein yield differences may be indicative of EV concentration rather than content, and will seek to confirm that in future experiments.

ApoA1 in EV research is used as a purity marker of isolated EV samples[69] and its presence confirms precipitation of non-EV co-isolates in our veicle preparations. ApoA1 is particularly relevant as a non-EV contaminant in plasma samples because it is the main apolipoprotein member of the plasma high-density lipoproteins (HDL)[163]. It has also been used as a biomarker of several diseases. First, ApoA1 has been shown to have a protective effect against cardiovascular disease, with higher levels of circulating ApoA1 correlated with lower risk of acute myocardial infarction, so that the ratio of ApoA1 to ApoB, the main apolipoprotein found in low-density lipoproteins, can be used as indicators of risk of acute myocardial infarction[164,165]. ApoA1 reduces inflammation in endothelial cells[166], which account for the production of a majority of plasma EVs, along with erythrocytes and platelets[167]. Thus, lower ApoA1 expression in our EV preparations from frail subjects could be due to less overall ApoA1 levels in the plasma of frail participants, who are also coincidentally at a higher risk for cardiovascular disease[143,168,169]. Previously, Muenchhoff et al. (2017) examined the relationship between apolipoproteins and frailty status and found no correlation between the two[170]. However, the frailty status of participants may vary between Muenchhoff et al. (2017)[170] who used solely the Fried Frailty Scale, and our current work using the Boreskie Frailty Index, which could explain the variance in observed ApoA1 levels in frail participants.

Cell count and MitoTracker staining did not change in F19 and F92 cells with any of the treatment conditions. A trend towards an increased cell count in F19 cells with EV treatment (from both young/robust and old/frails subjects) was apparent, though not statistically significant. Interestingly, in contrast to cell count, we found that cell viability was significantly reduced in F19 cells with plasma treatment from frail/ biologically old subjects, and significantly improved in F92 cells upon EV treatment from robust/biologically young subjects. We expected to see differences in mitochondrial content between F19 and F92 cells at baseline, and the lack of difference seen in our results may be due to two reasons. First, we used region of interest staining analysis, which allowed us to collect mitochondrial mass values irrespective of cell count which was higher in F19 cells. So, while per cell levels of MitoTracker staining may be similar, there is likely an overall decrease in mitochondrial content between young and old cells at baseline which was not captured by our analysis. Second, although mitochondrial dysfunction occurs with age[9], these changes may not be visible with mitochondrial mass staining alone. A comprehensive assessment of mitochondrial biogenesis usually involves the measurement of: 1) mRNA and proteins involved

in organelle synthesis, 2) activity of mitochondrial enzymes in the mitochondrial membranes and/or matrix, 3) respiration and ATP production by mitochondria, and 4) changes in dynamic processes such as mitochondrial protein import, assembly, mitophagy and movement. Measuring mitochondrial mass via MitoTracker staining is a crude, indirect assessment of mitochondrial content and function, and it is very likely that EV/plasma treatment affected some of the variables not measured in our study. We were limited by the slow cell growth and cell count in primary human skeletal muscle cells, particularly those from the 92-year-old donor and were thus unable to conduct a comprehensive assessment of mitochondrial biogenesis. Future experiments with skeletal muscle cell lines or *in vivo* analysis will be crucial in fully elucidating the effect of EV/plasma treatment on mitochondrial biogenesis as a function of biological age and frailty.

We found that treating both F19 and F92 cells with robust/young EVs significantly reduced SA-β-gal staining when compared to old/frail EV group. SA-β-gal staining is a gold standard marker for senescence due to changes in β-galactosidase pH associated with senescence, first discovered by Dimri et al. in 1995[171,172]. SA-β-gal was later found to reflect increased acidic β-gal activity in lysosomes indicative of increased lysosomal biogenesis that occurs in senescent cells[173]. We are the first to show that treatment with EVs from robust/biologically young subjects rescued recipient cell senescence, or the corollary, that EVs from old/frail subjects exacerbated cellular senescence. Further analysis of EV cargo - proteins, lipids, and genomic content - in tandem with careful analysis of cellular signalling changes in treated cells could help elucidate how EVs are exerting this effect on young and old cells alike. In 2005, Conboy et al showed that exposure to young plasma through parabiosis experiments in mice rejuvenated aged progenitor cells[174]. Our results support this conclusion as compared to old plasma/EV, treatment of F92 cells with young plasma/EV manifests as lower SA-β-gal staining. Further analysis is needed to determine whether young plasma/EV treatment is rejuvenating cellular senescence, or old plasma/EV is accelerating senescence in treated cells. Analysing treated cells for markers of DNA damage, increase in expression of senescence-associated proteins like p16 and p21[175] will provide critical insight into the possible mechanisms underlying our observations.

This work has some limitations which warrant consideration. First, EV samples isolated for biophysical characterization showed clear signs of apolipoprotein contamination as illustrated by the presence of ApoA1. Diluting starting volumes of plasma with PBS when comparing robust

and frail EVs in the future may eliminate this problem by facilitating the purification of intact, pure EVs. Furthermore, using alternative EV isolation methods such as differential ultracentrifugation, combined with other methods (e.g. ultrafiltration, SEC) or having a higher starting plasma volume to facilitate several sequential EV isolation methods may help improve EV purity. It is important to consider that while ApoA1 expression is traditionally classified as a non-EV precipitate/contaminant, expression of ApoA1 may be a physiologically-relevant EV-associated phenomenon. The ApoA1 that precipitates with EVs may be indirectly important for EV-induced cellular perturbations. Second, regarding co-culture experiments, we treated cells on day one and three of treatment. Treating cells with fresh plasma or EVs during each day of treatment may help evoke a stronger, more robust treatment response for any/all of the outcome variables. Further, cells were treated with 100 µl of plasma or EVs derived from the same volume of plasma. A dose-response curve for EV dosage (normalized by µg of EV protein, or by particle count) can establish the necessary minimum EV dosage required to show more robust changes in the outcome variables selected. However, it is encouraging that even with the chosen treatment we observed significant effects in the ability of young/robust EVs to modulate cellular senescence. Lastly, MitoTracker staining is an indirect measurement of mitochondrial biogenesis. We could not perform cellular respiration or enzyme assays to carefully ascertain the effect of EV treatment on metabolic profile as we were limited by the low cell counts and slow cell growth, especially in the F92 cell group. Indirect measurements of metabolic flux e.g. by measuring lactate levels in the conditioned media from treated cells can shed further light on any alterations in cellular metabolism with EV treatment in our study.

Conclusion

There are currently no universally accepted circulating biomarkers for frailty. Given the negative health outcomes associated with frailty, with poor prognosis for many age-associated chronic diseases, biomarkers to identify frailty status are sorely needed to identify at-risk patients and modify health care strategies accordingly. Here we show that EVs isolated from frail individuals contain significantly more protein and less ApoA1 than robust EVs in the demographics represented by our participants (i.e. post-menopausal Caucasian women over the age of ~65 years). While we did not observe any differences in size or zeta potential, EV plasma concentration and cargo content might be affected by frailty status. Further, the results may not extend to other

sociodemographic factions as race, sex/gender-specific effects on EV characteristics/cargo have been largely unexplored. More work is needed to determine if EVs and their biophysiological properties (e.g. concentration, cargo etc.) can be used as viable biomarkers of frailty. Furthermore, additional experiments are needed to confirm whether the differences in EV protein yield are the result of alterations in EV concentration, EV proteomic cargo, or both. There is a growing number of research studies that have identified the potential of using EVs as biomarkers of different chronic diseases. Thus research on whether EVs can be used as biomarkers of frailty status warrants further attention and in-depth exploration.

Furthermore, this work is the first to show that treating primary skeletal muscle cells with frail/biologically old EVs induces senescence when compared to robust/biologically young treatment. Since EVs are known to be involved in autocrine, paracrine, and endocrine signalling[68], this work gives insight into the potential effects of plasma EVs on recipient tissues. While anti-aging effects have been attributed to young EVs in literature, this is the first study to illustrate that biological age may be a powerful mediator of cellular senescence irrespective of chronological age. Future experiments focusing on identifying the genomic, proteomic, and lipidomic content of EVs would help elucidate the mechanisms by which EVs are inducing/mitigating senescence. More work is also needed to examine the effects of EV co-culture in proximal tissues to the circulation such as epithelial cells, instead of skeletal muscle cells, as the former would come into contact with EVs in circulation at a higher dosage, and increased primacy. Lastly, the mechanisms by which EVs are taken up in chronologically young and old cells, and whether differences between the two are contributing to the co-culture effects observed also remains to be investigated.

References

1. Van Rhee, J. A. An Aging Population. *Physician Assistant Clinics* **3**, xi–xii (2018).
2. Gems, D. What is an anti-aging treatment? (2014). doi:10.1016/j.exger.2014.07.003
3. Wick, G., Jansen-Dürr, P., Berger, P., Blasko, I. & Grubeck-Loebenstein, B. Diseases of aging. *Vaccine* **18**, 1567–1583 (2000).
4. *Health and Health Care for an Aging Population Policy Summary of The Canadian Medical Association Health and Health Care for an Aging Population*. (2013).
5. Research and Markets. Global Anti-Aging Market (2020 to 2030) - by Product, Treatment and Demography. (2020). Available at: https://ca.finance.yahoo.com/news/global-anti-aging-market-2020-112300989.html. (Accessed: 10th January 2021)
6. McCay, C. M., Maynard, L. A., Sperling, G. & Barnes, L. L. Retarded growth, life span, ultimate body size and age changes in the albino rat after feeding diets restricted in calories. *Nutr. Rev.* **33**, 241–243 (1975).
7. Lee, G. D. *et al.* Dietary deprivation extends lifespan in Caenorhabditis elegans. *Aging Cell* **5**, 515–524 (2006).
8. Fafián-Labora, J. A., Rodríguez-Navarro, J. A. & O'Loghlen, A. Small Extracellular Vesicles Have GST Activity and Ameliorate Senescence-Related Tissue Damage. *Cell Metab.* **32**, 71-86.e5 (2020).
9. López-Otín, C., Blasco, M. A., Partridge, L., Serrano, M. & Kroemer, G. The hallmarks of aging. *Cell* **153**, 1194 (2013).
10. Hayflick, L. & Moorhead, P. S. The serial cultivation of human diploid cell strains. *Exp. Cell Res.* **25**, 585–621 (1961).
11. Boccardi, V. & Mecocci, P. The Importance of Cellular Senescence in Frailty and Cardiovascular Diseases. in *Advances in Experimental Medicine and Biology* **1216**, 79–86 (Springer, 2020).
12. Calcinotto, A. *et al.* Cellular Senescence: Aging, Cancer, and Injury. *Physiol Rev* **99**, 1047–1078 (2019).
13. Kritsilis, M. *et al.* Ageing, cellular senescence and neurodegenerative disease. *International Journal of Molecular Sciences* **19**, (2018).
14. Severino, J., Allen, R. G., Balin, S., Balin, A. & Cristofalo, V. J. Is β-galactosidase staining a marker of senescence in vitro and in vivo? *Exp. Cell Res.* **257**, 162–171 (2000).
15. Juers, D. H., Matthews, B. W. & Huber, R. E. LacZ β-galactosidase: Structure and function of an enzyme of historical and molecular biological importance. *Protein Science* **21**, 1792–1807 (2012).
16. Pole, A., Dimri, M. & P. Dimri, G. Oxidative stress, cellular senescence and ageing. *AIMS Mol. Sci.* **3**, 300–324 (2016).
17. Seong, W. *et al.* Cellular senescence in aging and osteoarthritis Implications for cartilage

repair. *Acta Orthop.* **87**, 6–14 (2016).

18. He, S. & Sharpless, N. E. Senescence in Health and Disease. *Cell* **169**, 1000–1011 (2017).
19. Campisi, J. Cellular senescence: Putting the paradoxes in perspective. *Current Opinion in Genetics and Development* **21**, 107–112 (2011).
20. Di Micco, R., Krizhanovsky, V., Baker, D. & d'Adda di Fagagna, F. Cellular senescence in ageing: from mechanisms to therapeutic opportunities. *Nat. Rev. Mol. Cell Biol.* **22**, 75 (2021).
21. Ben-Porath, I. & Weinberg, R. A. The signals and pathways activating cellular senescence. *Int. J. Biochem. Cell Biol.* **37**, 961–976 (2005).
22. Mullenders, L. H. F. Solar UV damage to cellular DNA: From mechanisms to biological effects. *Photochemical and Photobiological Sciences* **17**, 1842–1852 (2018).
23. Bisht, S., Faiq, M., Tolahunase, M. & Dada, R. Oxidative stress and male infertility. *Nature Reviews Urology* **14**, 470–485 (2017).
24. Schumacher, B., Pothof, J., Vijg, J. & Hoeijmakers, J. H. J. The central role of DNA damage in the ageing process. *Nat. 2021 5927856* **592**, 695–703 (2021).
25. Pal, S. & Tyler, J. K. Epigenetics and aging. *Science Advances* **2**, (2016).
26. Yu, M., Hazelton, W. D., Luebeck, G. E. & Grady, W. M. Epigenetic aging: More than just a clock when it comes to cancer. *Cancer Research* **80**, 367–374 (2020).
27. Sen, P., Shah, P. P., Nativio, R. & Berger, S. L. Epigenetic Mechanisms of Longevity and Aging. *Cell* **166**, 822–839 (2016).
28. Jylhävä, J., Pedersen, N. L. & Hägg, S. Biological Age Predictors. *EBioMedicine* **21**, 29–36 (2017).
29. Quach, A. *et al.* Epigenetic clock analysis of diet, exercise, education, and lifestyle factors. *Aging (Albany. NY).* **9**, 419–446 (2017).
30. Zheng, Y. *et al.* Blood Epigenetic Age may Predict Cancer Incidence and Mortality. *EBioMedicine* **5**, 68–73 (2016).
31. Perna, L. *et al.* Epigenetic age acceleration predicts cancer, cardiovascular, and all-cause mortality in a German case cohort. *Clin. Epigenetics* **8**, (2016).
32. Lu, Y. *et al.* Reprogramming to recover youthful epigenetic information and restore vision. *Nature* **588**, 124–129 (2020).
33. Horvath, S. DNA methylation age of human tissues and cell types. *Genome Biol.* **14**, R115 (2013).
34. Bjornsson, H. T. *et al.* Intra-individual change over time in DNA methylation with familial clustering. *JAMA - J. Am. Med. Assoc.* **299**, 2877–2883 (2008).
35. Teschendorff, A. E. *et al.* Age-dependent DNA methylation of genes that are suppressed in stem cells is a hallmark of cancer. *Genome Res.* **20**, 440–446 (2010).

36. Christensen, B. C. *et al.* Aging and environmental exposures alter tissue-specific DNA methylation dependent upon CPG island context. *PLoS Genet.* **5**, (2009).

37. Unnikrishnan, A. *et al.* The role of DNA methylation in epigenetics of aging HHS Public Access. *Pharmacol Ther* **195**, 172–185 (2019).

38. Catic, A. Cellular Metabolism and Aging. in *Progress in Molecular Biology and Translational Science* **155**, 85–107 (2018).

39. Stefanatos, R. & Sanz, A. Mitochondrial complex I: A central regulator of the aging process. *Cell Cycle* **10**, 1528–1532 (2011).

40. Reichmann, D., Voth, W. & Jakob, U. Maintaining a Healthy Proteome during Oxidative Stress. *Molecular Cell* **69**, 203–213 (2018).

41. Gaschler, M. M. & Stockwell, B. R. Lipid peroxidation in cell death. doi:10.1016/j.bbrc.2016.10.086

42. Ljubicic, V. *et al.* Molecular basis for an attenuated mitochondrial adaptive plasticity in aged skeletal muscle. *Aging (Albany. NY).* **1**, 818–830 (2009).

43. Verdin, E. NAD+ in aging, metabolism, and neurodegeneration. *Science* **350**, 1208–1213 (2015).

44. van de Ven, R. A. H., Santos, D. & Haigis, M. C. Mitochondrial Sirtuins and Molecular Mechanisms of Aging. *Trends in Molecular Medicine* **23**, 320–331 (2017).

45. Yang, S. *et al.* Functional effects of muscle PGC-1alpha in aged animals. *Skelet. Muscle* **10**, 14 (2020).

46. Gill, J. F., Santos, G., Schnyder, S. & Handschin, C. PGC-1α affects aging-related changes in muscle and motor function by modulating specific exercise-mediated changes in old mice. *Aging Cell* **17**, e12697 (2018).

47. von Haehling, S., Morley, J. E. & Anker, S. D. An overview of sarcopenia: Facts and numbers on prevalence and clinical impact. *Journal of Cachexia, Sarcopenia and Muscle* **1**, 129–133 (2010).

48. Kurose, S. *et al.* Prevalence and risk factors of sarcopenia in community-dwelling older adults visiting regional medical institutions from the Kadoma Sarcopenia Study. *Sci. Rep.* **10**, 1–10 (2020).

49. Bachettini, N. P. *et al.* Sarcopenia as a mortality predictor in community-dwelling older adults: a comparison of the diagnostic criteria of the European Working Group on Sarcopenia in Older People. *Eur. J. Clin. Nutr.* **74**, 573–580 (2020).

50. Morley, J. E. *et al.* Frailty consensus: A call to action. *J. Am. Med. Dir. Assoc.* **14**, 392–397 (2013).

51. Fried, L. P. *et al.* Frailty in Older Adults: Evidence for a Phenotype. *Journals Gerontol. Ser. A Biol. Sci. Med. Sci.* **56**, M146–M157 (2001).

52. Mitnitski, A. B., Mogilner, A. J. & Rockwood, K. Accumulation of Deficits as a Proxy

Measure of Aging. **1**, 323–336 (2001).

53. Searle, S. D., Mitnitski, A., Gahbauer, E. A., Gill, T. M. & Rockwood, K. A standard procedure for creating a frailty index. *BMC Geriatr.* **8**, 24 (2008).

54. Jones, D. M., Song, X. & Rockwood, K. Operationalizing a Frailty Index from a Standardized Comprehensive Geriatric Assessment. *J. Am. Geriatr. Soc.* **52**, 1929–1933 (2004).

55. Canada Frailty Network. Frailty Matters - Canadian Frailty Network. Available at: https://www.cfn-nce.ca/frailty-matters/. (Accessed: 11th January 2021)

56. Rohrmann, S. Epidemiology of Frailty in Older People. in *Advances in Experimental Medicine and Biology* **1216**, 21–27 (Springer, 2020).

57. Zhang, Q., Guo, H., Gu, H. & Zhao, X. Gender-associated factors for frailty and their impact on hospitalization and mortality among community- dwelling older adults: A cross-sectional population-based study. *PeerJ* **2018**, (2018).

58. Lopes-Paciencia, S. *et al.* The senescence-associated secretory phenotype and its regulation. *Cytokine* **117**, 15–22 (2019).

59. Ritschka, B. *et al.* The senescence-associated secretory phenotype induces cellular plasticity and tissue regeneration. *Genes Dev.* **31**, 172–183 (2017).

60. Lau, L. & David, G. Pro- and anti-tumorigenic functions of the senescence-associated secretory phenotype. *Expert Opinion on Therapeutic Targets* **23**, 1041–1051 (2019).

61. Barinda, A. J. *et al.* Endothelial progeria induces adipose tissue senescence and impairs insulin sensitivity through senescence associated secretory phenotype. *Nat. Commun.* **11**, (2020).

62. Heinrich, P. C. *et al.* Principles of interleukin (IL)-6-type cytokine signalling and its regulation. *Biochem. J.* **374**, 1–20 (2003).

63. Alfaro, C. *et al.* Interleukin-8 in cancer pathogenesis, treatment and follow-up. *Cancer Treat. Rev.* **60**, 24–31 (2017).

64. Deshmane, S. L., Kremlev, S., Amini, S. & Sawaya, B. E. Monocyte chemoattractant protein-1 (MCP-1): an overview. *J. Interferon Cytokine Res.* **29**, 313–325 (2009).

65. Borghesan, M. *et al.* Small Extracellular Vesicles Are Key Regulators of Non-cell Autonomous Intercellular Communication in Senescence via the Interferon Protein IFITM3. *Cell Rep.* **27**, 3956-3971.e6 (2019).

66. Misawa, T., Tanaka, Y., Okada, R. & Takahashi, A. Biology of extracellular vesicles secreted from senescent cells as senescence-associated secretory phenotype factors. *Geriatrics and Gerontology International* **20**, 539–546 (2020).

67. Jakhar, R. & Crasta, K. Exosomes as Emerging Pro-Tumorigenic Mediators of the Senescence-Associated Secretory Phenotype. *Int. J. Mol. Sci.* **20**, (2019).

68. Safdar, A., Saleem, A. & Tarnopolsky, M. A. The potential of endurance exercise-derived

exosomes to treat metabolic diseases. *Nature Reviews Endocrinology* **12**, 504–517 (2016).

69. Théry, C. *et al.* Minimal information for studies of extracellular vesicles 2018 (MISEV2018): a position statement of the International Society for Extracellular Vesicles and update of the MISEV2014 guidelines. *J. Extracell. Vesicles* **7**, (2018).

70. Atkin-Smith, G. K. *et al.* A novel mechanism of generating extracellular vesicles during apoptosis via a beads-on-a-string membrane structure. *Nat. Commun.* **6**, (2015).

71. Phan, T. K., Ozkocak, D. C. & Poon, I. K. H. Unleashing the therapeutic potential of apoptotic bodies. *Biochem. Soc. Trans.* **48**, 2079 (2020).

72. Kent, J. E. *et al.* Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted. 16 (2020). doi:10.20944/preprints202007.0349.v1

73. Muralidharan-Chari, V., Clancy, J. W., Sedgwick, A. & D'Souza-Schorey, C. Microvesicles: Mediators of extracellular communication during cancer progression. *Journal of Cell Science* **123**, 1603–1611 (2010).

74. Tricarico, C., Clancy, J. & D'Souza-Schorey, C. Biology and biogenesis of shed microvesicles. *Small GTPases* **8**, 220–232 (2017).

75. V, M.-C. *et al.* ARF6-regulated shedding of tumor cell-derived plasma membrane microvesicles. *Curr. Biol.* **19**, 1875–1885 (2009).

76. Hakulinen, J., Sankkila, L., Sugiyama, N., Lehti, K. & Keski-Oja, J. Secretion of active membrane type 1 matrix metalloproteinase (MMP-14) into extracellular space in microvesicular exosomes. *J. Cell. Biochem.* **105**, 1211–1218 (2008).

77. Taraboletti, G. *et al.* Shedding of the matrix metalloproteinases MMP-2, MMP-9, and MT1-MMP as membrane vesicle-associated components by endothelial cells. *Am. J. Pathol.* **160**, 673–680 (2002).

78. Andreola, G. *et al.* Induction of lymphocyte apoptosis by tumor cell secretion of FasL-bearing microvesicles. *J. Exp. Med.* **195**, 1303–1316 (2002).

79. Huber, V. *et al.* Human colorectal cancer cells induce T-cell death through release of proapoptotic microvesicles: role in immune escape. *Gastroenterology* **128**, 1796–1804 (2005).

80. D'Souza-Schorey Crislyn, C. & Clancy, J. W. Tumor-derived microvesicles: shedding light on novel microenvironment modulators and prospective cancer biomarkers. *Genes Dev.* **26**, 1287 (2012).

81. Sidhom, K., Obi, P. O. & Saleem, A. A Review of Exosomal Isolation Methods: Is Size Exclusion Chromatography the Best Option? *Int. J. Mol. Sci.* **21**, 6466 (2020).

82. Van Niel, G., D'Angelo, G. & Raposo, G. Shedding light on the cell biology of extracellular vesicles. *Nature Reviews Molecular Cell Biology* **19**, 213–228 (2018).

83. Gruenberg, J. Life in the lumen: The multivesicular endosome. *Traffic* **21**, 76 (2020).

84. Raiborg, C. & Stenmark, H. The ESCRT machinery in endosomal sorting of ubiquitylated

membrane proteins. *Nat. 2009 4587237* **458**, 445–452 (2009).

85. Buschow, S. I. *et al.* MHC II in dendritic cells is targeted to lysosomes or T cell-induced exosomes via distinct multivesicular body pathways. *Traffic* **10**, 1528–1542 (2009).

86. Chairoungdua, A., Smith, D. L., Pochard, P., Hull, M. & Caplan, M. J. Exosome release of β-catenin: a novel mechanism that antagonizes Wnt signaling. *J. Cell Biol.* **190**, 1079 (2010).

87. Witwer, K. W. *et al.* Standardization of sample collection, isolation and analysis methods in extracellular vesicle research. *J. Extracell. Vesicles* **2**, (2013).

88. Hong, C. S., Funk, S. & Whiteside, T. L. Isolation of biologically active exosomes from plasma of patients with cancer. in *Methods in Molecular Biology* **1633**, 257–265 (Humana Press Inc., 2017).

89. Terlecki-Zaniewicz, L. *et al.* Small extracellular vesicles and their miRNA cargo are anti-apoptotic members of the senescence-associated secretory phenotype. *Aging (Albany. NY).* **10**, 1103–1132 (2018).

90. Tanaka, Y. & Takahashi, A. Senescence-associated extracellular vesicle release plays a role in senescence-associated secretory phenotype (SASP) in age-associated diseases. *J. Biochem.* **169**, 147–153 (2021).

91. Lehmann, B. D. *et al.* Senescence-associated exosome release from human prostate cancer cells. *Cancer Res.* **68**, 7864–7871 (2008).

92. Liu, Y. R. *et al.* Therapeutic effects and perspective of stem cell extracellular vesicles in aging and cancer. *Journal of Cellular Physiology* (2020). doi:10.1002/jcp.30212

93. Liu, Y., Shen, Q., Zhang, L. & Xiang, W. Extracellular Vesicles: Recent Developments in Aging and Reproductive Diseases. *Frontiers in Cell and Developmental Biology* **8**, (2020).

94. Alberro, A. *et al.* Inflammaging and frailty status do not result in an increased extracellular vesicle concentration in circulation. *Int. J. Mol. Sci.* **17**, (2016).

95. Eitan, E. *et al.* Age-Related Changes in Plasma Extracellular Vesicle Characteristics and Internalization by Leukocytes. *Sci. Rep.* **7**, (2017).

96. Davis, C. *et al.* MicroRNA-183-5p Increases with Age in Bone-Derived Extracellular Vesicles, Suppresses Bone Marrow Stromal (Stem) Cell Proliferation, and Induces Stem Cell Senescence. *Tissue Eng. - Part A* **23**, 1231–1240 (2017).

97. Lee, E. K. *et al.* The involvement of serum exosomal miR-500-3p and miR-770- 3p in aging: Modulation by calorie restriction. *Oncotarget* **9**, 5578–5587 (2018).

98. Terlecki-Zaniewicz, L. *et al.* Small extracellular vesicles and their miRNA cargo are anti-apoptotic members of the senescence-associated secretory phenotype. *Aging (Albany. NY).* **10**, 1103–1132 (2018).

99. Bertoldi, K. *et al.* Circulating extracellular vesicles in the aging process: impact of aerobic exercise. *Mol. Cell. Biochem.* **440**, 115–125 (2018).

100. Su, T. *et al.* Bone Marrow Mesenchymal Stem Cells-Derived Exosomal MiR-29b-3p Regulates Aging-Associated Insulin Resistance. *ACS Nano* **13**, (2019).

101. Huang, R. *et al.* Differential effects of extracellular vesicles from aging and young mesenchymal stem cells in acute lung injury. *Aging (Albany. NY).* **11**, 7996–8014 (2019).

102. Alberro, A. *et al.* T cells and immune functions of plasma extracellular vesicles are differentially modulated from adults to centenarians. *Aging (Albany. NY).* **11**, 10723–10741 (2019).

103. Zhang, X., Hubal, M. J. & Kraus, V. B. Immune cell extracellular vesicles and their mitochondrial content decline with ageing. *Immun. Ageing* **17**, (2020).

104. Willis, C. M. *et al.* Astrocyte Support for Oligodendrocyte Differentiation can be Conveyed via Extracellular Vesicles but Diminishes with Age. *Sci. Rep.* **10**, (2020).

105. Alibhai, F. J. *et al.* Cellular senescence contributes to age-dependent changes in circulating extracellular vesicle cargo and function. *Aging Cell* **19**, (2020).

106. Grenier-Pleau, I. *et al.* Blood extracellular vesicles from healthy individuals regulate hematopoietic stem cells as humans age. *Aging Cell* **19**, (2020).

107. Tsukamoto, H., Kouwaki, T. & Oshiumi, H. Aging-Associated Extracellular Vesicles Contain Immune Regulatory microRNAs Alleviating Hyperinflammatory State and Immune Dysfunction in the Elderly. *iScience* **23**, (2020).

108. Khanh, V. C. *et al.* Rejuvenation of mesenchymal stem cells by extracellular vesicles inhibits the elevation of reactive oxygen species. *Sci. Rep.* **10**, 17315 (2020).

109. Mensà, E. *et al.* Small extracellular vesicles deliver miR-21 and miR-217 as pro-senescence effectors to endothelial cells. *J. Extracell. Vesicles* **9**, (2020).

110. Sela, J., Bab, I. A. & Muhlrad, A. A comparative study on the occurrence and activity of extracellular matrix vesicles in young and adult rat maxillary bone. *Calcif. Tissue Int.* **33**, 129–134 (1981).

111. Wang, Y. *et al.* Differentially expressed microRNAs in bone marrow mesenchymal stem cell-derived microvesicles in young and older rats and their effect on tumor growth factor-β1-mediated epithelial-mesenchymal transition in HK2 cells. *Stem Cell Res. Ther.* **6**, 185 (2015).

112. Lötvall, J. *et al.* Minimal experimental requirements for definition of extracellular vesicles and their functions: A position statement from the International Society for Extracellular Vesicles. *Journal of Extracellular Vesicles* **3**, (2014).

113. Bertoldi, K. *et al.* Circulating extracellular vesicles in the aging process: impact of aerobic exercise. *Mol. Cell. Biochem.* **440**, 115–125 (2018).

114. Davis, C. *et al.* MicroRNA-183-5p Increases with Age in Bone-Derived Extracellular Vesicles, Suppresses Bone Marrow Stromal (Stem) Cell Proliferation, and Induces Stem Cell Senescence. *Tissue Eng. - Part A* **23**, 1231–1240 (2017).

115. Dutta, S. & Sengupta, P. Men and mice: Relating their ages. *Life Sciences* **152**, 244–248

(2016).

116. Sproti, R. L. *Development of Animal Models of Aging at the National Institute on Aging*. *Neurobiology of Aging* **12**, (Pergamon Press plc, 1991).

117. Sengupta, P. The laboratory rat: Relating its age with human's. *International Journal of Preventive Medicine* **4**, 624–630 (2013).

118. Liu, T. *et al.* EVmiRNA: a database of miRNA profiling in extracellular vesicles. *Nucleic Acids Res.* **47**, D89 (2019).

119. Kulkarni, R. *et al.* Intercellular Transfer of Microvesicles from Young Mesenchymal Stromal Cells Rejuvenates Aged Murine Hematopoietic Stem Cells. *Stem Cells* **36**, 420–433 (2018).

120. Yoshida, M. *et al.* Extracellular Vesicle-Contained eNAMPT Delays Aging and Extends Lifespan in Mice. *Cell Metab.* **30**, 329-342.e5 (2019).

121. Khayrullin, A. *et al.* Very Long-Chain C24:1 Ceramide Is Increased in Serum Extracellular Vesicles with Aging and Can Induce Senescence in Bone-Derived Mesenchymal Stem Cells. *Cells* **8**, 37 (2019).

122. Zhang, H. & Jin, K. Peripheral circulating exosomal miRNAs potentially contribute to the regulation of molecular signaling networks in aging. *Int. J. Mol. Sci.* **21**, (2020).

123. Weilner, S. *et al.* Vesicular Galectin-3 levels decrease with donor age and contribute to the reduced osteo-inductive potential of human plasma derived extracellular vesicles. *Aging (Albany. NY).* **8**, 16–33 (2016).

124. Lee, B. R., Kim, J. H., Choi, E. S., Cho, J. H. & Kim, E. Effect of young exosomes injected in aged mice. *Int. J. Nanomedicine* **13**, 5335–5345 (2018).

125. Jenike, A. E. & Halushka, M. K. miR-21: a non-specific biomarker of all maladies. *Biomark. Res.* **9**, 1–7 (2021).

126. Ye, D., Shen, Z. & Zhou, S. Function of microRNA-145 and mechanisms underlying its role in malignant tumor diagnosis and treatment. *Cancer Manag. Res.* **11**, 969 (2019).

127. Eitan, E. *et al.* Age-Related Changes in Plasma Extracellular Vesicle Characteristics and Internalization by Leukocytes. *Sci. Rep.* **7**, (2017).

128. Khayrullin, A. *et al.* Very Long-Chain C24:1 Ceramide Is Increased in Serum Extracellular Vesicles with Aging and Can Induce Senescence in Bone-Derived Mesenchymal Stem Cells. *Cells* **8**, 37 (2019).

129. Ipson, B. R., Fletcher, M. B., Espinoza, S. E. & Fisher, A. L. Identifying Exosome-Derived MicroRNAs as Candidate Biomarkers of Frailty. *J. frailty aging* **7**, 100–103 (2018).

130. Mao, G. *et al.* Exosomes derived from miR-92a-3poverexpressing human mesenchymal stem cells enhance chondrogenesis and suppress cartilage degradation via targeting WNT5A. *Stem Cell Res. Ther.* **9**, (2018).

131. Staines, K. A., Macrae, V. E. & Farquharson, C. Cartilage development and degeneration: A Wnt Wnt situation. *Cell Biochemistry and Function* **30**, 633–642 (2012).

132. Wang, G. *et al.* HucMSC-exosomes carrying miR-326 inhibit neddylation to relieve inflammatory bowel disease in mice. *Clin. Transl. Med.* **10**, (2020).

133. Zhu, G., Yang, X., Peng, C., Yu, L. & Hao, Y. Exosomal miR-532–5p from bone marrow mesenchymal stem cells reduce intervertebral disc degeneration by targeting RASSF5. *Exp. Cell Res.* **393**, (2020).

134. Picca, A. *et al.* Older Adults with Physical Frailty and Sarcopenia Show Increased Levels of Circulating Small Extracellular Vesicles with a Specific Mitochondrial Signature. *Cells* **9**, (2020).

135. Chen, B. *et al.* Human embryonic stem cell-derived exosomes promote pressure ulcer healing in aged mice by rejuvenating senescent endothelial cells. *Stem Cell Res. Ther.* **10**, 142 (2019).

136. Mas-Bargues, C. *et al.* Extracellular vesicles from healthy cells improves cell function and stemness in premature senescent stem cells by mir-302b and hif-1α activation. *Biomolecules* **10**, 1–16 (2020).

137. Wang, W. *et al.* Extracellular vesicles extracted from young donor serum attenuate inflammaging via partially rejuvenating aged T-cell immunotolerance. *FASEB J.* **32**, 5899–5912 (2018).

138. Alique, M. *et al.* Microvesicles from the plasma of elderly subjects and from senescent endothelial cells promote vascular calcification. *Aging (Albany. NY).* **9**, 778–789 (2017).

139. Xie, Y. *et al.* Involvement of serum-derived exosomes of elderly patients with bone loss in failure of bone remodeling via alteration of exosomal bone-related proteins. *Aging Cell* **17**, (2018).

140. Menzies, K. J., Singh, K., Saleem, A. & Hood, D. A. Sirtuin 1-mediated effects of exercise and resveratrol on mitochondrial biogenesis. *J. Biol. Chem.* **288**, 6968–6979 (2013).

141. Cantó, C. & Auwerx, J. Caloric restriction, SIRT1 and longevity. *Trends in Endocrinology and Metabolism* **20**, 325–331 (2009).

142. E, von E. *et al.* The Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) statement: guidelines for reporting observational studies. *J. Clin. Epidemiol.* **61**, 344–349 (2008).

143. Boreskie, K. F. *et al.* Standardization of the Fried frailty phenotype improves cardiovascular disease risk discrimination. *Exp. Gerontol.* **119**, 40–44 (2019).

144. Bergsma, T. & Rogaeva, E. DNA Methylation Clocks and Their Predictive Capacity for AgingPhenotypes and Healthspan. *Neurosci. Insights* **15**, (2020).

145. Levine, M. E. *et al.* An epigenetic biomarker of aging for lifespan and healthspan. *Aging (Albany. NY).* **10**, (2018).

146. Pierdoná, T. M. *et al.* Extracellular vesicles as predictors of individual response to exercise training in youth living with obesity. *bioRxiv* 2020.11.20.390872 (2020). doi:10.1101/2020.11.20.390872

147. Debacq-Chainiaux, F., Erusalimsky, J. D., Campisi, J. & Toussaint, O. Protocols to detect senescence-associated beta-galactosidase (SA-βgal) activity, a biomarker of senescent cells in culture and in vivo. *Nat. Protoc. 2009 412* **4**, 1798–1806 (2009).

148. Broquet, A. H., Thomas, G., Lle Masliah, J., Trugnan, G. & Bachelet, M. Expression of the Molecular Chaperone Hsp70 in Detergent-resistant Microdomains Correlates with Its Membrane Delivery and Release*. (2003). doi:10.1074/jbc.M302326200

149. Multhoff, G. & Hightower, L. E. Cell surface expression of heat shock proteins and the immune response. *Cell Stress Chaperones* **1**, 167 (1996).

150. Lancaster, G. I. & Febbraio, M. A. Exosome-dependent Trafficking of HSP70 A NOVEL SECRETORY PATHWAY FOR CELLULAR STRESS PROTEINS*. (2005). doi:10.1074/jbc.M502017200

151. R, R., NB, N., MP, M. & B, B. The Hsp70 chaperone network. *Nat. Rev. Mol. Cell Biol.* **20**, 665–680 (2019).

152. MP, M. & B, B. Hsp70 chaperones: cellular functions and molecular mechanism. *Cell. Mol. Life Sci.* **62**, 670–684 (2005).

153. M, G. *et al.* Tumor-specific Hsp70 plasma membrane localization is enabled by the glycosphingolipid Gb3. *PLoS One* **3**, (2008).

154. VL, V. *et al.* Hsp70 translocates into the plasma membrane after stress and is released into the extracellular environment in a membrane-associated form that activates macrophages. *J. Immunol.* **180**, 4299–4307 (2008).

155. Juan, T. & Fürthauer, M. Biogenesis and function of ESCRT-dependent extracellular vesicles. *Semin. Cell Dev. Biol.* **74**, 66–77 (2018).

156. Flower, T. G. *et al.* A helical assembly of human ESCRT-I scaffolds reverse-topology membrane scission. *Nat. Struct. Mol. Biol.* **27**, 570–580 (2020).

157. Colombo, M. *et al.* Analysis of ESCRT functions in exosome biogenesis, composition and secretion highlights the heterogeneity of extracellular vesicles. *J. Cell Sci.* **126**, 5553–5565 (2013).

158. Brosseau, C., Colas, L., Magnan, A. & Brouard, S. CD9 Tetraspanin: A New Pathway for the Regulation of Inflammation? *Front. Immunol.* **9**, (2018).

159. Mathieu, M. *et al.* Specificities of exosome versus small ectosome secretion revealed by live intracellular tracking of CD63 and CD9. *Nat. Commun.* **12**, (2021).

160. OO, G., NA, B. & BJ, N. Flotillin-1 defines a clathrin-independent endocytic pathway in mammalian cells. *Nat. Cell Biol.* **8**, 46–54 (2006).

161. A, B., A, T. & R, T. Functional aspects of membrane association of reggie/flotillin proteins. *Curr. Protein Pept. Sci.* **12**, 725–735 (2011).

162. Phuyal, S., Hessvik, N. P., Skotland, T., Sandvig, K. & Llorente, A. Regulation of exosome release by glycosphingolipids and flotillins. doi:10.1111/febs.12775

163. Cochran, B. J., Ong, K.-L., Manandhar, B. & Rye, K.-A. APOA1: a Protein with Multiple Therapeutic Functions. doi:10.1007/s11883-021-00906-7/Published

164. Karthikeyan, G. *et al.* Lipid Profile, Plasma Apolipoproteins, and Risk of a First Myocardial Infarction Among Asians: An Analysis From the INTERHEART Study. *J. Am. Coll. Cardiol.* **53**, 244–253 (2009).

165. K, S. *et al.* An association analysis between ApoA1 polymorphisms and the high-density lipoprotein (HDL) cholesterol level and myocardial infarction (MI) in Japanese. *J. Hum. Genet.* **49**, 433–439 (2004).

166. Pan, B. *et al.* A novel anti-inflammatory mechanism of high density lipoprotein through up-regulating annexin A1 in vascular endothelial cells. *Biochim. Biophys. Acta - Mol. Cell Biol. Lipids* **1861**, 501–512 (2016).

167. Hromada, C., Mühleder, S., Grillari, J., Redl, H. & Holnthoner, W. Endothelial Extracellular Vesicles—Promises and Challenges. *Front. Physiol.* **8**, 275 (2017).

168. N, V. *et al.* Risk of cardiovascular disease morbidity and mortality in frail and pre-frail older adults: Results from a meta-analysis and exploratory meta-regression analysis. *Ageing Res. Rev.* **35**, 63–73 (2017).

169. Boreskie, K. F. *et al.* Myokines as biomarkers of frailty and cardiovascular disease risk in females. *Exp. Gerontol.* **133**, 110859 (2020).

170. Muenchhoff, J. *et al.* Plasma apolipoproteins and physical and cognitive health in very old individuals. (2017). doi:10.1016/j.neurobiolaging.2017.02.017

171. Dimri, G. P. *et al.* A biomarker that identifies senescent human cells in culture and in aging skin in vivo. *Proc. Natl. Acad. Sci. U. S. A.* **92**, 9363 (1995).

172. Debacq-Chainiaux, F., Erusalimsky, J. D., Campisi, J. & Toussaint, O. Protocols to detect senescence-associated beta-galactosidase (SA-βgal) activity, a biomarker of senescent cells in culture and in vivo. *Nat. Protoc. 2009 412* **4**, 1798–1806 (2009).

173. Kurz, D. J., Decary, S., Hong, Y. & Erusalimsky, J. D. Senescence-associated (beta)-galactosidase reflects an increase in lysosomal mass during replicative ageing of human endothelial cells. *J. Cell Sci.* **113**, 3613–3622 (2000).

174. Conboy, I. M. *et al.* Rejuvenation of aged progenitor cells by exposure to a young systemic environment. *Nature* **433**, 760–764 (2005).

175. Hooten, N. N. & Evans, M. K. Techniques to Induce and Quantify Cellular Senescence. *J. Vis. Exp.* **2017**, 55533 (2017).