Effects of Low Testosterone on Frailty and a Selective Androgen Receptor Modulator on Frailty, Frailty Mechanisms, and Cardiac Structure and Function in Older Mice

By

Stefan D. Heinze-Milne

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To my parents,

who fostered in me

a sense of curiosity

and the

confidence to pursue inquiry.

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Abstract

Frailty is a state of increased risk to health issues and can arise in older age and during disease. Low circulating levels of testosterone, especially in older men, are thought to predispose toward a frail state due to links between low testosterone and mechanisms of frailty, including chronic inflammation, muscle wasting, and cardiac disease. However, results from a 6-month longitudinal study presented in this thesis suggested that chronic low levels of testosterone in older male C57BL/6 mice do not strongly predispose toward frailty, suggesting that low testosterone may correlate with frailty in older men but may not potently drive it. On the other hand, testosterone treatment improves aspects of frailty. An emerging class of drugs called selective androgen receptor modulators (SARMs) offer an exciting alternative to testosterone therapy due to their tissue selectivity in muscle and bone. A SARM was given (5 mg/kg/day of RAD140) to older male and female C57BL/6 mice for 6-weeks to investigate the impact on frailty, body composition, and chronic inflammation compared to vehicle treated mice. Not much is known about how SARMs affect the heart, but because low testosterone levels correlate with poor cardiac function, this also was assessed using echocardiography. Frailty levels were unchanged between treatment groups after 6-weeks, but SARM treated male mice had improvements in lean mass, bone mineral density, chronic inflammation, and cardiac function compared to control mice. SARM treated females only had better cardiac function, while demonstrating increased circulating levels of three pro-inflammatory cytokines compared to controls. These results suggested that the SARM RAD140 was able to beneficially change several bodily systems related to frailty in males, but not females. Future studies investigating RAD140 as a treatment for frailty are warranted, especially in males.

List of abbreviations used

Abbreviation	Full name
ACE	Angiotensin converting enzyme
BMD	Bone mineral density
B-mode	Brightness mode
CCL	Chemokine ligand
CCR5	C-C motif chemokine receptor type 5
cDNA	Complementary deoxyribonucleic acid
CO	Cardiac output
CXCL	Chemokine (C-X-C) motif ligand
DEXA	Dual-energy X-ray absorptiometry
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
EF	Ejection fraction
FI	Frailty index
FI-Lab	Frailty index based on laboratory values
FP	Frailty phenotype
FS	Fractional shortening
G-CSF	Granulocyte colony-stimulating factor
GDX	Gonadectomized
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HFpEF	Heart failure with preserved ejection fraction
HPrEF	Heart failure with reduced ejection fraction
IFN-γ	Interferon gamma
IL	Interleukin
IL-1ra	Interleukin 1 receptor antagonist
IVCT	Isovolumic contraction time
IVRT	Isovolumic relaxation time
KC	Keratinocyte-derived chemokine
MCP	Monocyte chemoattractant protein
M-CSF	Macrophage colony-stimulating factor
MIF	Macrophage migration inhibitory factor
MIP	Macrophage inflammatory protein
M-mode	Motion mode
mRNA	Messenger ribonucleic acid
mTOR	Mammalian target of rapamycin
NF-κB	Nuclear factor κ-B
PWD	Pulse-wave doppler
PWT	Pulse-wave tissue
RANTES	Regulated on activation, normal T cell expressed and secreted
RNA	Ribonucleic acid
RT-qPCR	Real-time quantitative polymerase chain reaction
SARM	Selective androgen receptor modulator
SASP	Senescence-associated secretory phenotype
SD	Standard deviation
TNF	Tumor necrosis factor
TNFR1	Tumor necrosis factor receptor 1

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Chapter 1 Introduction

This dissertation approaches complex questions about frailty in health, how it develops into old age, and how we can possibly prevent it. It asks whether we might ameliorate becoming frail later in life using androgen-like drugs. Frailty in a medical sense is defined as an increased susceptibility to adverse health outcomes (Rockwood & Howlett, 2018). People become frailer as they get older and when they accumulate health issues (Howlett et al., 2021; Fontana et al., 2014). Frailty can be quantified in humans but also in model organisms, such as mice. Importantly, model organisms share many of the same fundamental mechanisms underlying frailty. Chapter 2 deeply explores the concept of frailty, with an emphasis on modeling frailty in mice and other animals. The chapter ends with a first-ever systematic review on how a novel frailty assessment tool using routinely collected laboratory values can predict death and health problems in humans. Chapter 3 then discusses contributing mechanisms of frailty/aging, with an emphasis on age-related declines in skeletal muscle mass and strength, as well as "inflammaging", which is an age-related increase in chronic systemic low-grade inflammation. Inflammaging is an important pillar of aging and a fundamental mechanism that is directly linked with multiple pathologies and is reviewed in detail in Chapter 3. Age-related detrimental changes in bone and heart health are also linked with frailty and are reviewed briefly, as they are assessed in later chapters.

These chapters set the stage for Chapters 4 and 5, which report on original research investigating whether testosterone deficiency contributes to frailty progression and whether androgen-like drugs can prevent frailty or impact its mechanisms in a mouse model of aging. Low levels of circulating testosterone in older men correlate with mechanisms of frailty, including reduced muscle mass and higher chronic inflammation Mohamad et al., 2019; Saad et al., 2017). However, inferring causality between low testosterone and frailty is difficult because older age can relate to reduced testosterone levels (Tajar et al., 2011). Indeed, clinical evidence in humans suggests that low testosterone levels often do not relate with frailty once age is considered (Hsu et al., 2018). Despite this finding, other evidence suggests that low testosterone levels do relate with higher frailty, especially physical frailty, in older men to a certain degree (Peng et

al., 2022). To better understand how chronically low testosterone may impact frailty, Chapter 4 explores how inducing chronic androgen deficiency contributes to frailty in a mouse model of aging. These experiments help elucidate how testosterone deficiency impacts frailty progression in older males.

On the other hand, treating with testosterone can increase skeletal muscle mass and lower chronic inflammation, suggesting that androgen therapy may combat these mechanisms of frailty (Bianchi, 2018, Srinivas-Shankar et al., 2010), although its effectiveness is limited by increased hepatic and cardiovascular health risks (Cardona Attard & Fava, 2019). In Chapter 5 a newer class of drugs that act similarly to testosterone is introduced: selective androgen receptor modulators (SARMs), which could be of interest to treat frailty in men and women. As reviewed within, it is possible that SARMs can beneficially affect mechanisms of frailty, like muscle wasting, chronic inflammation, and bone health. Importantly, SARMs act similarly to testosterone on muscle and bone but less so in androgenic tissues (*e.g.*, prostate), making them an exciting new targeted therapeutic approach for these aspects of frailty (Narayanan et al., 2018). Chapter 5 includes results from experiments investigating the effect of a SARM on frailty, chronic inflammation, and body composition.

Additionally, frailty in humans is tightly linked with heart failure and the two have overlapping risk factors, including chronic inflammation, sarcopenia, and physical limitations like exercise intolerance (Pandey et al., 2019). The prevalence of frailty is higher in people with heart failure than those without heart failure, despite being the same age, and frailer heart failure patients fare worse in general (Pandey et al., 2019; Sanders et al., 2018). Low endogenous testosterone predicts poor cardiovascular outcomes in humans (*e.g.*, heart failure; Diaconu et al., 2021), suggesting a link between frailty, testosterone, and cardiac function. Links between low testosterone and worse heart function have also been demonstrated in older male mice and likely relate to testosterone's regulation of cardiac calcium handling and contraction (Banga et al., 2021; Ayaz & Howlett, 2015). Higher levels of pro-inflammatory cytokines have also been linked with worse myocardial function (Yan et al., 2010), possibly mediating the relationship between low testosterone and impaired cardiac contractility. It is therefore possible that SARM treatment can influence cardiac function in older mice. Because of

this, the effects of SARM treatment on cardiac function were also measured using echocardiography and are presented and discussed in Chapter 5.

Above all, the spirit of this research is to reduce suffering engendered by diseases typically seen in old age that relate to low levels of circulating androgens. It is possible that SARMs may help combat multiple mechanisms of frailty, which are discussed in this dissertation. In sum, the overarching objectives of this work were to:

1) Examine how chronic testosterone deficiency contributes to frailty in a mouse model of aging.

2) Explore how treatment with a SARM affects frailty and underlying mechanisms including body composition and chronic inflammation.

3) Investigate how treatment with a SARM affects cardiac left ventricular function in an aging mouse model.

Overall, it was hypothesized that low testosterone would increase frailty and that a SARM would improve frailty, body composition, chronic inflammation, and cardiac left ventricular function in an aging mouse model.

Chapter 2 Frailty and its assessment

Disclaimer: Portions of this chapter have been published previously in Heinze-Milne et al., 2019, published by S. Karger AG, Basel, reproduced here with permission, and Sapp et al. (unpublished). See Appendix A for author contributions in published work. My role in Sapp et al. (unpublished) was to manage, direct, and synthesize the collection of information on the FI-Lab brought together by authors D. G. Sapp & B. M. Cormier and to critically evaluate and edit the final version of the manuscript prior to its submission in April 2022.

2.1 Introduction

Wisdom dictates that people and animals 'age' at different rates. Someone may look 80 years old but may only be 60 years old (or *vice versa*). Intuitively, we understand that when people display signs of advanced age, they are more vulnerable to health issues. Such people are considered frail. This phenomenon is elegantly and quantitatively shown in Clegg et al. (2016), where they reported that frailer adults died sooner than those less frail at any given age. While this information is not new instinctively, the consideration, quantification, and integration of frailty into healthcare is (Dent et al., 2019; Checa-López et al., 2019; Hoogendijk et al., 2019). Interest in understanding and quantifying frailty has grown in part due to the aging population in many developed countries. Frailty prevalence increases exponentially with age and thus frailty is expected to become more common in tandem with longer average life expectancies and higher relative proportions of older people (*i.e.*, >80 years old) across the globe (Hoogendijk et al., 2019; Collard et al., 2012). While the enthusiasm to integrate frailty assessments into healthcare is high, it is limited by available evidence regarding how frailty develops and what can be done to manage it (Ambagtsheer et al., 2019).

2.1.1 Frailty and physical frailty: Definitions

Part of the challenge of utilizing and studying frailty is due to a history of uncertainty when defining frailty. A consensus meeting in Orlando, Florida in December 2012 was held with delegates from six major international and American societies to facilitate a solution (Morley et al., 2013). The proceedings from this conference articulated a theoretical divide between 'frailty' as a general state of health and 'physical

frailty', which can be considered a medical syndrome (Morley et al., 2013). Physical frailty as a medical syndrome is characterized by diminished strength, endurance, and physiologic function that increases someone's risk of dependency or death (Morley et al., 2013). The phrase "physical frailty" will be used when referring explicitly to the physical aspects of frailty. However, this definition lacks the nuance required to accurately define frailty as a construct. For the purposes of this dissertation, please refer to this definition of frailty, which captures the essence of frailty as an abstract but important phenomenon:

Frailty is a state of heightened vulnerability to adverse health outcomes compared to individuals of the same age.

This definition was adapted from Rockwood and Howlett (2018) and highlights two important components of frailty as a health state. The first is that there is an inherent risk associated with age. In other words, health is finite and bounded by time. The second is that health risks are heterogeneous between people. Implicitly, it is likely possible to modify the magnitudes of risk, given that heterogeneity arises at all. Thus, frailty in theory is an abstract phenomenon that reflects health risk, and any modification to frailty status has serious implications for our health and wellbeing.

2.1.2 Frailty and its reciprocal relationship with health deficits

Because frailty is a heightened state of health risk and it is instinctively known that sick people are more vulnerable, it makes sense to quantify frailty based on the relative presence of health problems. Frailty was first modeled using an 'accumulation of health deficits' approach in 2001 by Mitnitski et al. (2001). For reference, a health deficit in that paper was considered the presence of a deleterious health variable (*e.g.*, diabetes mellitus). The analysis essentially measured frailty as the presence of health deficits an individual displays compared to all health variables measured. This approach is explained in detail later, but all that must be known for now is that frailty scores increase as the number health deficits increase. The study by Mitnitski et al. (2001) was completed using data of older Canadians (range: 65-106 years; 81% were older than 85 years) from the Canadian Study of Health and Aging and included diverse health deficits such memory complaints, physical tremor, abnormal laboratory values (*e.g.*, elevated creatinine), and physical disabilities. The main finding was that frailty scores increased with age in an

exponential fashion (Mitnitski et al., 2001). Further, the distribution of frailty scores when displayed using a histogram displayed a gamma distribution, which had a shape of a heavy right tail.

What this meant practically was that very frail people, the individuals with very high frailty scores, were simply not nearly as common as those less frail. Moreover, time to death was far better explained by frailty score than age, with frailer people dying sooner (Mitnitski et al., 2001). With these two findings in mind, and the fact frailty increases exponentially with age, it can reasonably be said that health deficits act in a compounding manner to increase the risk of death. Otherwise, we would have a more even distribution of frailty scores. Similarly, the exponential relationship between health deficit accumulation and age suggests an accelerating, positive feedback relationship between frailty, morbidity, and subsequent mortality.

Since the proposition that frailty is a heightened state of risk, many clinical studies have recapitulated this phenomenon (Rockwood & Howlett, 2019). For example, frailty has been associated with heart failure (Pandey et al., 2019), major adverse cardiovascular events (Damluji et al., 2021), bone fractures (Li et al, 2019), hypertension (Vetrano et al., 2018), and dementia (Ward et al., 2021). It has also been shown that frailer breast cancer patients are more likely to die in general, but not from breast cancer, underscoring an independent risk from a single disease (Yan et al., 2021). It is therefore clear that frailty status is associated with multiple adverse health conditions. Further, the presence of these conditions increases the risk of developing more conditions. Contemporary estimations suggest the rate of health deficit accumulation in North Americans increases exponentially approximately 5% per year after the age of 50 (Abeliansky et al., 2020; Mitnitski & Rockwood, 2016). This leads to a doubling of health deficits present once every 15 years **(**Mitnitski & Rockwood, 2016**).** Interestingly, women tend to be frailer than men throughout all years in older age (Abeliansky et al., 2020). Sex differences in frailty will be discussed in greater detail later in this chapter. However, the important point here is that frailty is a state of increased risk for health issues relative to other people in the same group.

The development of frailty occurs with disease, but disease is not necessary for

frailty to occur (Fulop et al., 2010). However, as implied by the accelerated accumulation of health deficits into old age, the mechanisms of age-related declines in health are related to each other. There are common breakdowns in many fundamental molecular domains that relate to frailty and chronic disease, sometimes termed the 'pillars of aging' (Kennedy et al., 2014), which are discussed in Chapter 3. Issues in one area of the body can predispose to frailty across the body. For example, the presence of heart failure increases the risk of an individual becoming frailer (Pandey et al., 2019). Likewise, the presence of cancer increases the risk of becoming frail (Ness & Wogksch, 2020). These and many other health conditions have treatments and management strategies. This is an important concept, because it suggests that a contributing risk factor for frailty (*i.e.*, disease) is modifiable by interventions. Thus, the development of frailty is modifiable to an extent in this way. Newer thinking also suggests that frailty itself can be treated. In fact, the notion that frailty may be modifiable through treatments directly related to mechanisms of frailty is reflected in the Geroscience Hypothesis, which suggests that targeting the fundamental mechanisms of aging can reduce frailty (Kritchevsky & Justice, 2020). This may take the form of interventions like physical exercise or nutritional supplementation (Goh et al., 2021; Dent et al., 2019), caloric restriction (Belsky et al., 2017) or pharmaceuticals like metformin (Kulkarni et al., 2020). It therefore may be posited that therapeutic interventions designed to target disease or fundamental mechanisms of aging will reduce frailty. This line of thinking underpins the theoretical approach used when investigating the potential use of androgen-like drugs to prevent or manage frailty in Chapter 5.

2.1.3 How frailty interacts with treatment and treatment effectiveness

Because this dissertation involves the search for novel interventions to attenuate frailty progression, it is worthwhile to consider how treatments can themselves incidentally impact frailty progression. While effective management of disease may help prevent frailty, some therapies are harmful and predispose toward frailty. Further, frailty status can impact treatments decisions and effectiveness. For example, sometimes the use of a toxic therapy is necessitated by the disease severity. Many cancer therapies for example (*e.g.*, chemotherapy and radiation) have improved cancer treatment greatly but are still associated with long-term cardiovascular health risks (Kostakou et al., 2019).

This partly explains why cancer patients have an increased risk of becoming frail (Ness & Wogksch, 2020). On the other hand, frailty can moderate the effect of treatment. This is exemplified by the fact that adverse post-surgical outcomes are more common in frailer cancer patients (Chen et al., 2019; Lu et al., 2017). Frailer cancer patients also tend to be less tolerant to chemotherapy (Handforth et al., 2015).

The risk of developing frailty while taking several far less-toxic medications than chemotherapy is also higher. In practice, the prescription of multiple medications is called polypharmacy. The inherent risks of polypharmacy include increased risks for drug interactions and adverse drug events (Maher et al., 2014). Evidence suggests there is a bidirectional relationship between frailty and polypharmacy (Gutiérrez-Valencia et al., 2018). Although the mechanisms underlying the relationship between frailty and polypharmacy are likely complex, probable contributors include impaired drug metabolism and adverse effects from the medication (Villén et al., 2020). The relationship between polypharmacy and frailty is worth considering when evaluating drugs to manage frailty. This is mentioned explicitly in this dissertation, which is not on polypharmacy, because oral anabolic androgen drugs can be hepatotoxic (Abeles et al., 2020). Further, the prevalence of polypharmacy is quite high during ages where frailty is a concern. Estimates suggest that over half of people after the eighth decade are taking at least 4-6 medications (Payne, 2016). It is thus argued that any drug candidate aimed to help manage frailty must be viewed with the expectation that the recipient will be on polypharmacy. Risks of frailty arising either from the adverse effects of candidate drugs or subsequent polypharmacy must be considered.

2.1.4 Section summary

They key takeaway points moving forward from here are as follows. Firstly, frailty denotes an increased risk of acquiring new health problems relative to others with the same exposure. In practice this means that two people with a given condition can differ widely in their health risk profiles. Importantly, frailty and disease have a reciprocal relationship. The presence of frailty, and subsequently the presence of health deficit accumulation, increases exponentially with age and doubles once every 15 years. Additionally, treatments for ailments can themselves induce frailty and a prominent

example is polypharmacy. This suggests that drug interventions to prevent frailty must consider what impact it may have on the patient's current treatment plan and if it adds additional risk. Finding ways to attenuate the development of frailty would be incredibly valuable to the epidemiologist, the geriatrician/health practitioner, and the patient.

2.2 Frailty assessment in humans and model organisms

Several tools have been developed to quantify the heterogeneity in health risk into older age (*i.e.*, frailty). A systematic electronic search of peer-reviewed medial literature in September 2018 reported 51 different frailty assessment tools (Faller et al., 2019). Most frailty assessment tools developed thus far rely heavily or solely on physical manifestations of frailty, such as physical weakness, slowness, and fatigue, although there are many that include psychosocial, social, and environmental elements (Faller et al., 2019). This review highlighted two common approaches to frailty assessment, which they termed as 1) unidimensional, focussing on physical health; and 2) multidimensional, which includes non-physical elements (*e.g.*, psychosocial) as well as physical. This chapter section will first briefly review both types of assessments in humans. Then, a more detailed review of frailty assessments in model organisms is provided to showcase frailty research through a preclinical lens, which is used in this research.

2.2.1 Humans

Frailty has been explicitly studied in humans increasingly since the early 2000s. The two dominant methods to measure frailty in humans are the frailty phenotype (FP) proposed by Linda Fried (Fried et al., 2001) and the frailty index (FI) by Kenneth Rockwood (Mitnitski et al., 2001). The FP may be considered a unidimensional approach, as mentioned in Faller et al. (2019), while the FI is multidimensional in nature regarding which health variables are assessed. Differences between the two continue to be discussed (Proietti & Cesari, 2020), although it is recognized that both add value in defining frailty in their own way (Rockwood & Howlett, 2018). The FP approaches frailty as a medical syndrome and evaluates an individual's frailty based on five key areas: unintended weight loss, physical exhaustion, physical weakness, slow walking speed, and low physical activity (Fried et al., 2001). If an individual scores well on all categories, they are considered robust; if they score poorly on one or two, they are considered pre-frail; and if

they score poorly on three or more categories, they are considered frail. It is worth considering that despite being framed as unidimensional, the FP's criteria (*e.g.,* walking speed or weight loss) relate to multiple systems, such as the musculoskeletal system, nervous system, and metabolic system. On the other hand, the FI approaches frailty in a manner of deficit accumulation, as alluded to earlier. It differs from the FP by grading frailty on a continuum rather than categorically. It is calculated by dividing the number of health deficits present in an individual by the total number evaluated. This yields a value between 0 and 1 to provide an 'index' of frailty. Higher FI values are associated with an increased susceptibility to disease (Rockwood & Mitnitski, 2011). A strength of the FI approach is that there is no so-called 'ceiling effect', which denotes a loss of information beyond the maximum score on an assessment. People do not live long after accumulating enough deficits to achieve a score of ~0.7 (Rockwood et al., 2017), which does not exceed the limit of 1. This contrasts with the FP approach that categorizes individuals into broader groups and may be susceptible to a ceiling effect without recalibration (Sanders et al., 2011). In sum, several frailty assessment tools have been created to quantify frailty in people. Despite some differences in deciding who is frail, frailty assessments show that frailty increases non-linearly with age, and increased frailty associates with morbidity and mortality (Theou et al., 2014).

2.2.2 Preclinical models

2.2.2.1 Assessing frailty in animal models

Although the notion of frailty as a clinical concept was established in the early 2000s, the measurement of frailty in animals did not emerge until a decade later. Significant advancements have been made in clinical frailty research with both the FP (Fried et al., 2001) and the FI (Mitnitski et al., 2001), generating questions about how and why people become frail. This motivated the development of preclinical models to help answer these questions. Rodent models of aging have been extremely convenient for studying aging processes due to their relatively short lifespan and because of our ability to study their biology in detail. The growing interest in exploring frailty in preclinical models has paved the way to move the clinical FP and FI tools from the 'bedside to the bench.' The following sections briefly detail several preclinical methods of studying

frailty in animal models, categorizing them as either FP- or FI-based approaches.

2.2.2.2 Frailty Phenotype Approaches

The FP assessment developed by Fried et al. (Fried et al., 2001) has been modified for use in animal models. To relate to the clinical assessment of frailty with the FP, any frailty assessment based on the human FP approach will be labelled a preclinical FP model (rather than an index, as sometimes referred to in the literature). The FP approach was first applied to 27–28-month-old wild type C57BL/6 male mice by Liu et al. (2014). They based their frailty assessment on the FP approach and designed a clinically relevant FP assessment in mice. They assessed grip strength (via an inverted grip test), walking speed (rotarod test), physical activity (voluntary wheel running), and endurance (grip strength + rotarod test; see Table 2.1 for a summary). Animals are scored as non-frail, mildly frail, or frail based on how many criteria are greater than 1.5 standard deviations (SDs) below the cohort's mean. This method was based on an earlier FP-based approach, developed by the same group, called the neuromuscular healthspan measure (Table 2.1). This earlier, more invasive approach measured muscular force production and functional performance to reflect the degree of sarcopenia (Graber et al., 2013). Mice (C57BL/6) had their grip strength and walking speed tested using the same methods as Liu et al. (2014) to measure endurance, along with an *in vitro* muscle contraction test of the extensor digitorum longus. The strengths of these approaches are that there are clear cut-off values for the FP measurements and the neuromuscular healthspan reduces individual variability by combining the scores relative to the mean and predicted values as determined by multiple linear regression. However, unlike the human FP, weight loss is not considered in either model and, despite the combination of strength and walking speed to measure endurance may add measurement bias in the neuromuscular healthspan. The authors acknowledge that running to exhaustion may therefore be a better measure of endurance (Liu et al., 2014). In addition, non-physical frailty measurements, including blindness or deafness, are not assessed using this type of method. The use of within-group comparisons to derive frailty also limits its use when comparing between studies/interventions.

A different approach to measure frailty, called the Valencia Score (Table 2.1;

Gomez-Cabrera et al., 2017), has been developed based on the original human FP (Fried et al., 2001). It measures weight loss, endurance, slowness, weakness, and activity by using a grip strength test (peak force while pulling a bar) and an incremental/graded treadmill test. The lowest 20th percentile in grip strength, running time, and running speed are considered frail on those criteria. Mice that lost >5% body weight or failed a tightrope test were also considered frail. The Valencia Score is calculated by dividing the number of failed tests by total tests in each of the five categories. This frailty score is a relevant predictor of lifespan and physical activity can attenuate frailty measured with this score (Martinez de Toda et al., 2018; Gomez-Cabrera et al., 2017). The strength of this method is that is provides clear definitions of frailty assessment and it is non-invasive, although non-physical attributes of frailty are not assessed. As with the other FP methods, the interpretation of frailty is based on the cohort measured, which limits comparability.

The mouse FP has also been adapted for use in Fisher 344 rats (Miller et al., 2017) using the same four physical assessments used previously (Liu et al., 2014). Rats are considered frail if they scored below the 20th percentile in three or more measures, mildly frail if they were below the 20th percentile in two measures, and non-frail if they scored below the 20th percentile in fewer than two tests. Miller et al. (2017) assessed 133 older (17-month-old) rats using this FP technique and determined that 17% were mildly frail and 2% were frail. Like the other FP approaches, this method is useful given its straightforward cut-offs for frailty and its non-invasive nature. It is worth noting that this method also requires specialized equipment (*i.e.*, a treadmill) and is time-consuming. As for the FP approach designed by Liu et al. (2014), this technique is limited in that rats are classified as frail relevant to their own cohort, and thus comparison between studies is difficult.

The FP assessment has also branched out from rodent models. Hua et al. created an FP assessment for dogs (Table 2.1; Hua et al., 2016). They followed 116 guide dogs (typically golden or Labrador retrievers or crossbreeds, all spayed or neutered) from birth to death using a five-item FP approach. It evaluated muscular weakness, exhaustion, activity levels, nutrition, and mobility through surrogate measures, with some items adapted from clinical geriatric health evaluation scoresheets scored by veterinarians. This

categorized frailty-related items as either ‘present’ or ‘absent’ in the dog. Dogs that scored ‘present’ on two or more of the five categories were considered to have a frailty-related phenotype. Dogs presenting with one or fewer categories were considered not to have a frailty-related phenotype. The median follow-up time from the clinical geriatric assessment to death was 4.4 years. They showed that dogs with a frailty-related phenotype using this measurement were more likely to die than less frail dogs (adjusted hazard ratio = 3.9 (1.4, 10.9); Hua et al., 2016). The FP approach has also expanded into nonhuman primate research (Table 2.1; Yamada et al., 2018). Adult rhesus monkeys had their FP scores assessed after a caloric restriction intervention, which corroborated evidence in smaller organisms that caloric restriction can improve frailty (Yamada et al., 2018).

Overall, these assessments using the FP approach are physical in nature and enable comparison with the predominantly physical frailty assessment approaches used in humans. However, these measurements fail to account for other important signs or symptoms of frailty, including cognition, body composition, ocular deficits, etc. As stated by Liu et al. (2014), these other aspects of frailty are important to consider. In addition, these tests often require specialized equipment and the muscle isolation for the neuromuscular healthspan scoring is clearly invasive. The FP approaches for animal models are therefore useful for comparison to physical frailty in humans, although they lack a multi-system approach to frailty.

2.2.2.3 Frailty phenotypes in genetically altered mice

Genetic modification has been used previously to study a variety of age-related mechanisms and several genetic models of frailty have been developed. In 2008 Walston et al. repurposed a C57BL6-based homozygous interleukin- (IL-) 10 knockout mouse model (IL-$10^{tm/tm}$) to study frailty based on FP (Walston et al., 2008). Although originally developed as a model of inflammatory bowel disease, they found that IL-$10^{tm/tm}$ mice remained disease free when kept under sterile conditions and rapidly developed several characteristics of aging (*i.e.*, decreased muscle mass and inflammation). The premise of the model is that, without IL-10, there is a subsequent increase in nuclear factor-κB, which mediates several age-associated inflammatory cytokines (Walston et al., 2008). IL-

$10^{tm/tm}$ mice have increased levels of the aging-associated cytokine IL-6 and many alterations in skeletal muscle gene expression related to mitochondrial function and cellular apoptosis by 12 months but not at 2 months of age (Walston et al., 2008). The IL-$10^{tm/tm}$ knockout mice also declined in muscle strength faster than age- and sex-matched controls. In practice, these mice show several phenotypic signs of aging including poor muscular strength (Akki et al., 2014) and impaired cardiovascular health (Sikka et al., 2013). These findings suggest that an IL-$10^{tm/tm}$ mouse could model the multisystemic manifestations of frailty. However, some limitations exist when using this model. For example, the removal of IL-10 may lead to unforeseen differences in the biology of frailty in these mice versus naturally aging mice (*i.e.*, apoptotic pathways; Santiago-Lomelí et al., 2005). This model therefore exhibits physiologic and physical changes that accelerate frailty-like symptoms but may not exclusively use frailty pathways associated with 'natural' frailty progression. Further work with this model is needed to establish this.

Another genetically altered model that has recently been proposed for frailty research is the Cu/Zn superoxide dismutase knockout mouse (Deepa et al., 2017). This model is also based on the FP approach. Otherwise known as the Sod1KO mouse, it exhibits several phenotypical features of frailty such as weight loss, low physical activity, weakness, and exhaustion (Deepa et al., 2017). These mice also develop increased inflammation and sarcopenia, which are related to frailty in humans. There is also evidence that Sod1KO mice may become frail through proposed mechanisms of aging, including increased oxidative stress, mitochondrial dysfunction, and cellular senescence (Deepa et al., 2017). Furthermore, caloric restriction has been shown to delay/prevent frailty in this model similarly to what has been reported in wild type C56BL/6 mice (Kane et al., 2016). Thus, this model provides a multi-systemic approach to study physical frailty, although as with the IL-$10^{tm/tm}$ mice, it is not yet clear whether these pathways are representative of naturally aging/frail mice.

Other genetically modified mouse models of aging may provide additional insight into frailty. Although frailty has not been assessed, the mouse models of human Werner and Hutchinson-Gilford syndromes display premature aging characteristics (Mitchell et al., 2015). A knockout mouse model of the transcription factor NF-κB (nuclear factor

kappa-light-chain-enhancer of activated B cells knockout; *nfκb1*$^{-/-}$) also displays accelerated aging, like chronic inflammation, sarcopenia, cardiac hypertrophy, and cellular senescence (Jurk et al., 2014). Similarly, an inducible mouse model of IL-6 rapidly acquires frailty and impaired physical function (Jergović et al., 2021). These and other genetically modified models of frailty are reviewed in Chapter 3 regarding chronic inflammation and provide useful models for studying frailty. Such models may be important in uncovering the biology of frailty, although none may accurately represent the multiple factors that contribute to naturally occurring frailty in humans.

2.2.2.4 Frailty index approaches

The FI approach is unlike the FP approach in that it assesses many bodily systems to derive a multidimensional measure of frailty. It rates frailty on a continuum from 0 to 1 using deficit accumulation rather than a categorical classification. The FI approach was first applied to an animal model (C57BL/6 mice) in 2012 by assessing 31 possible health deficits spanning four categories (activity levels, hemodynamic measures, body composition, and basic metabolic status; Table 2.2; Parks et al., 2012). Each variable was scored using a graded scale determined by how many SDs away from the mean the mouse's score was. If the score was between 1-2 SD different than the reference mean, it would be given a frailty value of 0.25 for that health deficit (where 0 is non-frail). Two to 3 SDs from the mean scored a value of 0.5, 3-4 SDs scored 0.75, and >4 SDs scored 1.00. All 31 health deficit measures were added and divided by the total number of deficits assessed to determine the mouse's FI score between 0 and 1. This FI tool was sensitive enough to identify frailty despite a small sample size. Furthermore, it provided a measure of frailty based on many different health parameters. The major limitations to this tool are its time-consuming nature, invasiveness (blood draws), requirement of specialized equipment, and lack of cognitive assessment. To simplify the procedure, an 8-item FI measure was created by the same group (Parks et al., 2012). Seven items were activity-based and the eighth item measured weight. This method was sensitive enough to detect frailty with age in male mice and was less time consuming. However, this method only measures a small number of variables related to physical frailty and risked a ceiling effect akin to the FP. Frailty values obtained from this latter method were also highly variable.

A limitation to both the 31- and 8-item FI was their reliance on intra-group distributions to determine frailty. This makes between-study comparisons more difficult. Thus, further development of the mouse FI was needed.

To simplify frailty assessment, the mouse 'clinical FI' was created (Whitehead et al., 2014). The clinical FI is non-invasive, as along with assessing musculoskeletal health, it considers the integument, vestibulocochlear/auditory systems, ocular/nasal systems, digestive/urogenital systems, respiratory systems, and general signs of discomfort. Each deficit is assigned either a 0, 0.5, or 1 based on the severity of the deficit. Using this method, C57BL/6 mice demonstrated a graded increase in clinical FI scores between 5, 19, and 28 months (Whitehead et al., 2014). A strength of this method is its ability to model frailty in aging humans. During a comparison study these FI scores closely modelled the FI deficit trends in older humans and higher FI scores predicted a higher risk of mortality (Rockwood et al., 2017). Another strength of the clinical FI is that it has a high inter-rater reliability (Kane et al., 2017; Feridooni et al., 2015). For example, 233 C57BL/6 mice aged 11.5-14 months had their frailties assessed and compared between raters. FI scores were comparable regardless of rater ($p=0.802$) and differences between raters on individual deficit interpretations were ameliorated by refining techniques throughout the study (intra-class correlation coefficient (ICC) = 0.77; Feridooni et al., 2015). It was also found that the FI scoring method had high inter-rater reliability when assessing 45 mice across four people; two experienced and two inexperienced raters (Kane, Ayaz et al., 2017). An ICC of 0.80 was reported across all four raters, which remained high when split between rater experience (ICC=0.76) and rater sex (ICC=0.86; Kane, Ayaz et al., 2017). However, professional backgrounds have been identified as a major source of variation between assessors, which needs to be considered (Kane et al., 2015). Since its inception, the clinical FI has been modified slightly to create a similar 27 item clinical FI by another group (Suckoff Rizzo et al., 2018). Evidence therefore suggests that the clinical FI approach is adaptable, reliable between raters, non-invasive, simple, and considers factors outside of physical frailty, which makes it a useful tool for evaluating frailty in mice.

Interestingly, the FI and FP approaches categorize different mice as frail. Kane et

al. directly compared the FP (Liu et al., 2014) and FI (Whitehead et al., 2014) approaches in C57BL/6 mice and found that there was a 50% agreement between the two methods (Kane, Huizer-Pajkos et al., 2017). Thus, deciding on either approach may depend on the model of frailty used (*i.e.*, naturally aging wild type or a genetic model of frailty) and the study's primary outcomes (*e.g.*, physical fitness).

The robust nature of having many possible deficits to create an index lends itself well to adaptation. In addition to mice, the clinical FI approach has been modified for use in male Fisher 344 rats (Table 2.2; Yorke et al., 2017). It closely models the clinical FI for mice (Whitehead et al., 2014) and measures 27 health deficits in nine categories (integument, physical/musculoskeletal, vestibulocochlear/auditory, ocular/nasal, neurological, digestive/urogenital, respiratory, pain/discomfort, and other; Yorke et al., 2017). Like assessments in mice, deficits are scored as 0, 0.5, or 1 based on the absence or presence of mild or severe deficits. Notably, this model was developed using rats aged 6-21-months. Given that Fisher 344 rats can live longer than this, the authors noted that it would be interesting to investigate frailty in even older animals (Yorke et al., 2017). Furthermore, reliability studies and studies using female rats would strengthen its validity, as have been completed for the mouse clinical FI (Kane, Ayaz et al., 2017; Feridooni et al., 2015). This approach is limited in that it does not include a cognitive assessment. Nonetheless, because rats are often used in behavioural and aging studies, this translation into Fisher 344 rats is an important step in enabling frailty research in other preclinical models.

A clinical measures/biochemical approach has also been developed, whereby an FI assessment was created for C57BL/6 mice based solely on laboratory measurements of health deficits (Kane et al., 2019). This was inspired by an FI technique in humans called the FI-Lab that creates an FI based on common laboratory measurements (Howlett et al., 2014). This FI-lab in mice considered 23 items including blood pressure, metabolic markers (*i.e.*, serum cytokine concentrations), and echocardiographic measurements (Kane et al., 2019). In brief, younger mice (~12 months old) are used to collect reference values from all 23 items. Older mice are considered "normal" and score a 0 when a measurement's value falls within ±1.5 SDs from the younger cohort's mean, suggesting

no deficit. Values greater than ±1.5 SDs from the reference mean are considered to have a deficit and score a 1. Item scores are then summed and divided by the total number of assessments to calculate the FI for each mouse. Arguably the most important finding from the FI-lab is the positive sex-specific association between select pro-inflammatory cytokines and frailty in aging C57BL/6 mice. Like the original FI developed by Parks et al. (Parks et al., 2012), this method is limited in its need for specialized equipment and lack of cognitive assessment.

Thus far the FI approach has been used primarily in mice, although it has also been used in rats and dogs (Banzato et al., 2019). It is likely that the FI will be adapted for other preclinical models of aging in coming years. For example, assessing frailty in the nematode *Caenorhabditis elegans* has been suggested for both drug screening and understanding the biology of frailty, given its extensive use in science and possibility for frailty assessment (Matsunami, 2018). Fruit flies (Drosophila melanogaster) are another potential candidate for frailty research, with criteria based on feeding and locomotion (Le Bourge, 2013; McDonald et al., 2013). The FI approach can arguably be created for larger animals too, including livestock or zoo animals like nonhuman primates. Thus, the FI is a versatile tool for measuring frailty in preclinical models. Its potential for preclinical application is large and includes behavioural and pharmaceutical research aimed at the prevention of frailty (Banga et al., 2019).

2.2.3 Comparisons between mice and humans using a frailty index

Because the frailty assessments in experiments presented in Chapter 4-5 use the 31-item non-invasive FI (Whitehead et al., 2014), comparisons between frailty indices in humans and mice are the focus of this section. A key paper published in 2017 showed similarities in the development of frailty measured by a, FI in humans and mice (Rockwood et al., 2017). There were three shared fundamental characteristics of frailty progression using an FI. The first was that deficit accumulation rates are similar between species. Humans aged 20+ years accrued deficits on a natural logarithm scale at a slope of 0.029, whereas in mice it was 0.036. This translated to the second finding, which was that there was a strong relationship between higher FI scores and mortality. In fact, there was a higher mortality rate for mice than humans (1.15 (1.12-1.18) versus 1.05 (1.04-1.05))

using a Cox proportional hazard model per 0.01 unit increase in FI score. Thirdly, the FI approach had no ceiling effect (*i.e.*, death before the maximum score is reached) in mice and humans (Rockwood et al., 2017).

Using raw frailty-survival data available in our lab, which was originally published in Rockwood et al. (2017), FI scores and survival curves were recreated in this dissertation for illustrative purposes and subsequently fitted using different curves. For example, FI scores across the life course are shown in Figure 2.1, panel A. As shown, frailty scores increase over time from approximately 30-days until death. Interestingly, the accumulation of deficits in this dataset was best described by a Gompertz curve[1] (Gompertz, 1825). Summary statistics for five tested curves are in Table 2.3. This is in contrast to humans, which tend to increase exponentially (Mitnitski & Rockwood, 2016). Indeed, a flattening of the curve is evident between roughly between 600-800 days. It is worth noting that there is a second "rise" in frailty scores closer to 950 days of age (Figure 2.1B). This 'pause' in the accumulation of deficits may be a genuine characteristic of this FI in C57BL/6 mice, however. Publicly available data from a study involving older male C57BL/6Nia mice that assessed frailty until death starting just after 600 days of age suggest that frailty scores indeed continue rising past this point (Figure 2.2; data from Schultz et al., 2020). Interestingly, the cubic curve was again the best fit for the data (Table 2.3), hinting at such a plateau. The curve was arguably very close to being described exponentially too. Overall, frailty scores undoubtedly increase over time in C57Bl/6 mice.

Panels C and E in Figure 2.1 depict a frequency distribution of what was called a *mortality product* in Mitnitski and Rockwood (2001). This was an innovative approach to test the validity of an FI at predicting survival time from a survival function and worked quite well (the R^2=0.999). The mortality product is created by multiplying an FI score by

[1] Mr. Benjamin Gompertz, an elected member of the Royal Society, tabulated large amounts of mortality data to create a single function to predict mortality. His mathematical function grows quickly at first but tapers when it reaches a theoretical limit. When reflecting on causes of death leading to this curve, Mr. Gompertz eloquently said: "*It is possible that death may be the consequence of two generally co-existing causes; the one, chance, without previous disposition to death or deterioration; the other, a deterioration, or an increased inability to withstand destruction.*" Poetically, the latter postulation arguably reflects an early intuitive definition of modern medical frailty.

the time to death. The distribution shown in these panels is remarkably similar to those in humans (Mitnistski & Rockwood, 2001). In that seminal paper, the author's demonstrated that the probability of an individual surviving can be accurately predicted by using the cumulative survival function, if you know an individual's FI, independent of age. Survival probability graphs as a function of mortality product (rather than age, which is traditional) for young and older mice are shown in panels D and F in Figure 2.1, respectively. The younger mice (panel D) had a flatter curve than was described in humans. Interestingly, the curve for the older animals has a similar shape to that in Rockwood and Mitnistki (2001), which was generated by using data from people aged 82 years. This similarity arguably supports the similarities between humans and mice in this regard.

Frailty scores are relevant to survival in mice, as they are in humans, with higher frailty scores increasing the risk of dying (Rockwood et al., 2017). Figures 2.3 and 2.4 highlight statistically significant differences in survival probability for mice assessed aged <300 days and mice aged 300-430 days, respectively. In both figures, the survival curves demonstrate that mice with higher frailty scores died sooner, even from a younger age. It is notable that the ages of mice with higher frailty scores in both the younger and slightly older groups were older. However, this absolute difference in age simply does not offset the mean survival difference between the frailty groups. In younger mice, the mean age difference was eight days, while the mean difference in survival was 73 days. When assessed between 300-430 days, the mean age difference was 19 days, while the mean survival difference was 42 days. Thus, there is variance explained through quantifying frailty that does not overlap with age.

It is important to recognize that interventions designed to impact health are reflected by preclinical frailty quantification with an FI in an intuitive way (Howlett et al., 2021). Lifestyle modifications in the form of caloric restriction (Mitchell et al., 2019) or voluntary aerobic exercise (Bisset et al., 2022) can mitigate the rise of frailty into older age. More importantly for this dissertation is the fact that pharmaceutical interventions can impact frailty. Enalapril, an angiotensin converting enzyme inhibitor, ameliorated frailty in older male and female mice (Keller et al., 2019). Interestingly, FI scores can

correlate to different aspects of health without impacting lifespan in some populations. Correira-Melo et al. (2019) eloquently showed that rapamycin treatment attenuated the rise of frailty in the genetically modified *nfκb1*$^{-/-}$ mice compared to mutant controls without extending lifespan. Despite this, they reported attenuated memory loss and improved neuromuscular test scores in the rapamycin treated group. Due to these results, they argued that the 'healthspan' (the percentage of life where you are healthy) increased in these mice. Polypharmacy, which is mentioned in Chapter 2.1 regarding human frailty, also increases frailty in mice but can be reversed by deprescribing (Mach et al., 2021). Frailty scores also decrease concomitantly with increases in grip strength and balance/motor control (Mach et al., 2021). This is all to say that interventions that impact frailty scores in mice correlate meaningfully to important health measures.

2.2.4 Sex Differences in Frailty

In human populations females tend to live longer despite having higher frailty scores (Kane, Gregson et al., 2017). This has been referred to as the male-female health-survival paradox (Gordon et al., 2017). Several explanations have been offered to solve this paradox. For example, it is possible that males exhaust physiologic reserve by better optimizing fitness, that females having fewer children than in our evolutionary past may affect physiologic reserves, or that the frailty assessments in humans are biased for females over males (Gordon et al., 2017). Regardless of the reason, it is well documented that females live longer than males, albeit with poorer health.

Sex differences in frailty are similar in mouse models as they are in humans, with females tending to be frailer than males (Kane & Howlett, 2021). Studies that have explored male-female differences in frailty in mouse models are summarized in Table 2.6. Four studies (Bauman et al., 2019; Kane et al., 2019; Keller et al., 2019; Whitehead et al., 2014) report that aged (5-32 months) female C57BL/6 mice had higher clinical FI scores than males. Another also found that female Swiss mice, aged 6-24 months, had higher FI values than males (Antoch et al., 2017). However, other studies report no sex difference. Older (23–32-month-old) male and female C57BL/6 mice had similar clinical FI scores (Cole et al., 2022). The FI scores of aged (18 months old) C57BL/6 and DBA/2J male and female mice have also been similar (Kane et al., 2016). Parks et al. (2012) also found

no difference between aged (12–30-month-old) C57BL/6 mice, although the sample sizes may have been too small to demonstrate sex differences with age. On the other hand, a study using 6–35-month-old 3Tg-AD and B6129F2 mice found that males had higher FI scores than females, at least in these strains of mice (Kane et al., 2018). Interestingly, the female-male frailty trends reversed when using a laboratory-based FI (*i.e.*, serum inflammatory cytokine concentrations), where aging male mice were frailer than aging females Kane et al., 2019). As was expected from prior studies, female mice in the same study had higher clinical FI scores than males (Kane et al., 2019). Of note, similar results have been reported in older guide dogs of various breeds, where females had a higher FP compared to males (Hua et al., 2016). Thus, although most animal studies suggest that females are frailer than males, this may depend on the animal strain evaluated and on the frailty assessment tool employed. Additional work in this area may help clarify male-female differences in healthspan and identify underlying mechanisms. The sex differences in humans make these comparisons important when modelling frailty preclinically (Kane & Howlett, 2021).

2.2.5 Review conclusions

Preclinical research is crucial to better understand frailty. This review has highlighted and discussed current methods to quantify frailty in animals. The most common models use wild type or genetically altered rodents. Frailty assessments in other animals, such as dogs and nonhuman primates continue to be developed. Other preclinical models of frailty have been proposed or discussed in the literature (*i.e.*, nematodes (Matsuanami, 2018) and fruit flies (Le Bourge, 2013; McDonald et al., 2013), which may be used in future frailty research, particularly high-throughput screening. The two major types of frailty assessment, the FP and FI, each have their own unique strengths and weaknesses. The FP remains better at identifying the physical manifestations of frailty, while the FI employs a more rounded ‘systems’ approach that has wide applicability. Ultimately, discovering interventions that lengthen the healthspan of an individual, and not just their lifespan, are of upmost importance in geriatric medicine. Frailty approaches such as the FI and FP can serve as a surrogate measure to quantify the heterogeneity in aging.

These preclinical tools allow researchers to better understand the effects of behavioural, environmental, surgical, or pharmacologic interventions on frailty (Banga et al., 2019). In doing so the mechanisms of frailty can be uncovered and practical therapeutic interventions to extend an individual's healthspan can be found. Conversely, the effects of frailty on treatment choices (*i.e.*, drug therapies) are also important to investigate given the global risk of frailty and associated pharmacokinetic changes (Johnston et al., 2014). In addition, sex differences seen in humans are often but not always demonstrated in animal models. While human females are typically frailer than males, this does not hold true for all animal models. The underlying reasons are unclear and offer interesting research avenues, including the effects of parity on frailty in aging.[2]

The advancement of frailty research will require further application of frailty assessments to different models and interventional studies. Contemporary challenges include the comparison between the FI and FP approach to measuring frailty and the translation of frailty assessment into more preclinical research. To overcome these challenges, researchers need to determine the most appropriate frailty assessment methods for their area of research and apply them. Different frailty measurements also need to be directly compared to themselves to enhance our collective understanding of preclinical frailty models. Future studies should optimize known frailty tools and seek to apply them to better understand the mechanisms of frailty. The ability to quantify frailty in preclinical models enables the eventual translation of interventions to treat frailty into humans and will provide insight into the mechanisms underlying the development of frailty.

2.3 A systematic review and meta-analysis of frailty indexes based on laboratory measures

The following text is adapted from a currently unpublished systematic review on

[2] The idea that more births could lead to higher frailty was discussed by Hubbard and Rockwood (2011) when reasoning why women are frailer than men. They referenced a curious analysis by Westerndorp and Kirkwood (1998), who analyzed birth and death records from the British aristocracy (1500-1875). They reported that women having more children was related to lower longevity, while giving birth to a first child later in life related to extended longevity. This suggested a trade-off between reproduction and longevity. Out of interest, evidence using more recent data is supportive of this theory, but the magnitude has decreased into the modern era (Hsu et al., 2021).

FIs based primarily on laboratory-derived measures. This project was undertaken as part of my doctoral studies to provide a summary and critical analysis of this relatively new approach to measuring frailty. The paper is in the first round of revisions at *Age and Ageing* as of July 2022.

2.3.1 Introduction to a frailty index based on deficit accumulation

The FI is an instrument used to quantify frailty and is based on a deficit accumulation model (Mitnitski et al., 2001). To achieve this end, various clinical health measures across physiological systems are assessed dichotomously either as deficient or not, which are then summed and divided by the total number of assessments. This yields a score ranging from 0 (no deficits) to 1 (all deficits). The resultant FI score is a macroscopic variable that reflects the state of an individual's health irrespective of how chronologically old they are, sometimes referred to as 'biological age' (Mitnitski et al., 2002). In this way, the FI reduces dozens of dimensions into a single variable (Mitnitski et al., 2001).

A clinical FI is often constructed from a comprehensive geriatric assessment (Cooper et al., 2022; Howlett et al., 2021; Cohen et al., 2016; Jones et al., 2004). A more recent deficit accumulation FI method primarily employs laboratory data (FI-Lab) to substitute for, or complement the count of deficits (Howlett et al., 2021; Howlett et al., 2014). Laboratory derived components are employed as non-arbitrary physiologic measures that count as deficits when deviating from an acceptable range. The first FI-Lab was in a murine aging model (Parks et al., 2012), although not presented as such. In 2014, Howlett et al. developed the first formal FI-Lab using standard laboratory tests in humans (Howlett et al., 2014). This approach has subsequently been used in various human and preclinical aging studies, as reviewed here. The FI-Lab can be calculated readily, and its components can usually be obtained from commonly measured hospital tests. Indeed, basing an FI-Lab on routinely collected data was part of its inception (Howlett et al., 2014). Thus, operationalizing standard laboratory data into an FI-Lab may be a convenient and accessible way to assess frailty in a clinical setting. The subsequent FI-Lab score could then be used as a screening tool, as has been suggested (Rockwood et al., 2015).

Likely due to its relatively recent origins, we found no systematic reviews or meta-analyses that focus on the FI-Lab. To summarize the available evidence on the FI-Lab, we performed a systematic review and meta-analysis on studies involving the FI-Lab. Our primary objective was to assess the relationship between the FI-Lab and mortality in humans. Secondary objectives were to assess the FI-Lab in relation to other adverse events, examine sex differences in FI-Lab scores, and review whether preclinical studies that have used the FI-Lab offer results similar to human studies.

2.3.2 Methods

PRISMA guidelines

This systematic review and meta-analysis followed the PRISMA 2020 guidelines. The protocol was published on Open Science Framework. The most recent protocol is publicly available at: https://doi.org/10.17605/OSF.IO/2ASF9. All amendments are dated and explained in the protocol. The PRISMA 2020 checklist for this systematic review and meta-analysis is available in Appendix D (Sub-appendix A) at the end of this dissertation.

Data Source and Search Strategy

Two electronic literature searches were conducted in July 2020 and May 2021 by author DGS. Papers published in English from January 2010-May 2021 were searched for on electronic databases (PubMed, CINAHL, MEDLINE, EMBASED, Scopus, Web of Science). Specifically, we searched for ((“Frailty Index” AND Laboratory) OR (FI-LAB AND Frailty)) using Boolean-based terms. The searches were full text unless there were over 500 results from a single database.

Inclusion criteria:

An FI of at least ten measures, where 70% or more of the deficits measured must be laboratory data, defined as any non-arbitrary diagnostic measure including clinical measures (*e.g.*, hemodynamic measures).

Exclusion criteria:

Papers were excluded if they were case studies, reviews, conference reports/presentations/abstracts, opinion pieces, or unpublished data.

Data Collection and Management

Author DGS compiled a list of articles from all databases, removed duplicates, and completed a primary screening. Subsequently, authors DGS and BMC independently screened the remaining article titles and abstracts. Reviewer SEH arbitrated conflicts in screening; inconsistencies were resolved by discussion. If one study included multiple hazard ratios (HRs), then the closest to only age- and sex-adjusted models, closest to 1-year follow-up time, and most items measured were used for the meta-analysis. HRs were collected at the 0.01 or 0.1 decimal place, but always reported at the 0.01 level.

Subgroupings

Five subgroupings were created to categorize papers based on their findings, including: mortality; adverse events; sex differences (humans); preclinical models; and sex differences in preclinical models (Supplemental Figure 1, Appendix D). A single paper could exist in multiple subgroups. All papers were considered for subgroup analysis; however, two studies (Chao et al., 2020; Theou et al., 2016) did not fit any of the criteria and were not included in further subgroup analysis. The 'mortality' subgroup was further divided for dichotomous statistical comparisons considering study populations and design, including sample size; sex; age; items measured; and follow-up time (Supplemental Figure 2, Appendix D).

Risk of bias and certainty of evidence

Risk of Bias Assessment used a modified Newcastle-Ottawa scale (Sub-Appendix B, Appendix D). Studies were excluded from meta-analysis if they had 4 or fewer 'stars' across all categories (Supplemental Table 1, Appendix D). The Grading of Recommendations, Assessment, Development and Evaluations (GRADE) scale was used to determine the certainty of evidence. A detailed description of the meta-bias assessment and certainty of evidence can be found in the Supplemental Data.

Statistics

The inverse variance method was used to calculate effects using log-transformed HRs, based on a 0.01 FI-Lab unit change. Study heterogeneity was assessed using

Cochrane's Q and the I^2 test statistic. If heterogeneity was present, study populations were assessed using a random effects model rather than a fixed effects model. Detailed descriptions of the statistical approach can be found in the Supplemental Data, Appendix D.

2.3.3 Results

Search and selection

Two systematic searches across 6 electronic databases (PubMed, CINAHL, MEDLINE, EMBASE, Scopus, and Web of Science) identified 350 articles for the systematic review. The first search was in July 2020, followed by another search in May 2021. Figure 2.5 depicts the screening process.

Characteristics of human studies

Of 25 human studies (Table 2.7) the first was published in 2014 (Howlett et al., 2014) and 12 were published since 2019. Studies came about equally from the Asia-Pacific region (six from China (Gu et al., 2021; Hao et al., 2019; Ma & Sun et al., 2019; M & Lu et al., 2018; Wang et al., 2019; Yang et al., 2018); one from each of Australia (Theo et al., 2016), South Korea (Sohn et al., 2019) and Northern Taiwan (Chao et al., 2020), Europe (Blodgett et al., 2016; Ellis et al., 2020; Heikkila et al., 2021; Jäger et al., 2019; Klausen et al, 2017; Mitnitski et al., 2015; Nixon et al., 2019; and Ritt et al., 2017), and North America (five from the USA (Bello et al., 2018; Blodgett et al., 2019; Blodgett et al., 2017; Justice et al., 2019; and King et al., 2017), and three from Canada (Howlett et al., 2014; Rockwood et al., 2015; and Cheung et al., 2017)). The sample sizes ranged from 14 clinical trial participants (Justice et al., 2019) to 8898 in a repeat of sequential cross-sectional studies (Blodgett et al., 2019). The mean age range was from 49.4 years (Blodgett et al., 2019) to 93.6 years (Hao et al., 2019); four studies did not disclose a mean age of participants (Theou et al., 2016; Wang et al., 2019; Klausen et al., 2017; and Blodgett et al., 2019). The average percent female population across all studies was 49.7%.

Follow-up periods ranged from 3-weeks (Justice et al., 2019) to 18 years (Heikkila et al., 2021). Four studies included multiple follow-up periods (Theou et al., 2016;

Heikkila et al., 2021; Jäger et al., 2019; and Ritt et al., 2017); two studies were cross-sectional with mortality follow-up (Blodgett et al., 2019; Blodgett et al., 2017); five were cohort studies (Ma & Lu et al., 2018; Ma & Cai et al., 2018; Nixon et al., 2019; Bello et al., 2018; Cheung et al., 2017); and one was a retrospective observational study (Gu et al., 2021). Blodgett et al. (Blodgett et al., 2017) addressed mortality using national death records.

The number of items measured per FI-Lab ranged from 14 deficits (Heikkila et al., 2021) to 44 deficits (Wang et al., 2019). The average number of deficits across the 25 studies was 26.1 (±6.9 as standard deviation). The modal value of deficits was 23, and nine studies employed that many in their FI criteria (Howlett et al., 2014; Rockwood et al., 2015; Chao et al., 2020; Gu et al., 2021; Ma & Lu et al., 2018; Ma & Cai et al., 2018; Blodgett et al., 2016; Ritt et al., 2017; and Cheung et al., 2017). The items measured varied across studies and have been summarized in Supplemental Figure 3, Appendix D. Of the items used, 91% were from blood/urine tests and 9% were from physical measures (especially vital signs, *e.g.*, blood pressure, heart rate, and oxygen saturation).

Studies involving preclinical models

Three murine studies incorporated an FI-Lab (Table 2.7). The first was published in 2012 (Parks et al., 2012), while the others were published in 2017 and 2019, respectively (Antoch et al., 2017; Kane et al., 2019). Two studies were published in Canada (Kane et al., 2019; Parks et al., 2012), and the third in the USA (Antoch et al., 2017). One study did not disclose the percentage of female mice used (Antoch et al., 2017); the other two used approximately equal numbers of males and females (Kane et al., 2019; Parks et al., 2012). Mean ages were not disclosed, but the age range of mice used was 6-30 months. The number of the FI-Lab items used ranged from 16 variables (Antoch et al., 2017) to 31 (Parks et al., 2012). One study (Antoch et al., 2017) used 16 variables to construct the FI-Lab for male mice and 18 variables for female mice.

Certainty of evidence

Risk of bias was assessed using a modified 8-item Newcastle-Ottawa scale (Appendix B). The GRADE approach was used to assess the quality of evidence for each

of the subgroups, with evidence per subgroup ranging from very low to moderate (Table 2.8).

The FI-Lab as a predictor of mortality

Higher FI-Lab scores related to increased mortality risk in all included studies (Table 2.9). The relationship with mortality was assessed by a meta-analysis of studies that reported a HR based on a continuous 0.01 increase in FI-Lab scores. These effect sizes were heterogenous, as indicated by the I^2 (panel A, Figure 2.6) and the funnel plot with visual asymmetry (Supplemental Figure 4A, Appendix D). Despite the appearance of the funnel plot, Egger's (p=0.117) and Begg-Mazumdar's (p=0.144) tests for funnel plot asymmetry did not show significant risk of bias (Supplemental Figures 4B and 4C, Appendix D). However, the large I^2 value and visual inspection of the funnel plot supported the use of a random effects model for the meta-analysis.

Using a random effects model, the FI-Lab predicted mortality across all ages (panel A, Figure 2.6). To evaluate how the FI-Lab predicts mortality for older individuals, a subgroup analysis was performed that separated studies at a mean age of 80 years. The 80+ year old group had a more favorable GRADE score and a non-significantly higher HR. The mean age (or median for Wang et al. (2019)) of a study and the HR per 0.01 unit increase in FI-Lab scores were not significantly related (p=0.17; data not shown). We also separated studies by follow-up time (above/below 2 years); shorter follow-up times yielded a higher HR (panel B, Figure 2.6).

The number of FI-Lab items assessed (fewer or more than 25) had no effect on mortality risk (panel C, Figure 2.6). Likewise, the proportion who were community dwelling (Supplemental Figure 5A, Appendix D), the sample size (Supplemental Figure 5B, Appendix D), and percent female sex (data not shown) or age or sex adjustment (data not shown) demonstrated no significant differences between groups. Each mortality subgroup analysis demonstrated high heterogeneity, save for the community dwelling group which had a sample size of two.

Adverse events

Eleven studies investigated the relationship between FI-Lab scores and adverse

events (Table 2.8). Of these, ten showed that FI-Lab scores were related to adverse events. The FI-Lab predicted adverse events both a general population (*e.g.,* Blodgett et al. (Blodgett et al., 2019; Blodgett et al., 2016) and in clinical groups (like cancer patients (Wang et al., 2019) and chronic kidney disease patients (Nixon et al., 2019).

Sex differences in FI-Lab scores

Of six studies that assessed how FI-Lab scores differ by sex, four concluded that men had higher FI-Lab scores than women in older age (Hao et al., 2019; Bello et al., 2018; Blodgett et al., 2019; and King et al., 2017). Two studies observed no sex differences in FI-Lab scores (Ma & Lu et al., 2018; Cheung et al., 2017). One study (Blodgett et al., 2019) concluded that FI-Lab scores were higher in women aged 20-39 but higher in men aged 60+ years.

Preclinical models and the FI-Lab

Three studies used the FI-Lab in preclinical models (Table 2.7). Here too higher FI-Lab scores were associated with poorer health. Specifically, increasing FI-Lab scores related to obesity (Antoch et al., 2017), increased markers of inflammation (Kane et al., 2019), and ventricular contractile dysfunction (Parks et al., 2012). Sex differences were inconsistent: in one study, male mice had higher FI-Lab scores (Kane et al., 2019), in another higher scores occurred in female mice (Antoch et al., 2017). The third study did not identify a difference in FI-Lab scores between sexes (Parks et al., 2012).

2.3.4 Discussion

This systematic review of the FI-Lab, which was introduced in 2014, identified 28 studies. Of these, 25 investigated human health and 3 investigated murine health.

Mortality and adverse events

Our meta-analysis of the FI-Lab as a predictor of mortality in humans combined the HR per 0.01 change in FI-Lab scores across nine studies. A 0.01 change in FI-Lab score is roughly equivalent to a person gaining a quarter of a deficit, if 25 items were measured (or 1 deficit in 100 measures). Consequently, as in the overall deficit accumulation-based FI, a small change in risk is expected for a 0.01 change and becomes

larger when more deficits are present. Meta-analysis of 9 studies predicting mortality from an FI-Lab yielded an effect size as an HR of 1.04 (95%CI=1.03-1.05) per 0.01 increase in the FI score. Interestingly, this effect size is nearly identical to a meta-analysis of clinical FIs, which sometimes included some laboratory measures, to predict mortality, where the comparable HR was 1.04 (95%CI=1.03-1.04; Kojima et al., 2018). Together, this suggests the FI-Lab can predict mortality and under some circumstances, this effect may even be comparable to FIs not incorporating laboratory measures.

The relationship between the FI and mortality appears to hold in older adults. Our review did not identify much research using younger adults. One study suggested the prognostic value of an FI-Lab is not as strong a predictor of mortality in younger adults compared to older adults (Blodget et al., 2017). The improved predictive value in older adults might make sense, given the original criteria FIs are based upon (Searle et al., 2008), which suggests that deficits should increase with age and cover a range of systems. As suggested by Blodgett et al. (Blodgett et al., 2017), investigating the FI-Lab's relationship with adverse events in younger people, instead of mortality, may be more fruitful. The current state of evidence cannot answer this question, however.

Despite most study populations having a mean age of 80+ years, the lack of association between mean age and HR at the 0.01 level using weighted means is intriguing. At the least, this suggests an FI-Lab predicts mortality similarly for older populations, such as those analysed here. In addition, the fact that the FI-Lab was associated with incident mortality in both presentative population samples and in clinical/institutional samples suggests that it is a useful tool for diverse health populations.

We also examined how the number of deficits measured per FI-Lab affected its relationship with mortality, which demonstrated no differences in HRs for studies using 20-25 items versus those with 26-44 items. This supports the notion that the number and exact items measured are not important when comparing between samples, so long as they are ample enough and relate to a variety of physiological systems (Kojima et al., 2018). Sample size also did not seem to affect the FI-Lab's ability to predict mortality, suggesting that the included studies were powered appropriately.

The relationship between duration for follow-up time and mortality is difficult to interpret, as confounding factors may be in play. When comparing follow-up times, the subgroup with a shorter follow-up time had a higher HR. Although the reason is unclear, we suggest that this may be a consequence of study design, rather than an inherent feature of the FI-Lab. Studies conducted on populations at higher risk as here- nursing home (Yang et al., 2018) and hospitals (Ellis et al., 2020; Jäger et al., 2019; Ritt et al., 2017) may need shorter follow up times, because less time is necessary to achieve sufficient statistical power. Even so, there were no differences in HRs when separating community dwelling and institutionalized groups. Considering non-community dwelling populations included those living in long term care, nursing homes, or the hospital, this suggests that the FI-Lab's ability to predict mortality is not dependent on the initial risk of the sample.

Regarding non-fatal health issues, every study showed that the FI-Lab was associated with adverse events, except institutionalization in one study (Heikkila et al., 2021). It is unknown if this trend holds up to most adverse health events, but it is intriguing to think of the FI-Lab serving as a robust holistic health risk metric for non-fatal health issues.

Sex differences in the FI-Lab

There was a tendency to find increased FI-Lab scores in men, but not all studies agreed. Four of 6 studies that evaluated sex differences concluded that men have higher FI-Lab scores than women in older age. Notably, Bello et al. (Bello et al., 2018) found an age-frailty interaction, where women were frailer at younger ages but then became less frail compared to men at older ages. These findings are inconsistent with the male-female health survival paradox, which suggests that women have increased frailty, but they are more resilient than men and live longer (Kane et al., 2021; Gordon et al., 2016). With an FI-Lab it seems that men mostly have higher FI scores, so that the paradox is not present when using an FI-Lab. More dedicated studies, with less heterogeneity, are needed to establish whether there is a *bona fide* sex difference when using an FI-Lab. Whether the answer agrees with the male-female health survival paradox is unknown, although current evidence suggests it does not. Further, it will be important to identify whether the relationship between mortality and the FI-Lab as equal between men and women.

FI-Lab components

The deficits that make up the FI-Lab distinguish it from FIs based on clinical assessments. While both these tools function similarly, the FI-Lab is likely easier to automate and looks at frailty from a different perspective. In fact, the FI-Lab was able to improve the predictive power of a clinical FI through their combination or addition to a proportional hazard model (Rockwood et al., 2015). Even so, it is not clear whether this simply reflects the nature of the additional items, or that more items typically make for more informative FIs, especially after age 65 (Farrell et al., 2016). Standard laboratory tests can be core measures used to create an FI-Lab, as was suggested (Howlett et al., 2014), which operationalizes routinely collected data.

Preclinical models and the FI-Lab

Investigating the use of the FI-Lab in animal models is a narrow field. Only three studies were found, and they all used mouse models. However, it is theoretically transferrable to other preclinical frailty models within other species, such as rats or dogs (Heinze-Milne et al., 2019), to predict to a variety of diseases and adverse events, or mortality. All three murine studies reported a relationship between adverse health outcomes and FI-Lab scores. This is similar to humans and strengthens the case for preclinical application of FI-Labs.

Regarding sex differences, the murine studies did not corroborate the higher FI-Lab scores in males that trended in human studies. It is possible that the mice were not as relatively aged as the human males that were shown to be frailer, or the difference could lie in FI-Lab composition/measurements. More research is warranted to see if the relationship holds true across species, which has relevance for research into frailty interventions.

Quality of evidence

From the outset, we decided to focus on breadth for this systematic review and meta-analysis. This style has inherent benefits and limitations. We were able to collect all available information on the FI-Lab. However, the studies were quite diverse in nature, ranging widely in study populations. Our quality of evidence table, assessed by GRADE,

reflects the evolving literature and our broad inclusion criteria. Our meta-analysis for the overall mortality subgroup included low-quality evidence due to high statistical heterogeneity and the distribution of ages across studies. This portion of the meta-analysis lost quality because we sought to examine the FI-Lab's association with mortality in adults for all ages of 20 years or greater. However, the papers we identified were mostly older adults. In this way, the quality of evidence improved by reframing our question to older adults, but it identified that little is known about how the FI-Lab works in younger adults.

2.3.5 Conclusions

This systematic review and meta-analysis suggest that FI-Labs, made of diverse deficits, predict mortality, and other adverse health events in a variety of populations. FI-Lab scores tended to be higher in males, but not all studies agreed. This interestingly is opposite of what is found with clinical FIs, which typically find females to be frailer. Few preclinical studies have utilized an FI-Lab, but early results are promising.

Future research utilizing an FI-Lab may benefit from investigating the relationship between frailty in younger populations and subsequent health status changes. Additionally, there is emerging evidence that a more granular approach to health variable categorization (*i.e.*, a non-binary quantile approach) using an FI-Lab improves the model's accuracy (Stubbings et al., 2021) relative to dichotomizing variables. Both avenues deserve further attention. Lastly, we hope our review will draw more interest in FI-Labs in preclinical models, which will help support the discovery of frailty interventions.

2.4 Chapter summary

Frailty as a concept is similar but not quite analogous to disease, although like diseases, it may be treatable. Instead, it is a higher-level, more abstract continuous phenomenon that is a product of someone's state of health and health risk. Therefore, frailty is an important consideration in clinical practice; how frail someone is influences the seriousness of the risk to develop additional adverse health outcomes. Frailty is assessed clinically and preclinically using either a FP approach that focuses on physical frailty or a multidimensional FI approach, which incorporates measures of diverse health

systems. Importantly for results presented in Chapter 4 and 5, the FI approach in C57BL/6 mice shows remarkable similarities with clinical FIs. A newer type of FI, termed the FI-Lab that is calculated mostly using routinely collected laboratory values shows promise in predicting death and adverse events in humans but has been under-used in preclinical models.

2.5 Tables

(Continued on next page.)

Table 2.1 Frailty Assessment Models or Tools in Animals Based on the Frailty Phenotype Approach

Basis for Model	Species	Sex	Species/ Strain	Frailty Assessment	Strengths	Limitations	Ref.
IL-10 knock-out to mimic frailty phenotype (weight loss, weakness, low activity, muscle changes, inflammation)	Mouse	Female	IL-10$^{tm/tm}$ on C57BL/6 background	Not conducted	Can be used to investigate biological mechanisms of frailty	Does not model frailty in natural aging; models Crohn's disease; specific housing requirements	Walston et al., 2008
Neuromuscular healthspan scoring system to assess physical function & sarcopenia	Mouse	Male	C57BL/6	Functional assessment based on rotarod, inverted cling grip strength and *in vitro* muscle contractility	Combined score (grip strength, rotarod and contractility) reduces individual variability within groups	Focus on physical frailty only; specialized equipment required; invasive procedure; time-consuming	Graber et al., 2013
Frailty Phenotype (4 factors): grip strength, slow walking speed, low physical activity, endurance	Mouse	Male	C57BL/6	Inverted cling grip strength (weakness), rotarod (walking speed), voluntary wheel running (activity), grip test plus rotarod (endurance)	Evaluation is non-invasive; cut-off values to assess mice as frail or non-frail	Focus on physical frailty; specialized equipment; no weight loss factor; time-consuming; derived endurance factor	Liu et al., 2014
Frailty Phenotype (5 factors): grip strength, running time, weight loss, running speed, motor coordination	Mouse	Female	ICR/CDI	Grip strength (weakness), treadmill running time (endurance), weight loss, treadmill running speed (slowness), tight rope (activity)	Evaluation is non-invasive; cut-off values to assess mice as frail or non-frail	Focus on physical frailty only; specialized equipment required	Martinez de Toda et al., 2018
Frailty Phenotype (4 factors): strength, speed, physical activity, endurance	Rat	Male	Fischer 344	Forelimb wire suspension (strength), rotarod (speed), open field (physical activity), inclined screen (endurance)	Evaluation is non-invasive; cut-off values to assess mice as frail or non-frail	Focus on physical frailty; specialized equipment; no weight loss factor; time-consuming	Miller et al., 2017

Basis for Model	Species	Sex	Species/ Strain	Frailty Assessment	Strengths	Limitations	Ref.
Frailty Phenotype (5 factors): weakness, exhaustion, chronic undernutrition, low physical activity, poor mobility	Dog	Both sexes, neutered	Mostly Golden and Labrador Retrievers or crossbreed	Low muscle mass (weakness), fatigue/breathlessness (exhaustion), poor body condition (undernutrition), low perceived activity (activity) gait abnormalities/joint pain (mobility)	Evaluation is non-invasive; can be completed as part of routine veterinary care	Focus on physical frailty only; subjective criteria	Hua et al., 2016
Frailty phenotype (5 factors): weight loss, weakness, low activity, slowness, exhaustion.	Nonhuman primate	Both sexes	Rhesus monkey	Unintentional weight loss (weight loss), low estimated calorimetry (weakness), low total energy expenditure (low activity), low activity counts (slowness), impaired movement efficiency (exhaustion)	Accurate estimations of all five phenotype criteria	Invasive blood samples and expensive equipment	Yamada et al., 2018

Table 2.2 Use of the frailty index to assess frailty in rodents. DEXA = dual-energy x-ray absorptiometry; FI = frailty index; SD = standard deviation.

Species /Strain	Sex	Frailty Assessment	Frailty Scoring	Strengths	Limitations	Ref.
Mouse, C57BL/6	Both sexes	Score 31 health-related deficits based on activity levels (open field), body composition (DEXA scan) hemodynamics (blood pressure), and metabolism (blood tests).	Deficits coded by number of SD from mean values in young adults with >1 SD difference 0.25, and > 4 SD coded 1.0. FI score calculated as deficits in an individual divided by all deficits.	Assesses age-related deficit accumulation across a variety of health parameters	Does not assess cognitive function. invasive procedures (large blood samples); specialized equipment	Mitchell et al., 2015
Mouse, C57BL/6	Both sexes	Abbreviated 8-item FI from Parks et al. (2012) study based on activity levels (open field) only.	Deficits coded by number of SD from mean values in young adults with >1 SD difference 0.25, and > 4 SD coded 1.0. FI score calculated as deficits in an individual divided by all deficits.	Relatively easy to use	Focus on physical frailty only; specialized equipment required	Parks et al., 2012
Mouse, C57BL/6	Mostly female	Score 31 clinical measures across many systems including integument, ocular, nasal, musculoskeletal, digestive, urogenital, respiratory and vestibular systems.	Deficits coded with a simple scale. A score of 0 indicates no deficit, a score of 0.5 indicates a mild deficit, and a score of 1 designates a severe deficit.	Assesses deficits across many systems; convenient; rapid to administer; non-invasive.	Does not assess cognitive function	Whitehead et al., 2014
Rat, Fischer 344	Male	Modified from Whitehead et al. (2014) and adapted to rats. Score deficits based on 27 clinical measures across many systems.	Deficits coded with a simple scale. A score of 0 indicates no deficit, a score of 0.5 indicates a mild deficit, and a score of 1 designates a severe deficit.	Assesses deficits across many systems; convenient; rapid to administer; non-invasive.	Does not assess cognitive function	Yorke et al., 2017
Mouse, C57BL/6	Both sexes	Slight modification of the tool developed by Whitehead et al. (2014). Score deficits based on 27 clinical measures.	Deficits coded with a simple scale. A score of 0 indicates no deficit, a score of 0.5 indicates a mild deficit, and a score of 1 designates a severe deficit.	Assesses deficits across many systems; convenient; rapid to administer; non-invasive.	Does not assess cognitive function	Suckoff Rizzo et al., 2018
Mouse, C57BL/6	Both sexes	Modified from Parks et al. (2012). A 23-item FI-Lab was created from blood pressure, metabolism (blood tests) and echocardiography.	Deficits coded based on deviation from mean values for young adult mice. Values within ± 1.5 SD of the mean were scored 0 (no deficit) and values above or below the cut off were scored 1 (deficit).	Assesses deficits across many systems.	Does not assess cognitive function; focus on cardiac deficits; special equipment needed	Kane et al., 2019

Table 2.3 Summary statistics for curve fitting from panel A in Figure 2.1. The curve which fits the best is bolded.

Model	**R^2**	**SS**	**AICc**
Linear	0.8100	1.464	-3730
Quadratic	0.8480	1.172	-3866
Cubic	0.8510	1.150	-3875
Exponential	0.6950	2.350	-3437
Gompertz	**0.8532**	**1.131**	**-3887**

Notes: R=correlation coefficient; SS=sum of squares; AICc=Akaike Information Criterion (corrected). *NB:* The more negative the AICc, the better the data is predicted by the model.

Table 2.4 Summary statistics for curve fitting from panel B in Figure 2.1. The curve which fits the best is bolded.

Model	**R^2**	**SS**	**AICc**
Linear	0.2392	0.5712	-797
Quadratic	0.2438	0.5677	-795
Cubic	**0.2915**	**0.5319**	**-802**
Exponential	0.2435	0.5680	-798
Gompertz	N/A	N/A	N/A

Notes: R=correlation coefficient; SS=sum of squares; AICc=Akaike Information Criterion (corrected). *NB:* The more negative the AICc, the better the data is predicted by the model.

Table 2.5 Summary statistics for curve fitting from Figure 2.2. The curve which fits the best is bolded.

Model	**R^2**	**SS**	**AICc**
Linear	0.6112	1.111	-1483
Quadratic	0.6398	1.030	-1502
Cubic	**0.6463**	**1.011**	**-1505**
Exponential	0.6353	1.043	-1501
Gompertz	0.6351	1.043	-1499

Notes: R=correlation coefficient; SS=sum of squares; AICc=Akaike Information Criterion (corrected). *NB:*

The more negative the AICc, the better the data is predicted by the model.

Table 2.6 Sex differences in frailty in mouse models

Strain	Age	Frailty Assessment Tool	Sex Difference	Reference
C57BL/6	12 & 30 months	Frailty Index based on 31 health-related deficits in activity levels, body composition, hemodynamics and metabolism.	No significant sex difference reported; small sample size.	Parks et al., 2012
C57BL/6	5, 19, & 28 months	Clinical Frailty Index based on 31 health-related deficits across many systems (*e.g.*, integument, ocular, nasal, musculoskeletal, digestive, urogenital, respiratory and vestibular systems, etc.)	Females had higher Clinical Frailty Index scores than males, although this was not tested statistically.	Whitehead et al., 2014
C57BL/6 & DBA/2J	18 months	Clinical Frailty Index based on 31 health-related deficits across many systems (*e.g.*, integument, ocular, nasal, musculoskeletal, digestive, urogenital, respiratory and vestibular systems, etc.)	No significant difference between the sexes.	Kane et al., 2016
Swiss	6, 12, 18 & 24 months	Frailty Index based on 31 health-related deficits in activity levels, body composition, hemodynamics and metabolism.	Females had significantly higher Frailty Index scores than males.	Antoch et al., 2017
3xTg-AD & B6129F2 wild-type	6 to 35 months	Clinical Frailty Index based on 31 health-related deficits across many systems (*e.g.*, integument, ocular, nasal, musculoskeletal, digestive, urogenital, respiratory and vestibular systems, etc.)	Males (both 3xTg-AD & B6129F2 wild-type) had higher Clinical Frailty Index scores than females.	Kane et al., 2018
C57BL/6	17 & 23 months	Clinical Frailty Index based on 31 health-related deficits across many systems (*e.g.*, integument, ocular, nasal, musculoskeletal, digestive, urogenital, respiratory and vestibular systems, etc.)	Females had significantly higher Clinical Frailty Index scores than males.	Kane et al., 2019
C57BL/6	17 & 23 months	Modified from Parks et al. (2012). A 23-item Laboratory-based frailty index was created from blood pressure, metabolism (blood tests) and echocardiography.	Males had significantly higher Laboratory-based Frailty Index scores than females.	Kane et al., 2019
C57BL/6	9-13 & 16-25 months	Clinical Frailty Index based on 31 health-related deficits across many systems (*e.g.*, integument, ocular, nasal, musculoskeletal, digestive, urogenital, respiratory and vestibular systems, etc.)	Females had higher FI scores than males.	Keller et al., 2019
C57BL/6	17-32 months	Frailty Phenotype (5 factors): Walking speed, strength, endurance, and overweightness (2 or more factors out of 5 were considered frail).	Females were frailer than males at 26 months (but not at five other timepoints).	Bauman et al., 2019
C57BL/6	19 months	Clinical Frailty Index based on 31 health-related deficits across many systems (*e.g.*, integument, ocular, nasal, musculoskeletal, digestive, urogenital, respiratory and vestibular systems, etc.), and an 8-item performance-based Frailty Index.	Males were frailer than females at 19 months using both FI instruments.	Herrera et al., 2020
C57BL/6	23-32 months	Clinical Frailty Index based on 31 health-related deficits across many systems (*e.g.*, integument, ocular, nasal, musculoskeletal, digestive, urogenital, respiratory and vestibular systems, etc.)	No difference between males and females.	Cole et al., 2022

Table 2.7 Summary of study characteristics in the systematic review.

Author	Study Details/Location	Number of Participants	Female (%)	Age (mean) (range)	Follow-up Period	Number of Items in FI-Lab
Human Studies						
Bello et al. 2018	World Trade Center Health Program (WTCHP) New York, USA.	7346	16.7	51.0 40-85	-	33
Blodgett et al. 2016	European Male Ageing Study (EMAS). Europe.	Initial: 3369 At follow-up: 2933	0.0	60.2 40-79	4.4 years	23
Blodgett et al. 2017	National Health and Nutritional Examination Survey (NHANES). USA.	8888	51.7	49.4	-	32
Blodgett et al. 2019	National Health and Nutritional Examination Survey (NHANES) USA.	8898	51.7		-	32
Chao et al. 2020	Northern Taiwan.	33	55.0	69.5	2-3 years	LFI-1: 23 LFI-2: 32
Cheung et al. 2017	Canada.	221	47.5	76.8	-	23
Ellis et al. 2020	United Kingdom.	1580	55.3	84.8	21 months	27
Gu et al. 2021	China.	154	29.2	79.7	-	23
Hao et al. 2019	Project of Longevity and Ageing (PLAD). Sichuan Province, China.	736	67.5	93.6 90-108	4 years	22
Heikkila et al. 2021	Leito, Finland.	1153	58.0	73.6 64-100	10 and 18 years	14
Howlett et al. 2014	Canadian Study of Health and Ageing (CSHA). Canada.	Initial: 1013 Follow-up: 986	60.7	81.1	5 years	23
Jager et al. 2019	Erlangen, Germany.	Initial: 500 Follow-up: 494	67.4	82.8	6 months and 1 year	21

Author	Study Details/Location	Number of Participants	Female (%)	Age (mean) (range)	Follow-up Period	Number of Items in FI-Lab
Justice et al. 2019	North Carolina and Texas, USA.	14	14.3	70.8 55-85	1 month	34
King et al. 2017	Duke Established Populations for Epidemiological Studies of the Elderly (D-EPESE). North Caroline, USA.	1740	67.0	78.0 65-105	14 years	28
Klausen et al. 2017	Hvidovre, Denmark.	4005	49.7		3 years	17
Ma & Lu et al. 2018	Rugao Longevity and Ageing Study (RuLas). Jiangsu Province, China.	1463	57.8	77.4 70-84	-	23
Ma & Cai et al. 2018	Rugao Longevity and Ageing Study (RuLas). Jiangsu Province, China.	1780	52.8	77.0 70-87	-	23
Mitnitski et al. 2015	Newcastle 85+ Study. Newcastle, United Kingdom.	777	60.9	85.5 85	7 years	40
Nixon et al. 2019	United Kingdom.	90	50.0	69.0	-	27
Ritt et al. 2017	Erlangen, Germany.	Initial: 306 1-year follow-up: 304	67.6	82.9	6 months and 1 year	23
Rockwood et al. 2015	Canadian Study of Health and Ageing (CSHA). Canada.	595	67.9	82.7	6 years	23
Sohn et al. 2019	South Korea.	154	49.3	78.7	40 months	32
Theou et al. 2016	South Australia, Victoria, New South Wales.	53 6-month follow-up: 44 1-year follow-up: 36			3 months, 6 months, and 1 year	22
Wang et al. 2019	Chengdu, China.	1020	28.6		3.9 years	44
Yang et al. 2019	Chengdu, China.	329	68.1	85.2	1 year	30
Murine Studies						
Antoch et al. 2017	USA.	10-14 per group	-	6-30 months	-	PFI-M: 16 PFI-F: 18
Kane et al. 2019	Canada.	71	52.1	17 and 22.5 months	-	23
Parks et al. 2012	Canada.	12	50.0	12-30 months	-	31

Table 2.8 Grading of Recommendations, Assessment, Development and Evaluations (GRADE) for primary and secondary objectives.

OUTCOMES	**NO OF PARTICIPANTS (STUDIES) FOLLOW-UP RANGE**	**QUALITY OF THE EVIDENCE (GRADE)**
MORTALITY (META-ANALYSIS)	7909 (9 studies) 6 months – 7 years	⊕⊕⊖⊖ LOW Due to inconsistency[1], indirectness[2]
MORTALITY (META-ANALYSIS – AGE SUBGROUP)	5100 (7 studies) 6 months – 7 years	⊕⊕⊕⊖ MODERATE Due to inconsistency[1]
MORTALITY (WHOLE SUBGROUP)	23016 (15 studies) 6 months – 18 years	⊕⊕⊕⊖ MODERATE Due to indirectness[2]
ADVERSE HEALTH OUTCOMES	25625 (11 studies) 1 month – 18 years	⊕⊕⊕⊖ MODERATE Due to indirectness[3]
SEX DIFFERENCES	21271 (7 studies) N/A	⊕⊖⊖⊖ VERY LOW Due to risk of bias[4], inconsistency[5], indirectness[6]
SEX DIFFERENCES IN PRECLINICAL MODELS	111 (3 studies) N/A	⊕⊕⊖⊖ LOW Due to inconsistency[5], imprecision[7]

[1]Considerable heterogeneity amongst study results according to meta-bias tests.
[2]The studies are not wholly representative of our target age range of 20+ years.
[3]The studies are not wholly representative of our target age range of 20+ years. Each study identified a different adverse outcome.
[4]Risk of bias due to studies including men and women but not reporting sex-based analyses.
[5]Inconsistency in results across studies. More evidence is needed to make conclusions about sex differences.
[6]The populations tend to be on the older-end of the investigated age range (20+ years of age).
[7]It is unclear that the available preclinical evidence is sufficiently powered to detect sex differences.

Table 2.9 Subgroup summary data from studies included in each subgroup in the systematic review

Study	Mortality Hazard Ratios (95% CI)	Conclusions
Mortality		
Blodgett et al. 2016	1.04 (1.03-1.06). 0.01 change in FI-Lab score. Adjustments: age.	High FI-Lab scores associated with mortality.
Blodgett et al. 2017	1.63, 2.59, 3.62, 6.35. Age groupings: 0.1-0.2, 0.2-.03, 0.3-0.4, >0.4 Adjustments: age, sex.	High FI-Lab scores associated with mortality.
Ellis et al. 2020	Univariable: 1.51 (1.43-1.60). Multivariable: 1.45 (1.37-1.54). 0.1 change in FI-Lab score. Adjustments: age, sex, clinical frailty score, dementia, delirium, falls, residence at admission.	High FI-Lab scores associated with mortality. Higher mortality risk in females.
Gu et al. 2021	Groupings: ≤0.2, 0.2-0.39, 0.40-0.60, ≥0.69. Adjustments: unadjusted.	High FI-Lab scores predict in-hospital mortality in AECOPD patients.
Hao et al. 2019	1.33 (1.09-1.63). Adjustments: age, sex, educational levels.	Higher frailty proportions in mortality group. 53.4% rate of 4-year mortality.
Heikkila et al. 2021	1.69-3.75. Groupings: ≤ 0.08, 0.09-0.42, ≥0.43. Adjustments: age, sex.	High FI-Lab scores predict increase in mortality.
Howlett et al. 2014	(1.02-1.04). 0.01 change in FI-Lab score. Adjustments: age, sex.	High FI-Lab scores associated with mortality.
Jager et al. 2019	1.066 (1.051-1.081). 0.01 change in FI-Lab score. Adjustments: age, sex.	High FI-Lab scores associated with increased mortality rates for 6 months and 1-year after hospital re-admission.
Klausen et al. 2017	1.94 (1.57-2.40); 2.84 (2.31-3.49); 3.66 (3.00-4.48). Quartiles: 2;3;4. Adjustments: age.	Post-discharge mortality associated with FI-Lab scores.
Mitnitski et al. 2015	1.05 (1.04-1.07). 0.01 change in FI-Lab score. Adjustments: sex.	FI-Lab scores strongly associated with mortality.

Study	Mortality Hazard Ratios (95% CI)	Conclusions
Ritt et al. 2017	1.071 (1.05-1.093). 0.01 change in FI-Lab score. Adjustments: age, sex.	FI-Lab scores predict 6-month and 1-year mortality risk.
Rockwood et al. 2015	1.016 (1.007-1.025). 0.01 change in FI-Lab score. Adjustments: age, sex.	High FI-Lab scores associated with mortality,
Sohn et al. 2019	1.075 (1.040-1.111). Unadjusted.	High FI-Lab scores associated with early mortality in SAVR patients.
Wang et al. 2019	1.02 (1.01-1.03). 0.01 change in FI-Lab score. Adjustments: age, sex.	FI-Lab scores can predict mortality in lung cancer patients.
Yang et al. 2019	1.07 (1.05-1.09). 0.01 change in FI-Lab score. Adjustments: age, sex.	FI-Lab scores can predict 1-year mortality.

Study	Conclusion
Adverse Events	
Bello et al. 2018	FI-Lab scores are inversely associated with mental and physical health.
Blodgett et al. 2016	High FI-Lab scores are associated with frequent doctor visits, poor self-reported health.
Blodgett et al. 2019	High FI-Lab scores are associated with frequent doctor visits, poor self-reported health, and number of medications used.
Cheung et al. 2017	Pre-admission clinical frailty independently predicts adverse discharge destination in geriatric trauma patients.
Ellis et al. 2020	FI-Lab scores are associated with adverse outcomes, rates of hospital re-admission, and discharge location.
Heikkila et al. 2021	Laboratory index scores do not significantly predict institutionalization.
Justice et al. 2019	FI-Lab scores were associated with pro-inflammatory cytokines.
Ma et al. 2018b	FI-Lab scores are associated with QTc prolongation.
Nixon et al. 2019	FI-Lab scores are associated with worsening kidney function in CKD patients.
Sohn et al. 2019	FI-Lab scores are associated with short- and long-term outcomes after SAVR in elderly patients.
Wang et al. 2019	FI-lab scores are associated with uncontrolled diseases. FI-Lab scores can predict adverse outcomes in cancer patients.
Sex Differences	
Bello et al. 2018	Higher FI-Lab scores in males.
Cheung et al. 2017	No sex differences observed.
Blodgett et al. 2019	Higher FI-Lab scores in females aged 20-39, males aged 60+ years.

Sex Differences	
Hao et al. 2019	Higher FI-Lab scores in males.
King et al. 2017	Higher scores in males.
Ma et al. 2018a	No difference in FI-Lab scores.
Preclinical Models + Sex Differences in Preclinical Models	
Antoch et al. 2017	Higher PFI scores in female mice.
	Higher PFI scores in obese mice.
Kane et al. 2019	Higher FI-Lab scores in male mice.
	FI-Lab scores associated with IFN-ϒ and IL-9 levels.
Parks et al. 2012	FI scores associated with larger cardiomyocyte length.
	FI scores associated with cardiac contractile dysfunction.
	No sex differences identified.

2.6 Figures

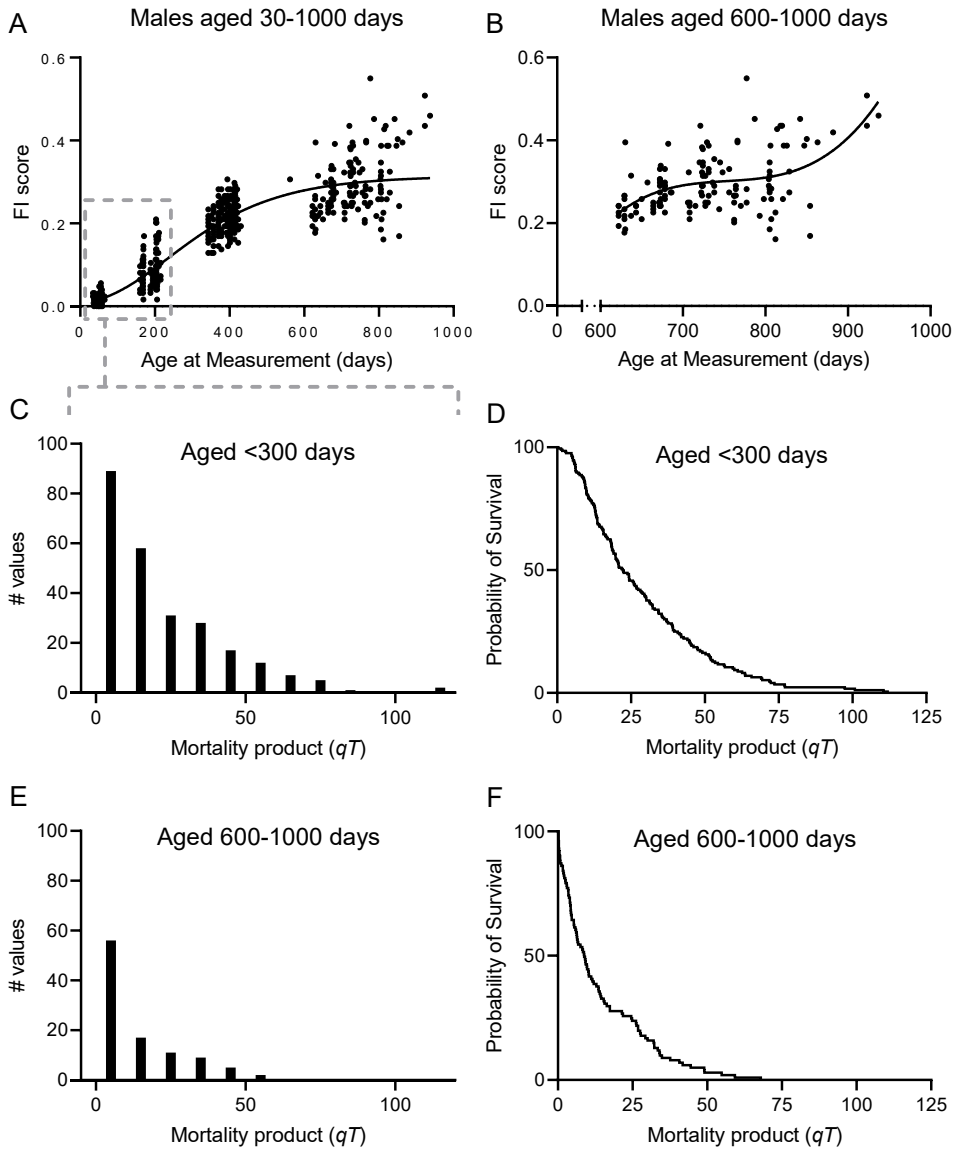


Figure 2.1 Frailty scores in mice increase with age and model human mortality products. A) Frailty scores from a 31-item non-invasive frailty index increased over time in male C57Bl/6 mice from a young age until death. A Gompertz curve was fitted and best described the data compared to four other curves (Table 2.1). B) The oldest grouping of mice (A) is analyzed the same way, but is best predicted by a cubic curve. A histogram (C) of mortality products (*qT*; frailty index score × days from assessment to death) for mice assessed <300 days shows a similar trend to humans (see Mitnitski et al., 2001). D) When fitted onto a survival probability curve, the relationship appears similar to that of humans, suggesting that frailty can predict the probability of survival for a given length of time. Mortality products were understandably lower (E), and the survival probability was steeper (F), which is expected given the shorter time-to-deaths expected for older mice.

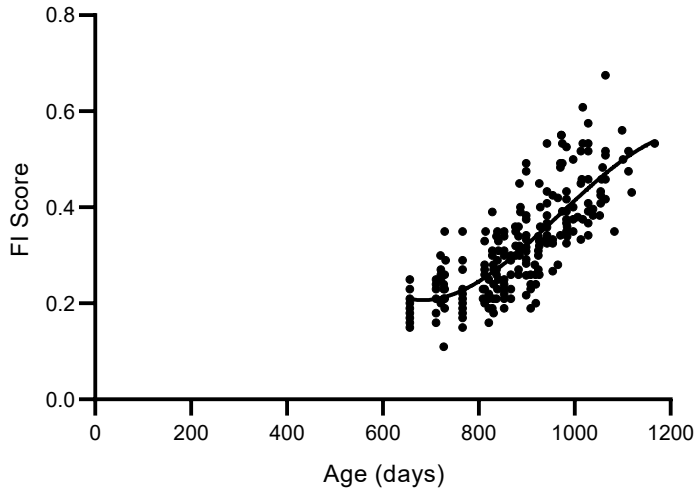


Figure 2.2 Frailty scores at 600+ days of age in C57BL/6 mice from a 31-item non-invasive frailty index increased over time in aged male C57Bl/6 mice until death. A cubic curve was fitted and best described the data compared to four other curves (Table 2.3). Data were drawn from publicly available data from a previously published manuscript (Schultz et al., 2020). The mice were euthanized only if moribund and expected to die within 48 hours.

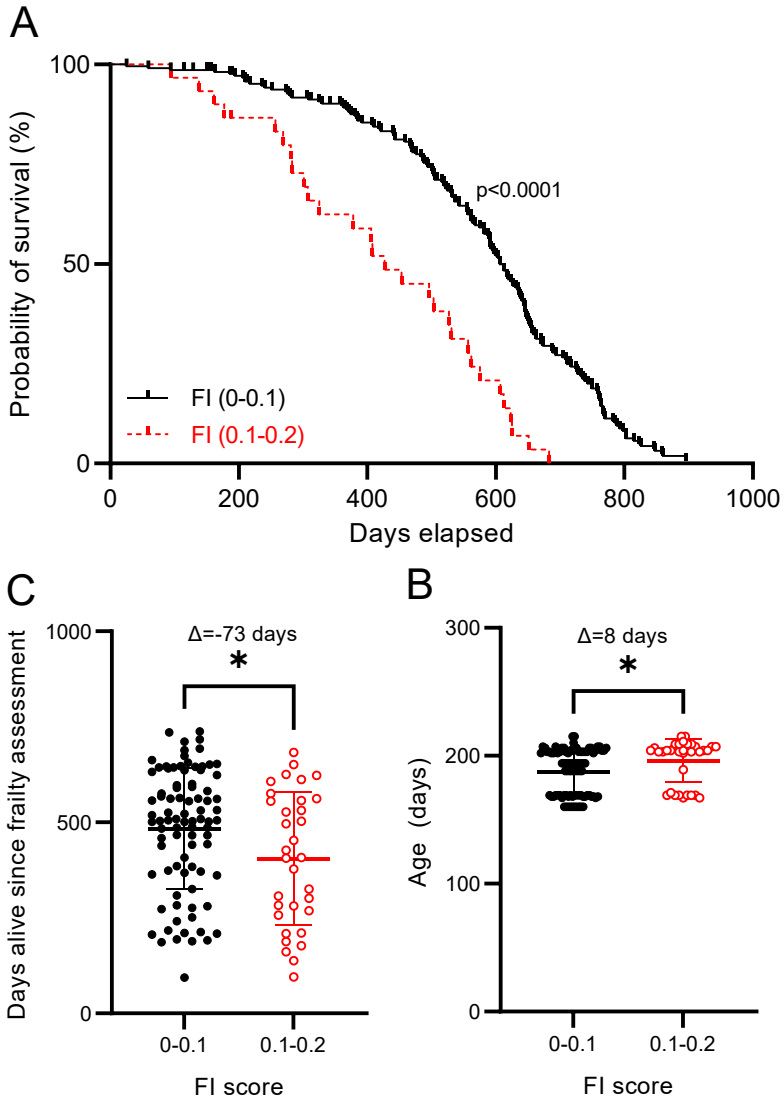


Figure 2.3 Survival curve, ages, and frailty scores for mice assessed <300 days of age.

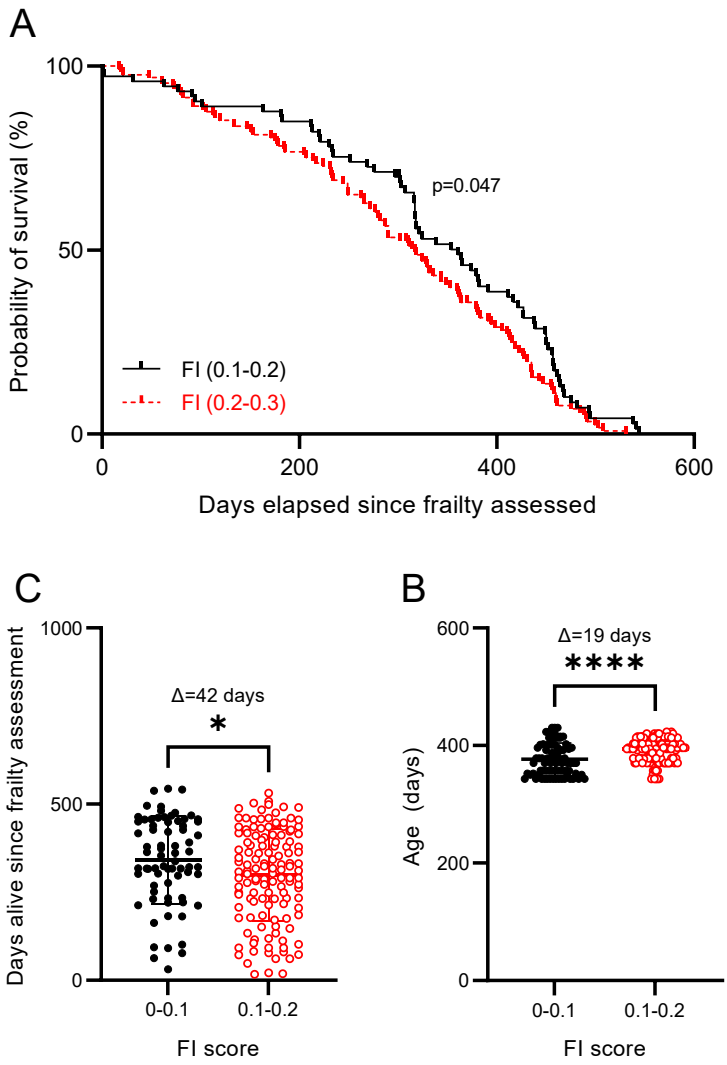


Figure 2.4 Survival curve, ages, and frailty scores for mice assessed 300-430 days of age.

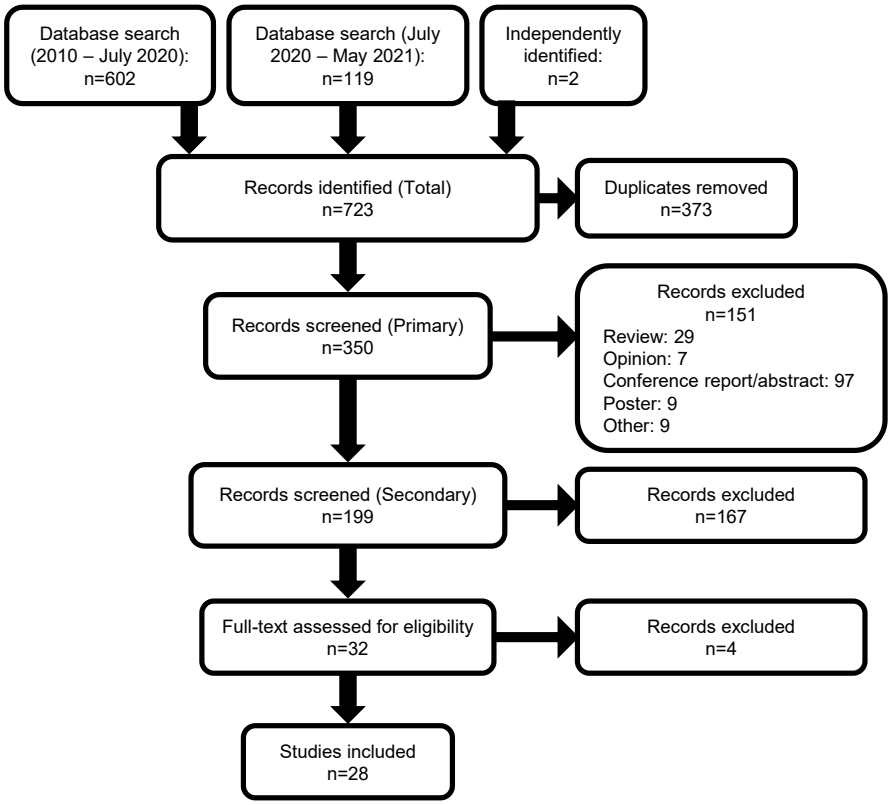
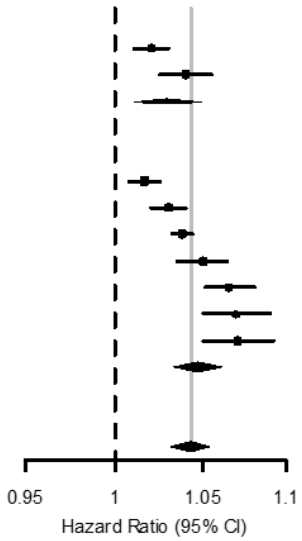


Figure 2.5 Search and screening results flow diagram of systematic review.

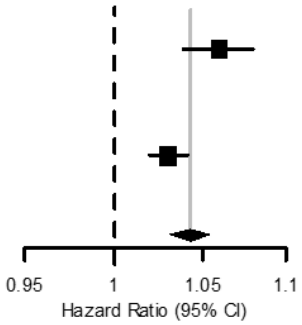
A

Subgroup	HR	95% CI	Weight
Age: 20-80 years			
Wang et al., 2019	1.02	[1.01; 1.03]	11.9%
Blodgett et al., 2016	1.04	[1.03; 1.06]	10.8%
Total (random effects)	1.03	[1.01; 1.05]	22.7%
Heterogeneity: $\chi^2_1 = 4.79$ ($P = .03$), $I^2 = 79\%$			
Age: 81+ years			
Rockwood et al., 2015	1.02	[1.01; 1.03]	12.1%
Howlett et al., 2014	1.03	[1.02; 1.04]	12.0%
Ellis et al., 2020	1.04	[1.03; 1.04]	12.7%
Mitnitski et al., 2015	1.05	[1.04; 1.07]	10.8%
Jäger et al., 2019	1.07	[1.05; 1.08]	10.9%
Yang et al., 2018	1.07	[1.05; 1.09]	9.6%
Ritt et al., 2017	1.07	[1.05; 1.09]	9.2%
Total (random effects)	1.05	[1.03; 1.06]	77.3%
Heterogeneity: $\chi^2_6 = 60.64$ ($P < .001$), $I^2 = 90\%$			
Total (random effects)	1.04	[1.03; 1.05]	100.0%
Heterogeneity: $\chi^2_8 = 71.51$ ($P < .001$), $I^2 = 89\%$			
Test for subgroup differences: $\chi^2_1 = 2.05$ ($P = .15$)			



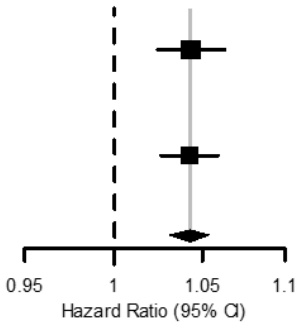
B

Subgroup	HR	95% CI	Weight
Follow-up (Years): 0-2			
Total (random effects)	1.06	[1.04; 1.08]	42.4%
Heterogeneity: $\chi^2_3 = 24.79$ ($P < .001$), $I^2 = 88\%$			
Follow-up (Years): 3+			
Total (random effects)	1.03	[1.02; 1.04]	57.6%
Heterogeneity: $\chi^2_4 = 20.16$ ($P < .001$), $I^2 = 80\%$			
Total (random effects)	1.04	[1.03; 1.05]	100.0%
Test for subgroup differences: $\chi^2_1 = 6.24$ ($P = .01$)			



C

Subgroup	HR	95% CI	Weight
Number of items: 10-25			
Total (random effects)	1.04	[1.02; 1.06]	55.0%
Heterogeneity: $\chi^2_4 = 46.14$ ($P < .001$), $I^2 = 91\%$			
Number of items: 26+			
Total (random effects)	1.04	[1.03; 1.06]	45.0%
Heterogeneity: $\chi^2_3 = 24.73$ ($P < .001$), $I^2 = 88\%$			
Total (random effects)	1.04	[1.03; 1.05]	100.0%
Test for subgroup differences: $\chi^2_1 = 0.00$ ($P = .96$)			



(Caption on next page)

Figure 2.6 Forest plots of mortality risk by hazard ratio (HR) according to a 0.01 increase in frailty measured by the FI-Lab. All forest plots used HRs from all studies included in the mortality subgroup meta-analysis. Data are presented on a log10 scale, the dotted-black line represents no effect, the solid-grey line indicates the overall effect of all studies. A) HR for all studies in the mortality subgroup. The mean age analysis is also represented with the respective effects of each age grouping. B) Analysis separated by follow-up time. C) Analysis separated by number of items measured. CI = confidence interval.

Chapter 3 Mechanisms underlying frailty

3.1 Introduction

Frailty is associated with age and disease, although neither are needed to become frail (Fulop et at., 2010). The biology of frailty is not yet well understood, although several fundamental mechanisms have been proposed. These include, but are likely not limited to genomic instability, chronic inflammation, cellular senescence, endocrine dysfunction, and mitochondrial damage (Goh et al., 2021; Bisset & Howlett, 2019). Overall, it is thought that frailty lowers physiologic 'reserves', which predisposes a frail person to a worse reaction to health challenges than a non-frail person (Nascimento et al., 2019). What these reserves are and how to keep them brimming into old age is a current topic of study in frailty/aging research. Despite different theories pointing to diverse mechanisms, it is interesting to reflect on how several age-related health problems share common risk factor like physical inactivity and obesity (Peters et al., 2019). A physical-functional mechanism of frailty that relates to these factors is sarcopenia, the age-related decline in muscle mass. Sarcopenia is notably downstream from fundamental molecular changes, like chronic inflammation, and will be reviewed here due to the proportional relationship between androgens and muscle mass and its clinical use as a screening tool for frailty (Finkelstein et al., 2013; Bhasin et al., 2001). Major putative molecular mechanisms are briefly reviewed here to contextualize this section in the broader realm of research on aging. Their relationships with androgens are touched upon for reference but are not explored in-depth, because they are not explicitly studied herein. Special emphasis will be given to chronic inflammation and sarcopenia, because they are mechanisms investigated in this dissertation. How they relate to testosterone will be discussed in Chapters 4-5.

3.2 Molecular mechanisms of frailty

Damage to cellular deoxyribonucleic acid (DNA) from exogenous (*e.g.*, radiation) or endogenous (*e.g.*, reactive oxygen species) sources has deleterious downstream effects that promote frailty (Schumacher et al., 2021). This damage is thought to accumulate over time and be a hallmark of aging (López-Otín et al., 2013). (Of note, the term "aging" with regards to the field of aging research can be re-framed as researching frailty (Howlett &

Rockwood, 2014)). Evidence supporting the theory that DNA damage precludes aging comes from studies on "accelerated aging" conditions like Hutchinson-Gilford or Werner syndromes, who have mutations for genes involved in DNA repair (Moskalev et al., 2013). Indeed, a potential model of frailty mentioned in Chapter 2.2.2.3 employs a genetically modified mouse model of Hutchinson-Guilford syndrome. Furthermore, accelerating DNA damage truncates lifespans, while promoting DNA repair can increase longevity in mice and *C. elegans* (Moskalev et al., 2013). Androgens contribute to mechanisms of DNA repair from such damage. In prostate cancer research, androgen deprivation therapy has been shown to impair DNA repair after radiation (Schiewer & Knudsen, 2019). This is in part because androgen receptor (AR) activation can lead to increased DNA repair mechanisms (Goodwin et al., 2013). Androgens therefore help regulate DNA repair mechanisms, although the implications regarding DNA repair into old age appear less studied.

DNA damage leading to epigenetic changes are also thought to contribute to aging. For reference, epigenetic changes refer broadly to modifications to histones, DNA, and noncoding ribonucleic acid (RNA) that lead to meaningful differences in gene expression (Zhang et al., 2020; Ferrucci et al., 2019). In cultured fibroblasts, late-passage cells, spanning population-doubling cycles of 80-85 times, had reduced histone synthesis and increased DNA methylation compared to early-passage cells (25-30 cycles; O'Sullivan et al., 2010). Notably, the location of chromatin modification is important when predicting the effects on aging. Epigenetic changes that lead to DNA segments with chromatin alterations reinforcing senescence (aptly termed DNA-SCARS) are common in senescent cells and are directly related to DNA damage (Rodier et al., 2011). Senescent cells, for reference, are cells in an ostensibly permanent and detrimental state of cell cycle arrest (Calcinotto et al., 2019). Similar epigenetic findings have been translated into humans, where methylated DNA levels from blood samples show highly predictive relationships with coronary heart disease, cancer, and mortality after adjusting for chronologic age (Lu et al., 2019). Interestingly, castration (*i.e.*, reduced androgen levels) led to a "feminization" of DNA methylation patterns in male sheep, which was associated with decelerated epigenetic aging patterns in adult sheep (Sugrue et al., 2021). The authors speculated that this may relate to sex differences in longevity, where females tend

to live longer than males (Lemaître et al., 2020).

Telomere deterioration has been posited as another mechanism of aging (López-Otín et al., 2013). Telomeres are made of thousands of TTAGGG repeats at the end of chromosomes that are integral for genomic stability (Rizvi et al., 2014). The degradation and damage of telomeres relates to overall DNA damage and increases with age (Schumacher et al., 2021; Rizvi et al., 2014). Importantly, damaged telomeres are considered to be irreparable and lead to cellular senescence (Fumagalli et al., 2012). Telomere health is therefore an important area of investigation when searching for mechanisms of aging. Inducing telomere elongation reversed age-associated epigenetic markers and restored histone synthesis in fibroblasts from a 92-year-old person to that of a 9-year-old (O'Sullivan et al., 2010). More recent evidence suggests that telomere length varies substantially between people and tissue types, despite a general correlation with age and several diseases associated with old age (Demanelis et al., 2020). Thus, telomere length and its degradation are important considerations as mechanisms of aging. Interestingly, androgen derivative therapy (*i.e.*, drugs that activate ARs) increased leukocyte telomere length in dyskeratosis congenita patients (Kirschner et al., 2021). This agrees with *in vitro* findings that testosterone increases telomerase activity in leukocytes, which is an enzyme that elongates telomeres (Calado et al., 2009).

It is therefore possible that androgens play key roles in putative, fundamental mechanisms of aging, although more research is needed to conclusively connect the two. Androgens appear to aid DNA repair and might help maintain telomere health, which would be beneficial in the setting of aging, although there may be deleterious effects on epigenetic aging. This research must be interpreted with caution though, given its relative incompleteness. Future studies in these areas will help illuminate any protective effects of androgens on these fundamental mechanisms of aging or otherwise. It must be also stated briefly that androgens have clear impacts on higher-level mechanisms such as mitochondrial dysfunction and protein synthesis dysregulation, which tend to be exacerbated by androgen deficiency in pathologic conditions (Rovira-Llopis et al., 2017; Rossetti et al., 2017), but this will not be discussed here in the interest of brevity.

3.3 Sarcopenia as a mechanism of frailty

Sarcopenia is an age-related syndrome characterized by decreased muscle mass and impaired strength or physical function (Cruz-Jentoft et al., 2010). Its relevance to frailty is outlined here, while the relationship between low testosterone levels and sarcopenic symptoms are explored in Chapter 4. Sarcopenia is highly related to physical frailty (Morley, 2016), which in turn is predictive of mortality in elderly patients (Chang & Lin, 2015). Sarcopenia has also been related to frailty when assessed using an FI (Feng et al., 2021). Many determinants of sarcopenia overlap with the molecular mechanisms of frailty reviewed briefly above, including DNA damage and cellular senescence (Csete, 2021; Nascimento et al., 2019). For this reason, it is possible to mistake frailty for sarcopenia or *vice versa*. It is indeed arguable that the similarities between sarcopenia and frailty destined the two fields to converge (Cesari et al., 2016). In a general sense, frailty can emerge from sarcopenia (Nascimento et al., 2019; Cesari et al., 2016), although there are many important facets of frailty that are largely independent of the physical limitations imposed by sarcopenia, including social factors like loneliness (Bessa et al., 2021). The consequences of sarcopenia on an individual's life nonetheless contribute substantially to the progression of frailty.

3.3.1 Impaired physical performance and reduced muscle mass in frailty

Before continuing, it is crucial to recognize that some frailty measurements (*e.g.*, FP approaches) utilize sarcopenic symptoms like muscle weakness in the assessment of frailty. On the surface this may seem like circular logic (*i.e.*, decreased strength ↔ frailty), but it is crucial to note that a physical frailty diagnosis with multiple physical issues is more predictive of adverse health outcomes, such as mortality, than any single physical deficit alone (Shamliyan et al., 2013). The relationship between sarcopenia and physical frailty is well exemplified by the percentage of overlap between the two conditions. In a study of German inpatients (n=100, mean age = 76.5 years), only 19% had frailty, measured by the FP, and sarcopenia together (Gingrich et al., 2019). Thus, having sarcopenia without the presence of frailty is not just possible, it can actually be common.

With that in mind, components of sarcopenia (muscle loss and weakness) both

relate to frailty. A study reporting on 560 outpatients (mean age=70 years, 60% female) in Vietnam analysed appendicular lean mass using dual-energy X-ray absorptiometry (DEXA) and evaluated its relationship with FP score (Nguyen et al., 2022). Lower appendicular lean mass was associated with higher frailty scores (Nguyen et al., 2022). A study on older Brazilian men and women (mean age=78, 56% women) also investigated the relationship between body composition using DEXA and frailty assessed using Fried's FP (Frisoli et al., 2021). It reported that having lower lean mass was significantly related to having a higher frailty score, even after adjusting for age, sex, osteoporosis, and diabetes mellitus (Frisoli et al., 2021). An interesting study performed in Toulouse, France, eloquently described the relationship between low lean mass and frailty (Fougère et al., 2018). Two-hundred and eighty-three people, 71% female and with a mean age of 82 years had their body composition assessed via DEXA upon admittance to a day hospital. The Fried FP approach was used to assess frailty. Five different methods of determining low lean mass were employed and only one found a statistically significant relationship between lean mass and FP score. Using this measurement, they reported that 36% of their population was both frail and had low lean mass, while 38% were otherwise frail and 26% had low lean mass but were not frail (Fougère et al., 2018). Thus, lower lean mass often relates to frailty in humans when assessed using the FP, although the relatively low amount of overlap between both suggests that lean mass alone cannot always accurately predict those that are frail.

Along with muscle mass, impaired physical fitness and frailty regularly correlate. Navarrette-Villanueva et al. (2021) systematically reviewed physical fitness tests to predict frailty and reported on 12 different types of physical tests. All dynamic or strength-based fitness tests regularly related to frailty, whereas more passive tests like flexibility were less reliable. Static balance appeared to be the least predictive in predicting frailty (Navarrette-Villanuev et al., 2021). Tests relating to physical performance can also be very easy to administer, like handgrip tests that simply require maximal voluntary squeezing of a handgrip dynamometer. Such tests not only relate to physical frailty or frailty assessed with an FI (Navarrette-Villanueva et al., 2021) but are thus an accessible piece of any frailty assay. The authors helpfully categorized tests by strength (handgrip or lower body), usual walking speed, maximum walking speed, and

aerobic capacity. Although handgrip was the least associated with frailty, all dynamic or strength-based tests were highly related to frailty scores. It must be noted that only four of the 20 included studies assessed frailty using an FI, however. Thus, reliability for physical tests to predict physical frailty was fairly convincing. On the other hand, more evidence is needed to understand the predictive role of formal physical fitness tests in multidimensional frailty assessments. This comprehensive analysis suggests overall that physical performance regularly predicts frailty status, especially physical frailty.

3.3.2 How sarcopenia contributes to frailty

The power of physical fitness tests to predict frailty is related to the natural decline in physical robustness with age but can also relate to non-physical frailty dimensions. Skeletal muscle strength (a surrogate measure for overall physical performance) declines steadily with age and exponentially from the sixth decade onward (Goodpaster et al., 2006). Perhaps more importantly, skeletal muscle power (the rate of force production) declines dramatically with age (Clark et al., 2010) and is highly predictive of reduced physical performance (Reid & Fielding, 2012). A concomitant reduction in muscle mass occurs but to a much smaller relative degree (Goodpaster et al., 2006). This reduction in muscle mass is due to reductions in muscular cross-sectional area (Goodpaster et al., 2008). Declines in muscular strength and size can be causally accompanied by excess adiposity, termed sarcopenic obesity, which can further impair muscle health and contribute to sarcopenia independently by attenuating protein synthesis and energy metabolism (Barazzoni et al., 2018). Indeed, high fat mass (but not fat-free mass) was associated with disability in men and women (n=4809) in the Cardiovascular Health Study (Visser et al., 1998). As mentioned earlier, sarcopenia does not merely relate to physical aspects of frailty. In fact, there is a bi-directional relationship between physical function and cognitive decline. In a study comparing autopsy results of patients with Alzheimer's disease pathology with physical frailty, it was concluded that physical frailty status was a better predictor of neuropathologic lesions than the presence of dementia (Buchman et al., 2008). Further, a meta-analysis reported that sarcopenia significantly related to cognitive impairment in both men and women (Chang et al., 2016). Low scores on several other physical fitness tests have also related to reduced cognition (Sui et al., 2020). Thus, age-related muscle wasting is related to physical

function (an intuitive conclusion) but is also related to cognitive decline, making it a multifactorial contributor to frailty.

3.3.3 Mechanisms underlying sarcopenia overlap with hallmarks of aging

A paper by López-Otín et al. (2013) highlights putative mechanisms of aging. Many of these mechanisms relate to sarcopenia and are briefly reviewed here alongside more muscle-specific changes to help contextualize assays of body composition and grip strength experiments in Chapter 5. The following text does not comprehensively cover all mechanisms but is a short narrative review on mechanisms of sarcopenia and their relationship with hallmarks of aging.

Increased levels of DNA damage in satellite cells, which are regenerative muscle stem cells, occur in older people versus young (Franco et al., 2018). These can lead to genomic issues that relate to the fundamental mechanisms of aging mentioned in Chapter 3.1 and impaired skeletal muscle reparation (Franco et al., 2018). For example, satellite cells from older humans (and mice) were more likely to be senescent than their younger counterparts (Sousa-Victor et al., 2014). Interestingly, telomere attrition (common in most organs) was not at all correlated with age in a sample of 952 human donors aged 20-70 years (Demanelis et al., 2020). It is notable however that age was significantly correlated with the *rate* of telomere shortening, inferred by correlating the difference in telomere lengths between paired muscle samples from the same donor (Demanelis et al., 2020). Thus, it is possible that muscles may be differentially affected by telomere shortening, but how this may relate to sarcopenia and frailty remains unanswered. On the other hand, methylated DNA was significantly elevated in older (~83 year old) people compared to younger (~27 year old) people (Turner et al., 2020). These epigenetic changes likely contribute to dysregulation in skeletal muscle metabolism seen into older age (Howlett & McGee, 2016; Zykovich et al., 2014). This metabolic dysregulation, in turn, is associated with sarcopenia, particularly via impaired insulin sensitivity (Nishikawa et al., 2021). Epigenetic changes in older individuals are seemingly prevented by regular physical activity, which helps to maintain healthy skeletal muscle metabolism in older age (Sailani et al., 2019).

Chronic, low-grade systemic inflammation, a mechanism of aging/frailty

(reviewed in Chapter 3.4), also plays a role promoting sarcopenia. Elevated levels the pro-inflammatory cytokines interleukin-6 (IL-6) and tumour necrosis factor-α (TNF-α) for example have been related to the presence of sarcopenia and muscle wasting (Tuttle et al., 2020; Bano et al., 2017). This is likely in part because chronic inflammation has been linked to catabolic mechanisms that lead directly to skeletal muscle atrophy (Jo et al., 2012). Issues also arise regarding the ATP-generating capacity of aging skeletal muscle mitochondria that can impair physical performance, a requisite of sarcopenia. An interesting study from the Baltimore Longitudinal Study of Aging assayed mitochondrial function from vastus lateralis muscle samples from participants aged 24-91 years (Gonzalez-Freire et al., 2018). They reported that older participants had significantly reduced mitochondrial respiratory capacity. Importantly, mitochondrial respiratory capacity predicted 400-m walk test performance, maximal oxygen uptake (during an *in vivo* cardiorespiratory fitness test), and skeletal muscle strength (Gonzalez-Freire et al., 2018). Thus, sarcopenic mechanisms relate to several hallmarks of aging, including genomic instability (including epigenetics and DNA damage), cellular senescence/stem cell exhaustion, chronic systemic inflammation, and mitochondrial dysfunction.

In addition to the above, deregulated nutrient (*i.e.*, protein) utilization and impaired protein synthesis are mechanisms of aging that relate to sarcopenia (Nascimento et al., 2019). The two are intimately related and ultimately their dysfunction contributes to muscle catabolism by preventing muscle regeneration. Although evidence is mixed, older adults seem to have a blunted anabolic response to dietary protein and may be susceptible to increase muscle protein breakdown (Wilkinson et al., 2018). For example, older adults need higher doses of protein per meal to stimulate skeletal muscle anabolism compared to younger adults (Katsanos et al., 2006). Further, there is evidence that the relative usage of ingested protein in older people versus younger people is reduced (Katsanos et al., 2005). This is important, because post-prandial protein synthesis appears to be the limiting factor for older adults instead of basal synthesis rates. In a study comparing basal and post-prandial protein synthesis in young (22 years old) and older (72 years old) men, it was reported that older men had a significantly blunted rise in protein synthesis despite there being no difference in basal synthesis rates (Wall et al., 2015). Following this, an unfortunate statistic is that about half of older community-dwelling adults and an even

greater percentage institutionalized residents do not consume the recommended amount of protein (Kisswetter, Sieber, & Volkert, 2020). It is important to note here that increasing the total amount of protein consumed can help prevent age-related muscle wasting in older adults (Ganapathy & Nieves, 2020; Thackler-Mercer e al., 2020).[3] Indeed, low protein intake can be related to the incidence of sarcopenia (Housten et al., 2017), although there is contradicting evidence that suggests protein intake alone is not enough to prevent sarcopenia (Granic et al., 2020; Verreijen et al., 2019). Thus, although impaired protein synthesis and reduced protein intake likely contribute to sarcopenia, age-related changes to muscle protein synthesis and contradicting results on protein supplementation suggest additional therapeutic strategies are needed to overcome barriers to stopping age-related muscle wasting.

On a more macroscopic level, age-related reductions in muscle power and function are related to changes in muscle fiber types. Muscle fibers are elementary units of a muscle that are essentially bundled together to form a single muscle. Muscle fibers can range from exhibiting low force generating capacity with long endurance (Type I) to high force generating capacity with little endurance (Type II), with hybrids in between (Zullo et al., 2020). Age-related declines in skeletal muscle cross-sectional area (and subsequent strength) are thought to be due to a reduction in the thicker, fast-twitch highly glycolytic Type II muscle fibers (Verdijk et al., 2014; Nilwik et al., 2013). On the other hand, the smaller, oxidative slow-twitch Type I muscle fibers do not change significantly with age, even into the eighth decade (Verdijk et al., 2014). These changes are highly related to reductions in the number of satellite cells, which decline significantly with age in Type II fibers but not Type I (Verdijk et al., 2014). These reductions are accompanied by increased myostatin levels (McKay et al., 2012). Myostatin is a negative modulator of muscle growth, suggesting that it may be a mechanism of sarcopenia (Sharma et al., 2015). Myostatin does this in part by inhibiting satellite cell proliferation and differentiation (McFarland et al., 2006). Overall, these changes lead to the reduced size and power of skeletal muscle in older age and contribute to sarcopenia.

[3] To reflect this finding, some scientists convincingly argue that recommended dietary protein intakes must be increased for older individuals from 0.8 g/kg/day to at least 1.0-1.2 g/kd/day (Bauer et al., 2013).

Thus, it is evident that aging can impair skeletal muscles at the molecular level and that there are many overlaps between generally accepted mechanisms of aging and sarcopenia. These changes underpin changes in muscle morphology and performance that are typical of sarcopenia or age-related skeletal muscle health decline. These changes are in turn related to frailty progression. Several of these changes are influenced by androgens and will be discussed in Chapter 4.

3.4 Chronic inflammation as a mechanism of frailty

This text was adapted from an invited narrative review article (Heinze-Milne, Banga, & Howlett, 2022) on the relationship between chronic low-grade blood borne inflammatory markers and frailty in humans and mice (see Appendix A for author contributions).

3.4.1 Introduction to "inflammaging"

The pathogenesis of frailty is not fully understood, but various mechanisms such as chronic inflammation, cellular senescence, oxidative stress, and other "pillars" of ageing are implicated (Goh et al., 2021). Of these various pillars, there has been considerable interest in the role of chronic inflammation or "inflammaging", as a key mechanism that drives accelerated ageing and frailty (Ferrucci & Fabbri, 2018). Chronic inflammation is linked to low circulating levels of testosterone, which is reviewed in Chapter 4, whereas this chapter focuses on the link between frailty and chronic inflammation. The age-associated increase chronic inflammation is distinct from acute inflammation, which is a transient process that arises in response to tissue damage and infection to protect the host by removing pathogens and facilitating tissue repair (Furman et al., 2019). Although sources of chronic inflammation are not fully understood, accumulation of a senescence-associated secretory phenotype (SASP), where senescent cells secrete proinflammatory factors like cytokines and chemokines, has been implicated (Rea et al., 2018; Furman et al., 2019). Cytokines bind to receptors on target cells to activate signalling pathways that alter gene expression to promote processes like differentiation, proliferation, and activation. A physically smaller sub-class of these are called chemokines, which are chemotactic cytokines that play a role in cell migration (Rea et al., 2018; Furman et al., 2019). Chronic exposure to these factors damages tissues and organs over time to increase the risk of disease, death, and frailty in older individuals

(Furman et al., 2019).

Here, we review key clinical evidence exploring links between cytokines and frailty in older adults, where frailty has been quantified most often using an FI or an FP instrument. In this sense, we are considering cytokines to be biomarkers of frailty. Biomarkers need to be related to the outcome of interest, like frailty, and be sensitive to changes in these outcomes (Justice et al., 2018). Notably, inflammatory biomarkers can be susceptible to measurement error and instability in circulation and when stored, which could obscure associations with clinical endpoints like frailty (Justice et al., 2018). Thus, we sought to summarize the regularity by which various circulating cytokines relate to frailty. We also investigate links between cytokines and measures of health or frailty in naturally ageing animals as well as in genetically manipulated models of chronic inflammation and frailty. Finally, we conclude by comparing relationships between cytokines and frailty in clinical populations and in mouse models.

3.4.2 The relationship between frailty and circulating inflammation in humans

Long-term immune system activation, which generates low-grade systemic inflammation that correlates with age, has been proposed as a mechanism underlying diseases and disabilities in older humans (Franceschi, 2000). This concept suggests that the immune response which helps young, fertile adults stay healthy, fails to control, and regulate the chronic inflammatory stimuli experienced into old age, which contributes to age-related health problems and possible frailty progression (Francheschi, 2007; Howlett et al., 2021). To compare to the mouse data presented later, Table 3.1 and Table 3.2 summarize key evidence linking circulating cytokines to frailty in humans. Studies included in our narrative review were found by searching published medical literature for studies involving cytokines/chemokines and frailty assessment in older humans. Brief informational summaries of cytokines and chemokines that most clearly relate to frailty are provided before discussing their relationship to frailty, to contextualize results in humans. Less commonly studied cytokines/chemokines are also highlighted to create a more diverse panel of potential frailty biomarkers that may overlap in humans and preclinical models.

IL-6 and its relationship with frailty in humans

Interleukin-6 (IL-6) is a member of the IL-6 cytokine family and influences diverse bodily functions, such as immunity, metabolism, and development (Kang et al., 2020). IL-6 has been identified as a major regulating factor of the SASP (Zhu et al., 2014) and its levels can be reduced by senolytic drugs (Zhang et al., 2022). Circulating IL-6 levels correlate with various disease states, like heart failure with preserved or reduced ejection fraction (Chia et al., 2021; Markousis-Mavrogenis et al., 2019), mitochondrial myopathy (Rue et al., 2014), Alzheimer's (Kim et al., 2017), and cancer development (Kumari et al., 2016). This has promoted interest in investigating whether IL-6 levels relate to frailty. Most studies (31 of 37) report that circulating levels of IL-6 increase as the degree of frailty increases (Table 3.1). This finding has been replicated across different frailty assessments, including the Fried FP (McKechnie et al., 2021) and the Rockwood FI (Collerton et al., 2012), along with other measurement tools such as the Liver FI (Mehta et al., 2021). IL-6 is related to higher frailty in community-dwelling men and women (Fried et al., 2009; Sammarath et al., 2019; Tembo et al., 2021) and in hospitalized people, such as cancer patients (Gilmore et al., 2020) and heart failure patients (Boxer et al., 2008). This suggests it may also be useful in clinical populations. Six of the 37 studies report no relationship between IL-6 and frailty, but none find an inverse relationship (Table 3.1). The reasons some studies do not report a link between frailty and IL-6 are unclear, although four of such studies examined relatively younger populations (Alberro et al., 2021; Hammami et al., 2020; Lu et al., 2016; Baylis et al., 2013). Overall, most evidence suggests IL-6 is a useful biomarker of frailty across sexes and in different participant populations. Notably, this relationship holds true across multiple types of frailty assessments.

TNF-α and its relationship with frailty in humans

Tumour necrosis factor-α (TNF-α) is a pleiotropic cytokine that contributes to multiple homeostatic and pathologic pathways throughout the body (Kalliolias & Ivashkiv, 2016). Importantly, increased expression of TNF-α is associated with the SASP (Kim et al., 2018). Its over-expression has been implicated in diseases including type II diabetes mellitus (Akash et al., 2018), Alzheimer's (Decourt et al., 2017), intervertebral disc degeneration (Risbud & Shapiro, 2014), and skeletal muscle wasting (Patel & Patel, 2017). These associations have likely spurred investigations into its relationship with

frailty. Interestingly, circulating TNF-α is generally higher in frailer individuals, although the results are less consistent compared to IL-6. Only nine of the 17 studies that investigate the relationship between TNF-α and frailty report a positive association (Table 3.1). This suggests that TNF-α may not be as reliable of a biomarker of frailty.

Male-female differences could help explain the heterogeneity observed in studies of TNF-α and frailty. In four studies involving only males, no relationship between TNF-α and frailty was seen (Buigues et al., 2020; Hsu et al., 2019; Navarro-Martínez et al., 2019; Lai et al., 2014). By contrast, Furtado, and colleagues (2020) found higher TNF-α levels in a study involving only women. A *post-hoc* analysis by Marzetti and colleagues (2019) showed that frailer males had lower TNF-α, which also supports the concept of a sex-dependent relationship between TNF-α and frailty. It is therefore possible that links between TNF-α and frailty are best interpreted in a sex-specific manner, although more evidence is required.

3.4.3 Relationships between other cytokines/chemokines and frailty in humans

There is growing interest to include additional cytokines in assay panels to predict frailty, which may have more sensitivity than any single analyte (Cardoso et al., 2018). Evidence suggests that some of these analytes hold promise as primary or adjunct factors for future frailty assay panels, as summarized in this section.

IL-8

IL-8 (a.k.a. CXCL8) is a chemokine that interacts with the CXCR1 and CXCR2 receptors in a multitude of organs in the body (Russo et al., 2014). Notably, activation of the CXCR2 receptor by IL-8 can promote the transition to a SASP (Acosta et al., 2008). Over-activation by IL-8 can facilitate the progression of numerous diseases, including cardiovascular disease, cancer, inflammatory bowel disease, and infections, among others (Russo et al., 2014). Although IL-8 has not been thoroughly investigated as a biomarker of frailty, a few recent studies have evaluated this. Higher serum IL-8 levels have been reported in frailer community dwelling men and women in three studies (Cybularz et al., 2021; Hammami et al., 2020; Hsu et al., 2019). However, one study found the opposite relationship, with frailer men and women having lower IL-8 levels (Marzetti et al., 2019). It must be recognized that Marzetti and colleagues' (2019) inclusion criteria included

only frail and non-frail individuals who were sedentary, with normal muscle mass and good physical performance. Selection for these attributes would be expected to influence the degree of frailty in the sample, given that these investigators used the physical FP tool, and may explain these divergent results. Results also have differed in prostate cancer patients where one study found a direct relationship between frailty and IL-8 and another did not (*cf.* Buigues et al., 2020 & Navarro-Martinez et al., 2019). It must be stated that both prostate cancer trials had a relatively small sample size (<50), which diminishes the power to detect relationships with cytokines and/or chemokines. Further, cytokine/chemokine measurements can vary between and within measurement types (Knight et al., 2020), which may help explain the discrepancy. Overall, IL-8 is a promising biomarker of frailty, but more work is needed to elucidate its usefulness.

IL-10

Il-10 is a powerful anti-inflammatory cytokine for many different types of immune and tissue cells and is crucial for homeostatic control of unnecessary inflammation (Wei et al., 2019). Because chronic, systemic inflammation is thought to be a pillar of frailty progression, it is possible that anti-inflammatory activation might combat pro-inflammatory mechanisms and help to attenuate frailty (Ferrucci & Fabbri, 2018; Bisset & Howlett, 2019). IL-10 is thought to mediate SASP progression in cells as well (Yeh et al., 2021), so it is an attractive candidate for assessing frailty status risk. Multiple studies have reported no relationship between circulating IL-10 levels and either the FP (Hsu et al., 2019; Nascimento et al., 2018; Su et al., 2017; Lu et al., 2016; Baylis et al., 2013) or FI (Lu et al., 2016). However, one study involving solely institutionalized elderly women found that higher IL-10 levels were directly proportional to frailty assessed with the FP approach (Furtado et al., 2020). Paradoxically, the relationship in this study was U-shaped, with median scores of IL-10 dropping from 15.6 pg/mL in non-frail women to 6.1 pg/mL in pre-frail women, while the frail category was reported to have 20.5 pg/mL. If the relationship between IL-10 and frailty is indeed non-linear, this will need consideration if IL-10 is included in a frailty assay panel.

MCP-1

Monocyte chemoattractant protein-1 (MCP-1; a.k.a. CCL2) is a chemokine that

regulates leukocyte chemotaxis (Bianconi et al., 2018). Due to its regulatory nature, higher levels of MCP-1 contribute to the progression of multiple inflammatory-mediated diseases and is associated with the SASP (Coppé et al., 2010). Higher MCP-1 levels either locally or systemically are associated with inflammatory diseases like rheumatoid arthritis, asthma, and systemic lupus erythematosus (Bianconi et al., 2018). Likewise, higher serum MCP-1 levels are linked to higher prevalence of and increased mortality from cardiovascular disease (Tajfard et al., 2017; Ding et al., 2015), as well as solid cancers (Wang et al., 2014). Given MCP-1's association with inflammatory diseases, it is not surprising that its relationship with frailty has been investigated. A significant positive relationship between MCP-1 levels and frailty has been found with the FP approach (Su et al., 2017; Liu et al., 2016). On the other hand, Hsu and colleagues (2019) found no significant relationship between MCP-1 and the FP, but only in men, and no relationship was seen in breast cancer patients evaluated using an 'oncogeriatric' frailty assessment tool (Brouwers et al., 2015). Two other studies (Marzetti et al., 2019; Lu et al., 2016) report that frail individuals have lower MCP-1 levels. On balance, while there is some evidence that MCP-1 is related to the degree of frailty, additional work in this area in clearly defined populations and using both sexes is required.

IL-1β

IL-1β functions as an amplifier for immune reactions and is tightly related to innate inflammatory, immune responses, and the SASP (Coppé et al., 2010; Dinarello, 2014). Like many other pro-inflammatory cytokines, it has links to cancer progression (Bent et al., 2018), cardiovascular diseases (Libby, 2017), and multiple autoimmune and inflammatory disorders (Galozzi et al., 2021). The wide-reaching influence of IL-1β, and indeed other members of the IL-1 family (Dinarello, 2014), make them important candidate biomarkers of frailty. However, the available evidence linking IL-1β to frailty is not convincing. Only one of five studies report a significant relationship between circulating IL-1β and frailty (Table 3.1; Furtado et al., 2020). This was the only study of the five to use an all-women sample (three used samples of all-men). Further, unlike most they used salivary samples to measure circulating levels of IL-1β. It is therefore possible that IL-1β is a better predictor of frailty in older women, although more evidence is needed to determine its utility, despite its clear value in the understanding and treatment

of conditions like cancer or cardiovascular disease (Libby, 2017).

Other candidate biomarkers of frailty in humans

Although some biomarkers have been studied extensively in the context of frailty (*e.g.* IL-6), there are several that have been explored rarely. The latter portion of Table 3.1 and all of Table 3.2 provide an overview of key cytokine and chemokine distributions in frailty research in humans. For brevity, each of the 50 additional cytokines or chemokines in these Tables are not reviewed individually, but we highlight select markers that have shown significant relationships with frailty so far. Is it also important to note that most cytokines in Table 3.2 come from the same study and are thus not independent observations evaluating the relationship between frailty and these cytokines. Caution is advised when interpreting each individual relationship for this reason.

Cytokines that have a direct proportional relationship with frailty: Two analytes are reported to be higher in frailer individuals, with no evidence to the contrary (Table 3.2). The chemokine I-309 (a.k.a. CCL1) is directly related to frailty assessed with an FI and FP in subjects in a Singaporean cohort study (Lu et al., 2016). In addition, higher tumour necrosis factor-β levels are present in older Brazilians with high FP scores (Nascimento et al., 2018). Although there is no conflicting evidence, these results should be interpreted cautiously, given the limited evidence available.

Cytokines with mixed relationships with frailty: Eleven additional cytokines/chemokines were reported at least once to relate to frailty, but there is contradicting evidence (Table 3.1). The endogenous immunomodulator interferon-γ (IFN-γ) is known to affect innate and adaptive immunity, so there has been interest in its relationship with frailty (Burke & Young, 2019). While one study reported that higher IFN-γ levels associated with frail community-dwelling adults using an FP approach (Mohamad et al., 2018), Marzetti and colleagues (2019) found the opposite relationship in men (and no relationship in women). Further, two other studies found no relationship between IFN-γ and the FP (Hsu et al., 2019; Lu et al., 2016). A more promising frailty biomarker is leptin, an adipokine (cytokine secreted by adipose tissue) that plays an important role in regulating metabolism (La Cava, 2017). Three studies have shown that higher levels of leptin were seen in frailer community dwelling participants using either an FI or an FP approach

(Sathyan et al., 2020; Lana et al., 2017; Lu et al., 2016). On the other hand, Hubbard and colleagues (2008) found that frailer individuals had lower leptin levels, although their inclusion criteria for frailty was based on hospital use status (*e.g.*, inpatients in long-term care wards versus community dwelling age-matched controls), making comparisons between studies difficult. On balance, leptin appears to be a promising and emerging candidate as a frailty biomarker.

Another analyte of interest is macrophage inflammatory protein-1 (MIP-1), which has two major forms: MIP-1α and MIP-1β. Marzetti and colleagues (2019) found that lower levels of MIP-1α were proportional to the degree of frailty in women, while lower MIP-1β levels related to frailty in men and women. By contrast, higher MIP-1β levels occur in frailer Chinese adults compared to those who were less frail (Su et al., 2017). Still, two studies report no relationship between frailty and MIP-1α (Hsu et al., 2019; Lu et al., 2016) or MIP-1β (Hsu et al., 2019; Lu et al., 2016). Thus, it difficult to conclude precisely how MIP-1α and MIP-1β relate to frailty.

Many other cytokines also display inconsistent results with respect to their relationship with frailty. Higher circulating eotaxin levels are associated with frailty in women (Marzetti et al., 2019), although this was not seen in a mixed (male/female) population (Lu et al., 2016) or in a study involving all-men (Hsu et al., 2019). This suggests that eotaxin may be a marker of frailty in women only, although additional studies would be required to test this. Similar sex-specific results are seen with respect to IL-17, which was higher in frail males in one study (Marzetti et al., 2019), but not in two others (Hsu et al., 2019; Lu et al., 2016). IL-1α was positively related to frailty in two studies (Sathyan et al., 2020; Nascimento et al., 2018), but not a third (Lu et al., 2016), although the latter study had less power compared to the first two (76 participants versus 901 and 200, respectively). One study involving CCL5 reported elevated circulating levels in frail individuals (Lu et al., 2016), while another did not (Hsu et al., 2019). This was also the case for CXCL10 (*cf.* Lu et al., 2016 and Qu et al., 2009). Likewise, some evidence suggests IL-23 may be lower in frailer people (Compte et al., 2013), but there is evidence to the contrary (Lu et al., 2016). Lastly, granulocyte-colony stimulating factor was reported to be lower in frail individuals in men only (Hsu et al., 2019), but not in a male-female sample (Lu et al., 2016).

Cytokines that have a neutral relationship with frailty: Table 3.1 and Table 3.2 also include results from 36 cytokines and chemokines that were reported not to relate to frailty. These analytes, however, are not well-studied and may yet prove useful biomarkers of frailty in humans if further investigated.

Summary: cytokines/chemokines and links to frailty in humans

Evidence linking cytokines to frailty in people continues to produce paradoxical and contradictory findings, with few cytokines holding any consistent relationship with frailty. The most compelling evidence is available for the pro-inflammatory cytokine IL-6, which exhibits a direct relationship to frailty in humans quite reliably. Additional cytokines that relate to frailty in humans include IL-1α, IL-1β, IL-8, Il-10, leptin, MCP-1, and TNF-α, although IL-1α, IL-8, TNF-α, and leptin are the most promising candidates and should be explored in more detail. Based on the evidence reviewed here, novel, and lesser-studied biomarkers such as I-309 and TNF-β should be investigated further. It is also important to note the high heterogeneity in results involving cytokines other than IL-6. This makes comparisons to preclinical models important, because discovering reliable shared relationships between frailty and circulating cytokines in humans and preclinical models would greatly enhance frailty research. Further, it would accelerate the search for drugs to combat mechanisms of frailty, otherwise known as "geroprotectors" (Trendelenburg et al., 2019). Finally, it seems clear that some cytokines and chemokines may relate to frailty in one sex but not the other. Future studies should focus on sex-specific differences in these potential biomarkers and how they relate to frailty in both sexes. Another important consideration in future studies assessing the relationship between frailty and cytokines is the possible bias within a dataset from patients taking anti-inflammatory drugs or from standardized practices within the assessments. For example, a significant portion of older adults in North America regularly take anti-inflammatory drugs (Davis et al., 2017), which may alter the relationship between circulating markers of inflammation and frailty.

3.4.3 Preclinical models of frailty and their importance

A major advance in the biology of frailty is the discovery that frailty can be quantified in preclinical models with assessment tools based on the FI and FP instruments

developed for use in humans (Howlett et al., 2021; Banga et al., 2019). This allows relationships between biomarkers of inflammation and frailty and the impact of interventions designed to modify the degree of frailty on these markers to be investigated in animals. Table 3.3 summarizes relationships between frailty and cytokines/chemokines in mice and the impact of interventions to modify frailty on these biomarkers of inflammation.

Links between frailty and cytokines/chemokines in naturally ageing mice

Relatively few preclinical studies have investigated relationships between frailty and markers of chronic inflammation. A study by Kane et al. (2019) addressed this question in ageing (17 & 23 months old) male and female C57BL/6 mice. This study used a clinical FI, a laboratory-based FI and combined FI tool. As shown in Table 3.3, they found that combined FI scores increased as levels of the proinflammatory cytokines IL-6, IL-9 and IFN-γ increased in aged female mice (Kane et al., 2019). By contrast, the combined frailty score had a positive relationship with only IL-12(p40) in older male mice (Kane et al., 2019). These observations indicate that links between frailty and serum cytokines/chemokines are sex specific and further studies in older mice with a wider range of frailty scores are warranted.

3.4.4 Interventions to modify frailty: impact on cytokines

Many researchers have investigated the impact of interventions designed to treat frailty on circulating cytokine and chemokine levels in mouse models. These interventions have included exercise, dietary supplements/modifications, and pharmacological treatments, as reviewed in below (Table 3.3).

Links between frailty, cytokines, and exercise

Aerobic exercise is a lifestyle intervention that reduces frailty levels in clinical studies and in preclinical models (Bisset & Howlett, 2019; Woolford et al., 2020). Studies have used both the FP and FI approaches to demonstrate that voluntary aerobic exercise and high intensity interval training can attenuate the degree of frailty in mice (Bisset et al., 2022; Gomez-Cabrera et al., 2017; Graber et al., 2015; Seldeen et al., 2018). Only one

study, however, has explored the effects of exercise on serum cytokine and chemokine levels in a murine model. Bisset et al. (2022) showed that voluntary aerobic exercise (wheel running) reduced frailty in aged (21 to 26 months) male and female mice and that the volume of exercise performed (number of bouts) correlated directly with several pro-inflammatory cytokines/chemokines. Interestingly, they found that circulating IL-2, IL-3, IL-5, IL-6, IL-9 and IFN-γ levels increased as frailty increased in female mice, but this was not seen in male mice. The chemokine KC (mouse equivalent to IL-8 in humans) also increased as exercise volume increased in females (Bisset et al., 2022). Despite increasing a marker of inflammation, exercise still had a beneficial effect on frailty (Bisset et al., 2022). This suggests exercise may affect frailty in female mice at least in part by modifying mechanisms other than inflammation, although additional studies are needed to explore the complex relationships between systemic inflammation, exercise, and frailty in old age.

Relationships between frailty, cytokines, and dietary supplements

Another approach to treat frailty is to use dietary modifications and some of these studies have investigated effects on cytokines and chemokines. Alpha-ketoglutarate (AKG) is an important metabolite of the citric acid cycle that has been shown to extend lifespan in Drosophila (Chin et al., 2014). Recent work by Shahmirzadi and colleagues showed that supplementing the diet with AKG can extend healthspan (decrease frailty) in aged mice of both sexes (Shahmirzadi et al., 2020). Of note, this was accompanied by an increase in the anti-inflammatory cytokine IL-10, but only in female mice. AKG treatment also caused a global reduction in proinflammatory cytokines and chemokines (*e.g.* IL-12(p70), IL-17, and CCL-2), but this was more prominent in females than in males (Shahmirzadi et al., 2020). These findings suggest that AKG attenuates frailty in females at least in part by reducing chronic inflammation through effects on both pro- and anti-inflammatory cytokines. Other frailty mechanisms may be targeted by AKG in males.

Nitrates and nitrites are popular nutritional supplements used to enhance exercise performance and cardiometabolic health (Lundberg et al., 2018). Justice et al. (2015) explored the ability of dietary nitrites to improve measures of physical performance in

ageing and whether these effects may be mediated by actions on cytokines in skeletal muscle. Although they did not measure the FP itself, they did measure factors that make up the phenotype including body weight, grip strength, locomotor activity, and rotarod performance (constant speed or accelerating). They showed that sodium nitrite supplementation improved these performance measures in older male mice; females were not investigated (Justice et al., 2015). In addition, muscle IL-1β, IFNγ, and TNF-α were reduced by nitrites, although IL-6 was not changed significantly by this intervention (Justice et al., 2015). This suggests that reducing inflammation may play a role in health benefits of these supplements, although additional mechanistic studies in both sexes are needed. It also would be important to use a validated frailty assessment tool to determine if this intervention improves frailty.

Roda et al. (2021) treated older male mice with *Hericium erinaceus*, an edible mushroom with medicinal properties, to determine if this improves neurological deficits in ageing. They created a "locomotor frailty index" to evaluate activity, speed, and resting time of the mice. Treatment with *H. erinaceus*, improved locomotor function and reduced cerebellar IL-6 (Roda et al., 2021). It would be interesting to evaluate whether *H. erinaceus* has beneficial effects beyond the brain, and whether it reduces systemic inflammation. Additional studies in both sexes and the use of a validated frailty assessment tool would be of important.

Associations between frailty, cytokines, and pharmacological interventions

Some studies of novel drug therapies to attenuate frailty have explored effects on markers chronic inflammation. The angiotensin converting enzyme (ACE) inhibitor enalapril is commonly used to treat cardiovascular diseases like hypertension and heart failure, but it also has anti-inflammatory effects and beneficial effects on muscle strength (Dandona et al., 2007; Marzetti et al., 2013). Keller et al. (2019) investigated whether chronic treatment with enalapril attenuated frailty and whether these effects were mediated by actions on serum cytokines or chemokines. They found that enalapril reduced FI scores in older mice of both sexes, but that associated effects on inflammation were sex specific. Enalapril reduced the proinflammatory cytokine IL-1α and the

proinflammatory chemokines MCP-1 and MIP-1α in older female mice but increased the anti-inflammatory cytokine IL-10 in males (Keller et al., 2019). Another approach to inhibit the renin angiotensin system is to use an angiotensin II type I receptor blocker (ARB). Lin et al. (2014) evaluated the effect of chronic treatment with the ARB losartan on signs of physical frailty including grip strength, body weight, treadmill endurance and activity levels, although frailty itself was not measured (Lin et al., 2014). They found that losartan improved these physical performance indices in aged female mice, in conjunction with a decrease in serum IL-6 levels. Additional studies to investigate the impact of drugs that inhibit the renin angiotensin system on frailty and markers of inflammation in both sexes would be important, as these drugs could be rapidly re-purposed for use clinically.

There is growing evidence that inflammaging may be facilitated by the SASP, whereby senescent cells secrete proinflammatory cytokines and chemokines (Rea et al., 2018; Furman et al., 2019). Xu et al. (2015) proposed that the JAK/STAT pathway might play a direct role in the SASP in ageing. They treated 24-month-old male mice with the JAK (1/2) inhibitor ruxolitinib and investigated whether this drug could improve functional impairment in ageing including activity, grip strength, hanging time and rotarod coordination, all parameters associated with the FP (Xu et al., 2015). Ruxolitinib improved these performance measures, reduced the SASP and lowered circulating levels of proinflammatory cytokines and chemokines including IL-6, IL-17, CXCL-1, CXCL-10, G-CSF, and GM-CSF (Xu et al., 2015). IL-6 and TNF-α declined in adipose tissue after treatment (Xu et al., 2015). These results suggest that inhibiting JAK improved physical function by reducing inflammation and suggest this pathway may be excellent candidate for drug development to target inflammaging and frailty. Further studies exploring the impact on both sexes, along with assessment of frailty will be of interest.

Another approach to determine if compounds that attenuate the SASP improve health and reduce chronic inflammation is to use senolytic drugs to clear senescent cells (Xu et al., 2018). Xu et al. (2018) used a battery of functional measures such as speed, endurance, grip strength, activity, and body weight to look at the impact of senescent cells and senolytic drug treatment on overall health in mouse models. When senescent cells were transplanted into 5-month-old healthy male mice, physical function declined while

IL-6 and TNF-α increased (Xu et al., 2018). When older (24 months) male and female mice were treated with a senolytic cocktail (dasatinib & quercetin), physical performance measures improved markedly and markers of inflammation (*e.g.* IL-6 and CXCL-1) declined in adipose tissue (Xu et al., 2018). This shows that induction of senescence increases inflammation and promotes signs of physical frailty, while clearing senescent cells reduces inflammation and measures associated with physical frailty. Additional studies with a validated frailty assessment tool and other senolytic therapies are needed.

Summary: cytokines/chemokines, links to frailty and responses to interventions in mice

The studies presented in Table 3.3 demonstrate the relationship between various cytokines/chemokines and frailty, or measures of physical performance. Circulating IL-6 levels were most associated with increased frailty. Further, localized (*i.e.,* within tissue samples) IL-6 levels were associated with frailer mice in three out of four studies. There is also evidence that circulating or localized levels of CXCL-1, G-CSF, MCP-1, and TNF-α are associated with frailty and physical performance, although not all studies agree. Most notably, circulating TNF-α levels never related to frailty, but localized levels (*e.g.*, in muscle or fat) always did. It is also clear that lifestyle interventions such as exercise and diet, as well as pharmaceutical interventions, can reduce frailty (or improve physical performance) through effects on chronic inflammation. Many of these studies have not yet measured frailty directly, so it will be important in future studies to quantify the degree of frailty with a validated frailty assessment instrument. It also is noteworthy that many studies have used only one sex of animal, usually males. This represents an important knowledge gap as studies that have used both sexes most often find that effects on inflammation are sex specific. Studies that explore the impact of dietary restriction on cytokine production and frailty status also are lacking. One limitation to exploring frailty's relationship with cytokines in mice is the small volume of extractable blood that can limit the number of analytes assessed. Still, the growing availability of multiplex assays can help overcome this, as only a small volume (*e.g.*, 15 μL) of serum/plasma is needed to assay dozens of inflammatory mediators. Additional mechanistic preclinical studies in this area would be welcome.

3.4.5 Studies relating inflammation to frailty in preclinical models of frailty: genetic manipulation to promote inflammation

Chronic inflammation is strongly implicated in the pathogenesis of accelerated ageing and frailty (Goh et al., 2021; Bisset & Howlett, 2019). Because of this, researchers have created mouse models of chronic inflammation by genetic manipulation of pathways involved in inflammation. These mice have been used as models of frailty and have shed light on mechanisms involved in cardiac, vascular, and skeletal muscle dysfunction in the setting of chronic inflammation.

3.4.5.1 The IL-10 knockout mouse model of inflammation and frailty

Development of the model

The first and most widely used frailty model focused on inflammation is the IL-10 knockout (IL-$10^{tm/tm}$) mouse (Table 3.4). This model was developed based on idea that the principal actions of IL-10 are anti-inflammatory (Rennick and Fort, 2000; Rasquinha et al., 2021). Indeed, the IL-$10^{tm/tm}$ mouse develops chronic enterocolitis with early mortality (Kuhn et al., 1993), unless they are maintained under strict barrier conditions (Bristol et al., 1997). Walston and colleagues (2008) investigated these mice as a model of frailty and showed that they have higher serum Il-6, worse grip strength and lower activity levels than wildtype (WT) controls. Based on this they proposed the IL-$10^{tm/tm}$ mouse as a model of human frailty (Walston et al., 2008).

Signs of inflammation and impact on frailty or function in IL-$10^{tm/tm}$ mice

Several investigators have examined the nature of the inflammation seen in IL-$10^{tm/tm}$ mice and its relationship with age (Table 3.4). Walston and colleagues showed that circulating levels of the pro-inflammatory cytokine IL-6 increased between 2 and 12 months of age in female WT mice but this increase was much greater in IL-$10^{tm/tm}$ mice (Walston et al., 2008). They later extended this work to show that IL-6 continues to increase up to 20 months of age in female IL-$10^{tm/tm}$ mice (Westbrook et al., 2017). Others report that serum levels of the pro-inflammatory cytokine IFN-γ increase from 3 to 6 months of age in IL-$10^{tm/tm}$ mice of both sexes (Pini et al., 2009). Serum levels of many pro-inflammatory cytokines (*e.g.* IL-6, IL-1β, TNF-α, IFN-γ but not IL-12p70), along

with the chemokine KC, are markedly higher in knockout mice compared to WT at 21 months of age regardless of sex (Ko et al., 2012). These inflammatory markers may arise, at least in part, from a larger number of senescent cells available to secrete chemokines and cytokines in visceral adipose tissue from IL-10$^{tm/tm}$ mice when compared to WT (Xu et al., 2018). Although frailty was not assessed in any of these studies, functional measures including grip strength, overall activity levels and oxygen consumption declined with age in knockout mice, at least in females (Walston et al., 2008; Westbrook et al., 2017). These observations suggest that signs of chronic inflammation are present in IL-10$^{tm/tm}$ mice of both sexes.

Interventions to modify inflammation, improve frailty and function in IL-10$^{tm/tm}$ mice

Several studies have used different intervention strategies to modify inflammation in variably aged IL-10$^{tm/tm}$ mice (Table 3.4). Pérez-Martínez et al. (2020) reasoned that because the C-C motif chemokine receptor type 5 (CCR5) is overexpressed in frail individuals (De Fanis et al., 2008), drugs that block this receptor or reduce its expression could be useful in treating frailty. They treated 1.5-month-old male IL-10$^{tm/tm}$ mice with a CCR5 antagonist (maraviroc), the mTOR inhibitor rapamycin (which also reduces CCR5 expression), or both for 5.5 months (Pérez-Martínez et al., 2020). Treatment did not reduce markers of inflammation (*e.g.,* IL-1β, IL-6, or TNF-α) and had no effect on either frailty or physical function (Pérez-Martínez et al., 2020). It is possible that beneficial effects on function were not observed because they used young adult (<7 months) knockout mice with low levels of frailty. It would be interesting to use this intervention with older animals of both sexes.

A novel approach to inhibit inflammation and improve function in IL-10$^{tm/tm}$ mice is to create a double knockout mouse with deletion of both IL-10 and the pro-inflammatory cytokine IL-6 to produce IL-10$^{tm/tm}$/IL-6$^{tm/tm}$ mice (Ma et al., 2021). Deletion of IL-6 in the IL-10$^{tm/tm}$ mice improved physical performance as it reduced the number of falls and increased running distance in both sexes (Ma et al., 2021). Paradoxically however, mortality was markedly increased in the IL-10$^{tm/tm}$/IL-6$^{tm/tm}$ mice, highlighting the complex nature of interventions that modify inflammation (Ma et al.,

2021). This latter observation suggests that, although deletion of key pro- and anti-inflammatory cytokines has some beneficial effects, this adversely affects health and may increase frailty, although this remains to be demonstrated.

Links between inflammation and stress responses have only recently been explored. Three to 21-month-old WT and IL-10$^{tm/tm}$ mice of both sexes were exposed to cold stress (4ºC) for 5 hours to determine if chronic inflammation exaggerates stress responses (*e.g.* circulating corticosterone and IL-6 levels) (Ge et al., 2020). They found that cold stress increased corticosterone in WT male mice and in all knockout mice regardless of age or sex, with few effects on IL-6 (Ge et al., 2020). The authors propose this as an interesting model to explore links between frailty and resiliency, although effects on function are unclear because they did not measure frailty or any other functional outcome in this work.

Impact of IL-10 deletion on skeletal muscle

Skeletal muscle weakness and sarcopenia are thought to play a key role in physical frailty in humans (Picca et al., 2020). To understand skeletal muscle weakness in the presence of chronic inflammation, investigators have explored mechanisms underlying muscle weakness in IL-10$^{tm/tm}$ mice (Table 3.4). Early work showed that many genes related to mitochondrial function and apoptosis are differentially expressed in skeletal muscle from IL-10$^{tm/tm}$ mice when compared to WT controls and these changes occur as early as 2 months of age (Walston et al., 2008). At least some of these changes are likely detrimental to muscle function as they correlate with reduced strength and activity levels in affected mice (Walston et al., 2008). Reduced ATP synthesis and a decrease in the free energy released from ATP hydrolysis in skeletal muscle also occur in ageing male IL-10$^{tm/tm}$ mice (Akki et al., 2014). Additionally, IL-10 knockout exacerbates age-related mitochondrial damage and alters the removal of damaged mitochondria through autophagy (mitophagy) in muscle from ageing female mice (Ko et al., 2016). Abadir et al. (2017) showed that the chemokine-like proinflammatory cytokine MIF (macrophage migration inhibitory factor) is co-expressed with NIP3-like protein X (Nix) in skeletal muscle from older mice, especially older IL-10$^{tm/tm}$ mice. As MIF has anti-apoptotic properties and Nix is a pro-apoptotic mitochondrial protein, these findings

suggest muscle weakness in IL-10$^{tm/tm}$ mice is associated with inhibition of apoptosis and increased mitochondrial death signaling (Abadir et al., 2017). There is also evidence that treatment with either an antioxidant/anti-inflammatory compound or a CCR5 antagonist, mTOR inhibitor or both reduces inflammation in muscle in IL-10$^{tm/tm}$ mice (Wang et al., 2014; Pérez-Martínez et al., 2020). This suggests that targeting inflammation may be a useful intervention. Together, these factors offer some mechanistic insights into skeletal muscle weakness in chronic inflammation and potentially help explain physical weakness in murine and human frailty.

Impact of IL-10 deletion in other tissues

Inflammation is known to play a crucial role in the development of age-associated cardiovascular diseases including atherosclerosis, myocardial infarction, and heart failure (Ruparelia et al., 2017; Murphy et al., 2020). Still, few laboratories have examined the heart and vasculature in the IL-10$^{tm/tm}$ mouse model (Table 3.4). Echocardiography has shown cardiac hypertrophy in IL-10$^{tm/tm}$ mice, with an increase in both heart mass and dilation (Sikka et al., 2013). These structural changes impair both systolic and diastolic function, especially in older mice (Sikka et al., 2013). In addition, myocardial oxidative phosphorylation is impaired in IL-10 knockout mice (Ma et al., 2021). There is also evidence that blood pressure is elevated in IL-10$^{tm/tm}$ mice compared to WT controls. Together, these changes could contribute to the development of heart failure and vascular disease in the setting of chronic inflammation and potentially frailty.

3.4.5.2 The Nfκb1$^{-/-}$ knockout model of inflammation and frailty

The transcription factor NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) is a critical mediator of inflammatory responses that is implicated in chronic inflammation associated with ageing (Liu et al., 2017; García-García et al., 2021). It induces expression of many different genes that promote inflammation, including those encoding chemokines and cytokines (Liu et al., 2017). Based on this, the *nfκb1* subunit of NF-κB was knocked out, which results in the absence of the NF-κB1 p50 subunit that normally represses NF-κB activity (Jurk et al., 2014). These *nfκb1*$^{-/-}$ mice exhibit enhanced activation of NF-κB, increased plasma IL-6 and accelerated ageing with increased mortality, loss of body mass, sarcopenia, impaired neuromuscular function, and

cardiac hypertrophy (Jurk et al., 2014).

This model has been used to determine whether the mTOR inhibitor, rapamycin, improves lifespan and healthspan in the presence of genetically enhanced NF-κB activity (Correia-Melo et al., 2018). This investigation showed that rapamycin enhanced long-term memory, improved neuromuscular coordination, reduced tissue damage and attenuated frailty in ageing male mice (Correia-Melo et al., 2018). Interestingly however, rapamycin had no impact on lifespan and did not attenuate the high serum levels of pro-inflammatory cytokines (*e.g.,* IL-6 and TNF-α) seen in these mice. These findings indicate that the beneficial effects of rapamycin on healthspan and frailty are not attributable to effects on chronic inflammation, at least in male animals (Correia-Melo et al., 2018). This suggests that inflammation and overall health/frailty may not always be linked, although additional work in naturally ageing animals will be required to test this concept and studies in females will be crucial.

3.4.5.3 Other genetically modified mouse models of inflammation and frailty

Three additional genetically modified mouse models of frailty have been identified beyond the IL-$10^{tm/tm}$ and *nfκb1*$^{-/-}$ models. Future use of these models may help diversify preclinical frailty research by targeting different pathways. More specifically, the use of additional and diverse models could help isolate different parts of the inflammation-frailty relationship and help discover future therapies targeting inflammaging, as discussed below.

The copper-zinc superoxide dismutase knockout mouse model of frailty

The copper-zinc superoxide dismutase knockout ($Sod^{-/-}$) mice have also been proposed as a model of frailty (Deepa et al., 2017). They display accelerated age-related skeletal muscle loss, low physical activity, and poor muscle strength (Deepa et al., 2017). This is accompanied by hearing loss (Keithley et al., 2005), cataracts (Olofsson et al., 2007), spontaneous skin damage/impaired wound healing (Iuchi et al., 2010), and a reduced lifespan (Zhang et al., 2013). Although mice share phenotypic traits associated with frailty in humans, the degree of frailty has not been measured (Deepa et al., 2017).

$Sod^{-/-}$ mice have high circulating levels of the pro-inflammatory cytokines/chemokines IL-6, eotaxin, KC, and G-CSF (Zhang et al., 2017), which suggests they may model inflammaging and frailty. There were non-significant changes in blood-borne GM-CSF, IL-1α, IL-1β, MCP-1, MIP-1, and TNF-α (Zhang et al., 2017). It must be noted however that all cytokines but IL-1α and MIP-1 showed a trend to increase in the $Sod^{-/-}$ mice (n=6) relative to age-matched WT controls (n=5). These conclusions were thus drawn from small samples and may belie the true potential of these mice to model the relationship between frailty and inflammation. Further studies using these mice may benefit from including more mice and using a mouse FI or an FP approach to characterize the association between frailty and cytokines.

An inducible IL-6 mouse model of frailty

The strong association between circulating IL-6 and frailty in humans suggests that IL-6 may be a causal factor. An inducible IL-6 expression mouse model ($IL\text{-}6^{TET\text{-}ON/+}$ mice) has been used to assess whether elevated IL-6 levels can induce frailty (Jergović et al., 2021). When these mice are treated with doxycycline to increase circulating IL-6 levels, results show that 10-month-old $IL\text{-}6^{TET\text{-}ON/+}$ mice have frailty scores like those seen in 23-month-old WT mice (Jergović et al., 2021). Furthermore, these mice have reduced grip strength after only 3-weeks of IL-6 induction (Jergović et al., 2021). The authors also measured the concentration of nine different cytokines in spleen homogenates to see if IL-6 induction may have affected the release of other cytokines. Doxycycline-treated mice had higher IL-6 and IL-10 levels and lower IL-1β levels compared to young WT controls, but no other changes were reported in IL-17A, IL-23, IL-27, MCP-1, IFN-γ, TNF-α, or GM-CSF. Overall, this study convincingly demonstrated that systemic IL-6 induction can markedly accelerate frailty progression in mice.

Intestinal alkaline phosphatase knockout mice as a model of frailty

Declines in gastrointestinal health often relate to increased frailty (An et al., 2018). Intestinal alkaline phosphatase (IAP) is an anti-inflammatory enzyme that helps the gut microbiome manage pro-inflammatory bacterial stimuli (Chen et al., 2010). IAP

levels decline with age, which may contribute to chronic inflammation and facilitate frailty (Kühn et al., 2020). To explore this, Kühn and colleagues (2020) used an IAP knockout mouse model developed previously (*AKP3*$^{-/-}$; Narisawa et al., 2003). They found that IAP knockout mice had higher frailty scores than age-matched WT controls at 12- and 24-months of age (Kühn et al., 2020). Ileal messenger RNA (mRNA) levels of IL-6 and TNF-α were higher in the IAP knockout mice, along with elevated serum TNF-α levels. A key mechanistic finding from this study was that oral IAP supplementation helped to reverse the impact of IAP knockout on frailty. Further, serum levels of IL-6 and IL-1β were reduced in IAP-treated knockout mice compared to vehicle-treated controls. The knockout mice also had a shorter average lifespan, which was reversed and indeed prolonged with IAP supplementation (Kühn et al., 2020). The IAP knockout additionally induced microbiome dysbiosis, which was reversed through IAP supplementation. This suggests that changes in systemic inflammatory markers related to gut metabolism can associate with frailty progression in a preclinical model and provides a unique opportunity to study inflammaging and frailty related to gastrointestinal microbiome dysfunction.

3.4.5.4 Limitations of genetically manipulated models of inflammation and frailty

The studies reviewed here demonstrate that mouse models of chronic inflammation can be created by genetic manipulation of key pathways involved in inflammation and that these models exhibit several of the changes associated with frailty in older people. For instance, both IL-10$^{tm/tm}$ and *nfκb1*$^{-/-}$ mice exhibit impaired neuromuscular function (Walston et al., 2008; Jurk et al., 2014), muscle atrophy/sarcopenia (Jurk et al., 2014; Wang et al., 2014), increased mortality (Ko et al., 2012; Jurk et al., 2014), and signs of cardiac hypertrophy (Sikka et al., 2013; Jurk et al., 2014). On the other hand, there are limitations to the use of these models to explore the biology of frailty and ageing. For example, as discussed earlier, the IL-10$^{tm/tm}$ mice are a model of enterocolitis, so they must be housed under stringent pathogen-free barrier conditions to prevent the development of enterocolitis, anemia, and early mortality (Bristol et al., 1997; Walston et al., 2008). There is also limited information about male-female differences in these models. Most studies that have used IL-10$^{tm/tm}$ mice did not

explore sex differences. As illustrated in Table 3.4, investigators sometimes used only one sex, pooled data from males and females, or did not clearly specify the sex used. What is more, work with the *nfκb1*$^{-/-}$ mice and IAP knockout mice used only males and studies in the Sod$^{-/-}$ mice did not specify the sex used. This lack of sex specific data represents an important knowledge gap that should be addressed in future studies. In addition, because these models are genetically manipulated, it is not clear whether they model the development of frailty in naturally ageing humans. It is likely genetically modified mouse models of frailty are cost-effective and can save time. However, it is unclear how closely these models reflect ageing and frailty, so naturally ageing mice may be necessary to capture frailty in a preclinical model.

An additional consideration is that relatively few studies have used a frailty assessment instrument to measure frailty in genetically modified mice. Only one study quantified frailty in IL-10$^{tm/tm}$ mice and found similar FI scores in knockout and WT mice (Pérez-Martínez et al., 2020). However, this study used young adult mice (*e.g.* <5.5 months) so it is unclear if high levels of frailty would be expected. FI scores did increase with age between 15 and 18 months in *nfκb1*$^{-/-}$ mice and this was attenuated by rapamycin at 18 months. Still, whether these scores were high relative to WT mice of the same age has not been investigated (Correia-Melo et al., 2018). It is equally important for interventional studies to ask explicitly if frailty relates to cytokine levels. Multiple studies reported only the intervention's effect on cytokines or on surrogate measures of frailty (Shahmirzadi et al., 2020; Justice et al., 2015; Roda et al., 2021; Keller et al., 2019; Lin et al., 2014; Xu et al., 2015; and Xu et al., 2018). Without plotting frailty as a function of cytokines, important dose-dependent effects of interventions on frailty may be lost. Lastly, because sample can play a significant role in how cytokines relate to frailty (*e.g.*, TNF-α related to frailty measures in fat/muscle but not serum), it is important to consider both tissue samples and circulating levels when assessing their relationship to frailty. Additional studies in this area could be illuminating.

3.4.6 Conclusions

Although clinical and preclinical studies provide evidence for relationships between circulating cytokines and frailty, these relationships are complex and can be

inconsistent. Despite this, there are promising overlaps between humans and mice, and future research may elucidate even better shared mechanisms of frailty. We conclude this review by contrasting relationships between circulating cytokines in humans and mice and by suggesting future research directions.

3.4.6.1 Consistencies between humans and mice when relating circulating cytokines to frailty

The discovery of overlapping relationships between circulating cytokines and frailty in mice and humans could help address meaningful mechanistic questions regarding inflammaging. One valuable outcome from discovering such cytokines would be the creation of an assay panel to assess frailty or frailty risk. As reviewed here, very few analytes share similar relationships to frailty in mice and humans, although three do emerge (Figure 3.1). In humans, higher circulating IL-6 levels relate to a frailer state, which was corroborated by preclinical frailty studies in Section 2 and mechanistic evidence in Section 3. Thus, we suggest that IL-6 is currently the leading translational frailty biomarker across humans and mice to study inflammaging and frailty. The chemokines MCP-1 and CXCL-10 also show promise, but evidence linking these to frailty in humans or mice is limited and equivocal. Interestingly however, the strength of association between other cytokines and frailty sometimes differs between humans and mice. For example, while IL-8 and leptin demonstrate fairly consistent relationships with frailty in humans, in mice a homologue of IL-8, KC, has not shown the same directional relationship and leptin has not been investigated. Likewise, circulating TNF-α tended to relate to frailty in humans but not in mice. Additional work characterizing these relationships would be helpful in terms of translating preclinical research to clinical/population-level frailty interventions.

A key difference between preclinical and clinical/population-level research is the ability to tightly control experimental conditions before isolating samples to process and assay. This difference may help to explain differing results between humans and mice linking cytokines to frailty. For example, TNF-α from tissue samples (*e.g.*, skeletal muscle or adipose tissue) related to frailty in mice (Table 3.3), suggesting that TNF-α is

involved in the mechanism of frailty progression despite the inconsistent findings relating circulating TNF-α to frailty in humans. It is possible that cytokines contributing to frailty progression may affect tissues at the local level, yet their systemic changes are too small to be sensitive in a frailty biomarker assay. If proven, this nuanced relationship between cytokine location and frailty must therefore be considered when discussing the mechanistic impact cytokines have on frailty.

Lastly, it is possible that I-309 and TNFβ are biomarkers of frailty in humans and mice, but they have only rarely been assayed in humans and never in mice (Figure 3.1). By contrast, higher circulating MCP-1 often related to frailty in mice and in humans. This suggests that MCP-1 may be of interest as a biomarker of frailty, although more studies are needed to validate this.

3.4.6.2 Future directions

Future work should validate circulating biomarkers that share the same relationship with frailty between humans and mice. Different inflammatory responses to similar stimuli have been reported between mice and humans in response to health perturbations such as trauma or sepsis (Seok et al., 2013). This differential inflammatory response could result in different effects on frailty in mice and humans. In the same vein, establishing the potency of cytokines between humans and mice is necessary to accurately interpret preclinical findings. It is theoretically possible for a cytokine to precipitate frailty in humans and not in mice. Thus, detailing these relationships is necessary before comparisons between mice and humans can be interpreted accurately. Currently, IL-6 is the most promising translational frailty biomarker between humans and mice. CXCL-10 and MCP-1 were the only other biomarkers that showed a similar relationship with frailty in humans and mice, but supporting evidence is limited.

The creation of a blood-borne biomarker assay panel for frailty including these analytes and others may prove fruitful in the search for a sensitive frailty assay panel. However careful attention must be given to the specificity of cytokines for frailty due to substantial between-subject variability in circulating cytokines. It will also be important to consider the highly interdependent relationships inflammatory cytokines have with one

another when creating these assay panels (Chen et al., 2017). It is possible that this complexity can be harnessed to yield a more sensitive assay by considering multiple connected analytes. For this reason, it may be best to combine cytokines with other laboratory measures related to inflammation (*e.g.*, C-reactive protein, Cu/Zn ratios *etc.*) to create a frailty assay panel. In combination with the above suggestions to confirm relative potency, such panels considering inter-connectivity may be able to weight variables accordingly to accurately predict frailty from analytes in inflammatory networks.

There is also a need to consider sex when discovering translational frailty biomarkers and creating a frailty assay panel, as results can vary between males and females (*e.g.,* TNF-α in Table 3.1). Finally, determining how localized inflammation versus systemic/blood-borne inflammation affects frailty progression would prove useful in understanding mechanisms of frailty and which circulating analytes may fit well within a frailty assay panel.

3.5 Other bodily systems involved in frailty

Although chronic inflammation and declines in physical function and/or skeletal muscle quality contribute to frailty, detrimental changes in many other bodily systems do too. Experiments in Chapters 5 investigate two other systems in relation to frailty: skeletal health and cardiac function. Accordingly, such systems are briefly reviewed here in the context of frailty. Both systems are also responsive to testosterone, which are reviewed in their appropriate chapters. Cardiac function is explored in much more detail in Chapter 5 than skeletal health and is therefore reviewed in more detail than skeletal health.

3.5.1 Bone health and frailty

The risk of breaking your bones increases into older age in correlation with declines in bone density (McClung, 2005). This is therefore a very literal form of frailty. The density of your bones is a product of how tight the mineralized matrix of bone fits together and its age-related decline has led to screening recommendations for people over the age of 65 years to check for bone weakness (Lupsa & Insogna, 2015). The association between poorer bone health, aging, and fracture risk is moderated by frailty. A report from the European Male Aging Study provides interesting evidence linking frailty levels to aspects of bone health, including bone mineral density (BMD). Men with a mean age

of 60 years (n=3231) had their frailty assessed using the Fried FP and FI (39 variables) along with their BMD and a quantitative ultrasound measurement of their heel (Cook et al., 2017). Men with higher FP and FI scores had significantly worse scores from the ultrasound measurements in a frailty severity-dependent manner. Further, those with higher FP and FI scores had significantly lower measures of BMD (Cook et al., 2017). Similar findings occurred in a mixed-gender population. A Canadian prospective cohort study involving 3149 older adults with a mean age of 65 years and comprising 70% women investigated the relationship between frailty (as a 30-item FI) and fracture risk (Li et al., 2019). Using a Cox proportional hazard model, for every three deficits present on the FI, people had a 20% higher risk of any kind of fracture (Li et al., 2019).

These relationships between frailty and bone fractures can predict increased risks of fractures later in life, too. One study reported the relationship between a 13-item FI and subsequent fall risk in a prospective study of 1044 community dwelling women approximately 75 years old. This study reported the risks of bone fracture at two-, five-, and 10-years post-assessment were significantly higher in frailer women, even after adjusting for BMD (Bartosch et al., 2021). These findings were akin to those by Kennedy et al. (2014), who reported that higher frailty levels assessed using their 30-item FI were related to bone fractures in their sample of 9423 participants (mean age=62 years; 69% women). These findings cumulatively suggest that frailty is related to poorer bone health, which subsequently increases the risk of bone fractures into older age and that frailty is even predictive of fractures on its own.

3.5.2 Cardiac health and frailty

The heart is the primary organ responsible for supplying blood to all parts of the body. Issues regarding the heart's ability to meet this demand pose a health threat and are a major source of frailty. Heart failure is a clinical syndrome associated with fatigue caused by structural or functional abnormalities in ventricular filling or ejection of blood (Bozkurt et al., 2021).[4] Heart failure is a multiply determined condition that tends to be the endpoint from a variety of cardiac issues arising from genetic or acquired factors

[4] Defined from a consensus conference in August, 2020, involving the Heart Failure Society of America (HFSA), the Heart Failure Association of the European Society of Cardiology (HFA/ESC), and the Japanese Heart Failure Society (JHFS).

(Ziaeian & Fonarow, 2016). Frailty and heart failure regularly co-exist, likely because both conditions are associated with higher total comorbidities and functional decline (Pandey et al., 2019). Indeed, a systematic review with a meta-analysis including 26 studies (total n=6896) reported that 45% of people living with heart failure are frail (Denfeld et al., 2017). This was true both for phenotypical frailty assessments and multidimensional ones. In fact, multidimensional frailty prevalence (47%) was higher in heart failure patients than physical frailty prevalence (43%; Denfeld et al., 2017). Notably, all 26 studies reported a statistically significant prevalence (*i.e.*, non-zero) of frailty in people with heart failure. Within-study confidence intervals ranged from 10% to 95% in multidimensional frailty assessments (*e.g.*, an FI) and 16-52% for physical frailty assessments (Denfeld et al., 2017). Other cardiovascular issues relate to frailty too. In men aged 71-92 years (mean=78 years; n=865), higher carotid intima media thickness, the thickness of the inner part of the carotid artery, was associated with a higher risk of becoming frail after 3-years (McKechnie et al., 2021). This was found in a population of 'pre-cardiovascular disease' patients that were not frail to begin with, which provides evidence that even sub-clinical cardiovascular disease can relate to frailty progression (McKechnie et al., 2021). Higher frailty status has also been related to issues in the cardiac conduction system. Patients with atrial fibrillation have been shown to have a higher prevalence of frailty, assessed via FI, compared to age- and gender-matched controls (Polidoro et al., 2013). Frailty status is therefore highly related to cardiac dysfunction, which is fundamentally demonstrated by the robust relationship between heart failure and frailty (Pandey et al., 2019).

For this section, the focus is on left-sided heart failure due to the relevance to experiments evaluating left ventricular function in Chapter 5. A common measurement used when characterizing heart failure is "ejection fraction" (EF). It is calculated by dividing the volume of blood ejected from the left ventricle by the total volume in the heart prior to the beat. Although there are subtle differences across medical governing bodies worldwide, there is a general consensus that the two major types of heart failure are heart failure with preserved EF (HFpEF) and heart failure with reduced EF (HFrEF). European and Japanese guidelines also include a mid-ranged EF etiology, known as heart failure with mid-range EF (Bozkurt et al., 2021). A study of 202 heart failure patients (96

HFpEF and 206 HFrEF) reported no between-group differences in frailty scores using a FP approach, suggesting that frailty prevalence is similar between aetiologies (Warraich et al., 2018). In a study using HFpEF patients with a mean age of 72 years, higher FI quartiles were related to significantly higher rates of HF-related hospitalization and mortality, along with all-cause mortality (Sanders et al., 2018). A different study involving 4741 HFrEF patients reported that higher FI scores related to worsening HF events, hospitalization, cardiovascular death, or all-cause mortality (Butt et al., 2022). Thus, frailty significantly relates to heart failure regardless of EF. However, a report from the Cardiovascular Health Study investigated the relationship between left ventricular EF and frailty in participants without heart failure (Tan et al., 2021). Frailty was assessed using a calibrated FP approach. A total of 3206 participants enrolled, had a mean age of 72 years, and 63% were female. Results were stratified by frailty status. There was a significant relationship between higher frailty scores and lower EFs, even after adjusting for 18 covariates (Tan et al., 2021). Thus, it appears that frailty is significantly related to adverse outcomes in heart failure in both HFpEF and HFrEF aetiologies, but may also relate to lower EFs in people without HF.

Although EF is a commonly studied variable relating to heart function, there are other important metrics to consider that also relate to frailty. One such metric that has great prognostic value is left ventricular strain analysis. Such analysis tracks speckles during *in vivo* imaging to calculate the biomechanical strain (*i.e.*, the change in length relative to baseline) of the left ventricle. The study by Tan et al. (2021) also investigated the relationship between myocardial strain and frailty. They reported that higher frailty scores related to worse strain in unadjusted models or after adjusting for covariates. It was also reported that more favorable strain rates (*i.e.*, the change in strain over time of the heart muscle during contraction) related to lower frailty scores, although this became non-significant after adjusting for 18 covariates (Tan et al., 2021). Interestingly, worse myocardial strain and worse frailty scores independently predicted reduced survival times, suggesting they are only partially overlapping risk factors.

Age-related changes in temporal elements of the cardiac cycle are also important. Although these variables have not been thoroughly studied in relation to frailty, their

relevance to heart failure and its relationship with frailty make their consideration worthwhile. Hearts of older people tend to take longer to begin filling after left ventricular ejection. The transition time between ejection and filling is termed the 'isovolumic relaxation time' (IVRT) and increases significantly with age (Vancheri et al., 2016; Carrick-Ranson et al., 2012). This change happens in parallel with the decline in early filling of the ventricle with age (Vancheri & Heinin, 2018). This is important, because this filling accounts for most of the ventricle's end-diastolic blood volume (Fukuta & Little, 2008). IVRT levels negatively correlate with early filling velocity (Peverill, 2019; Brecker et al., 1992) and are an important determinant of the pressure decline in the left ventricle after contraction, otherwise called τ (Bai & Wang, 2010). Patients with diastolic heart failure have a prolonged τ relative to healthy controls and longer IVRTs have been associated with hospitalization in heart failure (Correale et al., 2014; Zile et al., 2004). Further, a longitudinal study of 1892 patients (mean age=58; 58% female) reported that longer IVRT times were associated with increased risk of developing heart failure, although the relationship disappeared after 10 covariates were adjusted for (Alhakak et al., 2020). Thus, changes in IVRT levels have important implications in heart function and heart failure, which relates to frailty.

A similar metric is isovolumic contraction time (IVCT), which denotes the time the ventricle spends transitioning between filling and contraction (Bair & Wang, 2010). The duration of IVCT increases significantly with age too, although this result is not always found (*cf.* Vancheri & Henein, 2018 and Vencheri et al., 2016). Importantly, longer IVCTs significantly relate to heart failure, as was reported in the trial by Alhakak et al. (2020). This finding held in both of their adjusted models, which added 10 and 14 co-variates, respectively (Alhakak et al., 2020). When IVRT and IVCT are summed and then divided by total ejection time, this is considered to be a myocardial performance index (MPI). The MPI values (*i.e.*, a longer relative time transitioning between contracting and relaxing) of heart failure patients are significantly longer than those of healthy age-matched controls (Duzenli et al., 2009). This suggests that even relative to the time spent contracting, the transition times between contraction and relaxation of the left ventricle increase as heart function decreases. Overall, both the IVRT and IVCT are factors worthy of consideration alongside EF and strain analysis when evaluating heart

function, as both relate to heart failure, and are considered in Chapter 5.

Mechanisms contributing to frailty overlap with those of heart failure, with specific emphasis on both skeletal muscle dysfunction and chronic inflammation (Pandey et al., 2019). For example, heart failure is commonly associated with accelerated lean muscle mass loss. One study involving 2815 participants (52% women, mean age=72 years), 111 of whom had heart failure, tracked body composition parameters over 6-years using DEXA (Forman et al., 2017). Those with heart failure lost significantly more total lean mass and appendicular lean mass than those without heart failure. This was true in both men and women, although the effect size was larger in men (Forman et al., 2017). The declines in skeletal mass during heart failure translate to poorer fitness levels in patients, as occurs in frail individuals. Sixty patients with HFpEF with a mean age of 70 years (68% women) alongside 40 age- and gender-matched controls had their body composition measured via DEXA and aerobic exercise capacity measured using a graded cycler ergometer test (Haykowsky et al., 2013). Patients with heart failure had lower relative lean mass and lower aerobic fitness and peak power levels (Haykowsky et al., 2013). Further, both lower aerobic fitness and lean mass measures correlated with poorer physical performance in a performance test battery. These findings suggest that heart failure patients, similar to frail people, have reduced lean mass and impaired physical performance.

The mechanisms underlying the acceleration of cardiac 'aging' are demonstrably like frailty and one important mechanism is the accumulation of senescent cells into older age and with cardiac disease (Lewis-McDougall et al., 2019). So-called inflammaging is another important contributor to the development of cardiovascular diseases that overlaps with the mechanisms of frailty (Ferrucci & Fabbri, 2018). Senescent cells within the myocardium can contribute to inflammaging, as they exhibit a SASP that produces elevated pro-inflammatory cytokines and impairs the heart's regenerative ability (Lewis-McDougall et al., 2019). Pro-inflammatory markers originating from outside the heart are arguably even more important, with adipose tissue and multi-organ dysfunction playing key roles in the progression of heart failure (Mesquita et al., 2021). Elevated circulating levels of pro-inflammatory cytokines are a common occurrence in heart failure (Ferrucci

& Fabbri, 2018). Further, elevated levels of pro-inflammatory cytokines are not only related to the development of cardiac dysfunction, but they also relate to poorer prognoses in both HFpEF and HFrEF. Higher serum levels of pro-inflammatory cytokines IL-6 and TNF-α were related to a higher morbidity and mortality risk score (the MAGGIC[5] score) in a study of 379 HFpEF patients (46% women) with a mean age of 70 (Chirinos et al., 2020). Notably, IL-6 and TNF-α significantly predicted heart-failure related hospital admittance even after adjusting for MAGGIC scores, suggesting an added influence of these markers on disease severity beyond more traditional risk factors (Chirinos et al., 2020). In patients with HFrEF, similar patterns emerge. When stratified by IL-6 levels, a study involving 2329 patients with HFrEF (mean age=69 years; 26% female) reported that higher IL-6 levels were associated with more heart failure hospitalization, cardiovascular mortality, and non-cardiovascular mortality (Markousis-Mavrogensis et al., 2019). A different study comparing HFrEF patients (n=100, mean age=65 years) compared TNF-α levels, and levels of two of its receptors, to diastolic function and heart failure grade and reported that higher serum TNF receptor levels related to worse heart failure grades (Putko et al., 2014). This was also true for HFpEF patients. It is notable that not all studies report that these cytokines are elevated in heart failure patients, as the study from Putko et al. (2014) reported no significant difference in serum IL-6 or TNF-α levels in HFpEF or HFrEF patients versus 50 younger controls.

Chronic inflammation has also been associated with frailty in patients with atherosclerotic cardiovascular disease. In a cross-sectional study by McKechnie et al. (2022) involving men with (n=303) and without (1096) cardiovascular disease, it was reported that higher IL-6 levels were associated with frailty. Because IL-6 is related to cardiovascular disease risk and progression (Libby, 2021), these results help to confirm the link between inflammation, cardiovascular disease, and frailty. Chronic low-grade circulating inflammation therefore appears to be tightly linked to an increased risk for cardiac disease, especially a failing heart. As reviewed previously in this chapter, chronic inflammation also has strong ties in the development of frailty. Thus, it would be beneficial to consider the impact on the heart of any drug that may help manage frailty,

[5] Meta-Analysis Global Group in Chronic (MAGGIC) heart failure risk score (Rich et al., 2018).

because overlapping mechanisms might suggest pluripotent efficacy.

3.6 Chapter summary

Generally accepted mechanisms of aging drill down to the root of cellular function. These take the form of genomic instability and ultimately leads to the deterioration of our bodies at the molecular level. This translates to meaningful macroscopic changes in our physiologic robustness. Sarcopenia is one such change. With age, our physical capabilities decline at an exponential rate. Our ability to produce muscular force declines and our muscles shrink. These changes are intimately tied to our metabolic and neurological systems, which concurrently decay over time. We are then predisposed to become frail and more likely to "break", so to speak, when our health is challenged. Preclinical evidence suggests this is also the case in mice (reviewed in Chapter 2). Higher chronic levels of low-grade blood borne inflammatory markers are also present in older age. For example, the cytokine IL-6 has strong ties to frailty when it is elevated in the blood chronically, likely due to its relationship with cellular senescence. This chronic inflammatory state can further enable the progression of several disease states. This may be re-phrased as enabling frailty. Importantly, preclinical mouse models of aging reflect relationships between frailty and cytokines, especially IL-6, and components of frailty. In addition to sarcopenic traits and elevated chronic inflammation, the age-related decline in cardiac function and bone health play important roles in the development of frailty later in life. Further, deleterious changes in both systems relate to chronic inflammation. Discovering ways to mitigate all such changes would likely prove useful in the treatment of frailty.

3.7 Tables

Table 3.1 Cytokines/chemokines and their relationship with frailty in humans

Cytokine	Authors	Location	Study type	Study details	N (% F)	Age (Mean unless stated otherwise)	Frailty assessment tool	Relation with frailty
IL-6	Alberro et al., 2021	Spain	Cross-sectional	Community-dwelling	256 (59%)	69.6	Tilburg Frailty Indicator and Gerontopole Frailty Screening Tool	↔
	McKechnie et al., 2021	Britain	Longitudinal	Community-Dwelling	981 (0%)	77.7	Fried Frailty Phenotype	↑
	Mehta et al., 2021	USA	Cohort	Community-Dwelling	1208 (56%)	69.5	Liver Frailty Index	↑
	Tembo et al., 2021	Australia	Cross-sectional	Cohort	581 (0%)	74 (median)	Fried Frailty Phenotype	↑
	Buiges et al., 2020	Spain	Cross-sectional	Prostate cancer patients	39 (0%)	71.9	Fried Frailty Phenotype	↑
	*Furtado et al., 2020	Portugal	Cross-sectional	Nursing home residents	358 (100%)	88 (median)	Fried Frailty Phenotype	↑
	Gilmore et al., 2020	USA	Cross-sectional	Cancer patients	144 (100%)	59.5	Fried Frailty Phenotype	↑
	Hammami et al., 2020	Tunisia	Cross-sectional	Institutionalized	141 (43%)	66-85 (range)	Modified Short Emergency Geriatric Assessment Score	↔
	Badrasawi et al., 2016	Malaysia	Cross-sectional	Community-dwelling	92 (52%)	68	Fried Frailty Phenotype	↑
	Hsu et al., 2019	Australia	Cross-sectional	Community-dwelling	901 (0%)	81.3	Fried Frailty Phenotype	↑
	Navarro-Martínez et al., 2019	Spain	Cross-sectional	Nursing home and prostate	46 (0%)	72.2	Fried Frailty Phenotype	↑

Cytokine	Authors	Location	Study type	Study details	N (% F)	Age (Mean unless stated otherwise)	Frailty assessment tool	Relation with frailty
				cancer patients				
	Semmarath et al., 2019	Thailand	Cross-sectional	Community-dwelling	118 (37%)	68.1	Modified Fried Frailty Phenotype	↑ (M), ↑(F)
	Ma et al., 2018	China	Cross-sectional	CHCAS Study	130 (38%)	72.8	Fried Frailty Phenotype	↑
	Marcos-Pérez et al., 2018	Spain	Cross-sectional	Community-dwelling	259 (67%)	79.4	Modified Fried Frailty Phenotype	↑
	Nascimento et al., 2018	Brazil	Cross-sectional	Community-dwelling	347 (57%)	70.1	Modified Fried Frailty Phenotype	↑
	Yang et al., 2018	China	Cross-sectional	Hospitalized	435 (26%)	81.6	FRAIL scale	↑
	Liu et al., 2016	USA	Cross-sectional	Framingham heart study	1919 (54%)	70.9	Fried Frailty Phenotype	↑
	Lu et al., 2016	Singapore	Cross-sectional	Cohort	76 (59%)	68.4	Rockwood Frailty Index and Fried Frailty Scale	↔
	Arts et al., 2015	Iceland	Cohort	Depression	378 (64%)	70.6	Modified Fried Frailty Phenotype	↑ (performance-based component)
	Browers et al., 2015	Belgium	Cross-sectional	Cancer patients	244 (100%)	(medians) Young=40, Older=76	Balducci and Leuven Oncogeriatric Frailty Score	↑
	Piggott et al., 2015	USA	Cross-sectional	HIV	1326 (34%)	48 (median)	Fried Frailty Phenotype	↑
	Singer et al., 2015	USA	Cross-sectional	Lung transplant candidates	345 (45%)	59	Fried Frailty Phenotype	↑
	Darvin et al., 2014	USA	Cross-sectional	Community-dwelling	65 (60%)	80.6	Fried Frailty Phenotype	↑

Cytokine	Authors	Location	Study type	Study details	N (% F)	Age (Mean unless stated otherwise)	Frailty assessment tool	Relation with frailty
	Lai et al., 2014	Taiwan	Cross-sectional	Institutionalized	386 (0%)	81.5	Fried Phenotype	↑
	Baylis et al., 2013	UK	Longitudinal	Community-dwelling	254 (40%)	67.1	Fried Frailty Phenotype	↔
	Collerton et al., 2012	UK	Cross-sectional	Community-dwelling	811 (RFI; 61.7%) and 552 (FFS; 60.1%)	85↑	Rockwood Frailty Index and Fried Frailty Scale	↑
	Kalyani et al., 2012	USA	Cross-sectional	Community-dwelling	56 (100%)	84-95 (range)	Fried Frailty Phenotype	↔
	Leng et al., 2011	USA	Cross-sectional	Community-dwelling	133 (80%)	84	Fried Frailty Phenotype	↑
	Rønning et al., 2010	Norway	Cross-sectional	Cancer patients	137 (55%)	80 (median)	Fried Frailty Phenotype and Comprehensive Geriatric Assessment	↑
	Fried et al., 2009	USA	Cross-sectional	Cardiovascular health study	704 (100%)	70–79	Fried Frailty Phenotype	↑
	Hubbard et al., 2009	England	Case-control	Nursing home residents	110 (60%)	↔82.4	Modified Fried Frailty Phenotype and Frailty Index	↑
	Leng et al., 2009	USA	Cohort	Women's health and ageing studies	558 (100%)	73.9	Fried Frailty Phenotype	↑
	Qu et al., 2009	USA	Cross-sectional	Community-dwelling	32 (88%)	83	Fried Frailty Phenotype	↑
	Boxer et al., 2008	USA	Cross-sectional	Heart failure patients	100 (28%)	77	Fried Frailty Phenotype	↑
	Barzilay et al.,	USA	Cross-	Community-	2826 (62%)	71.8	Fried Frailty Phenotype	↑

Cytokine	Authors	Location	Study type	Study details	N (% F)	Age (Mean unless stated otherwise)	Frailty assessment tool	Relation with frailty
	2007		sectional	dwelling				
	Leng et al., 2004	USA	Cross-sectional	Community-dwelling	51 (76%)	82.6	Fried Frailty Phenotype	↔
	Leng et al., 2002	USA	Cross-sectional	Community-dwelling and hospitalized	30 (77%)	↔83.1	Fried Frailty Phenotype	↑
TNF-α	Alberro et al., 2021	Spain	Cross-Sectional	Community-dwelling	256 (59%)	69.6	Tilburg Frailty Indicator and Gerontopole Frailty Screening Tool	↔
	Buiges et al., 2020	Spain	Cross-sectional	Prostate cancer patients	39 (0%)	71.9	Fried Frailty Phenotype	↔
	Furtado et al., 2020	Portugal	Cross-sectional	Nursing home residents	358 (100%)	88 (median)	Fried Frailty Phenotype	↑
	Hammami et al., 2020	Tunisia	Cross-sectional	Institutionalized	141 (43%)	66-85 (range)	Modified Short Emergency Geriatric Assessment Score	↑
	Badrasawi et al., 2016	Malaysia	Cross-sectional	Community-dwelling	92 (52%)	68	Fried Frailty Phenotype	↑
	Hsu et al., 2019	Australia	Cross-sectional	Community-dwelling	901 (0%)	81.3	Fried Frailty Phenotype	↔
	Marzetti et al., 2019	Italy	Cross-sectional	Case-control	200 (63%)	76.2	Physical Frailty & Sarcopenia	↓ (M)
	Navarro-Martínez et al., 2019	Spain	Cross-sectional	Nursing home and prostate cancer patients	46 (0%)	72.2	Fried Frailty Phenotype	↔

Cytokine	Authors	Location	Study type	Study details	N (% F)	Age (Mean unless stated otherwise)	Frailty assessment tool	Relation with frailty
	Marcos-Pérez et al., 2018	Spain	Cross-sectional	Community-dwelling	259 (67%)	79.4	Modified Fried Frailty Phenotype	↑
	Nascimento et al., 2018	Brazil	Cross-sectional	Community-dwelling	347 (57%)	70.1	Modified Fried Frailty Phenotype	↑
	Lu et al., 2016	Singapore	Cross-sectional	Cohort	76 (59%)	68.4	Rockwood Frailty Index and Fried Frailty Scale	↔
	Lai et al., 2014	Taiwan	Cross-sectional	Institutionalized	386 (0%)	81.5	Fried Phenotype	↔
	Tsai et al., 2013	Taiwan	Cross-sectional	Hospital-based	168 (51%)	76.9	Modified Fried Frailty Index	↔
	Collerton et al., 2012	UK	Cross-sectional	Community-dwelling	811 (RFI; 61.7%) and 552 (FFS; 60.1%)	85↑	Rockwood Frailty Index and Fried Frailty Scale	↑
	Rønning et al., 2010	Norway	Cross-sectional	Cancer patients	137 (55%)	80 (median)	Fried Frailty Phenotype and Comprehensive Geriatric Assessment	↑
	Hubbard et al., 2009	England	Case-Control	Nursing home residents	110 (60%)	↔82.4	Modified Fried Frailty Phenotype and Frailty Index	↑
	Serviddio et al., 2009	Italy	Cross-sectional	Hospitalized	62 (59%)	76.7	Modified Fried Frailty Phenotype	↑
IL-8	Cybularz et al., 2021	Germany	Cross-Sectional	Hospitalized	120 (30%)	80.3	Eyeball assessment	↑
	Buiges et al., 2020	Spain	Cross-sectional	Prostate cancer patients	39 (0%)	71.9	Fried Frailty Phenotype	↑
	Hammami et al., 2020	Tunisia	Cross-sectional	Institutionalized	141 (43%)	66-85 (range)	Modified Short Emergency Geriatric Assessment Score	↑
	Hsu et al., 2019	Austrailia	Cross-	Community-	901 (0%)	81.3	Fried Frailty Phenotype	↑

Cytokine	Authors	Location	Study type	Study details	N (% F)	Age (Mean unless stated otherwise)	Frailty assessment tool	Relation with frailty
			sectional	dwelling				
	Marzetti et al., 2019	Italy	Cross-sectional	Community-dwelling	200 (63%)	76.2	Physical Frailty & Sarcopenia	↓
	Navarro-Martínez et al., 2019	Spain	Cross-sectional	Nursing home and prostate cancer patients	46 (0%)	72.2	Fried Frailty Phenotype	↑ (but not in cancer patients)
	Lu et al., 2016	Singapore	Cross-sectional	Cohort	76 (59%)	68.4	Rockwood Frailty Index and Fried Frailty Scale	↔
IL-10	Furtado et al., 2020	Portugal	Cross-sectional	Nursing home residents	358 (100%)	88 (median)	Fried Frailty Phenotype	↑
	Hsu et al., 2019	Australia	Cross-sectional	Community-dwelling	901 (0%)	81.3	Fried Frailty Phenotype	↔
	Nascimento et al., 2018	Brazil	Cross-sectional	Community-dwelling	347 (57%)	70.1	Modified Fried Frailty Phenotype	↔
	Su et al., 2017	China	Cross-sectional	Community-dwelling	306 (60.1%)	70.5	Modified Fried Frailty Phenotype	↔
	Lu et al., 2016	Singapore	Cross-sectional	Cohort	76 (59%)	68.4	Rockwood Frailty Index and Fried Frailty Scale	↔
	Baylis et al., 2013	UK	Longitudinal	Community-dwelling	254 (40%)	67.1	Fried Frailty Phenotype	↔
MCP-1	Marzetti et al., 2019	Italy	Cross-sectional	Community-dwelling	200 (63%)	76.2	Physical Frailty & Sarcopenia	↓
	Hsu et al., 2019	Austrailia	Cross-sectional	Community-dwelling	901 (0%)	81.3	Fried Frailty Phenotype	↔
	Su et al., 2017	China	Cross-sectional	Community-dwelling	306 (60.1%)	70.5	Modified Fried Frailty Phenotype	↑
	Liu et al., 2016	USA	Cross-	Framingham	1919 (54%)	70.9	Fried Frailty Phenotype	↑

Cytokine	Authors	Location	Study type	Study details	N (% F)	Age (Mean unless stated otherwise)	Frailty assessment tool	Relation with frailty
			sectional	heart study				
	Lu et al., 2016	Singapore	Cross-sectional	Cohort	76 (59%)	68.4	Rockwood Frailty Index and Fried Frailty Scale	↓
	Browers et al., 2015	Belgium	Cross-sectional	Cancer patients	244 (100%)	[Medians] Young=40, Older=76	Balducci and Leuven Oncogeriatric Frailty Score	↔
IL-1β	Buiges et al., 2020	Spain	Cross-sectional	Prostate cancer patients	39 (0%)	71.9	Fried Frailty Phenotype	↔
	*Furtado et al., 2020	Portugal	Cross-sectional	Nursing home residents	358 (100%)	88 (median)	Fried Frailty Phenotype	↑
	Hsu et al., 2019	Australia	Cross-sectional	Community-dwelling	901 (0%)	81.3	Fried Frailty Phenotype	↔
	Nascimento et al., 2018	Brazil	Cross-sectional	Community-dwelling	347 (57%)	70.1	Modified Fried Frailty Phenotype	↔
	Navarro-Martínez et al., 2019	Spain	Cross-sectional	Nursing home and prostate cancer patients	46 patients (0%), 46 controls	72.2	Fried Frailty Phenotype	↔ (cancer patients)
IFN-γ	Hsu et al., 2020	Australia	Cross-sectional	Community-dwelling	901 (0%)	81.3	Fried Frailty Phenotype	↔
	Marzetti et al., 2019	Italy	Cross-sectional	Community-dwelling	200 (63%)	76.2	Physical Frailty & Sarcopenia	↓ (M)
	Mohamad et al., 2018	Egypt	Cross-sectional	Community-dwelling	80 (53%)	64.7	Fried Frailty Phenotype	↑
	Lu et al., 2016	Singapore	Cross-sectional	Cohort	76 (59%)	68.4	Rockwood Frailty Index and Fried Frailty Scale	↔
Leptin	Sathyan et al.,	USA	Cross-	Community-	800 (54.8%)	75.4	Frailty Index	↑

Cytokine	Authors	Location	Study type	Study details	N (% F)	Age (Mean unless stated otherwise)	Frailty assessment tool	Relation with frailty
	2020		sectional	dwelling				
	Lana et al., 2017	Spain	Longitudinal	Community-dwelling	1801 (52%)	68.6	Modified Fried Frailty Phenotype	↑
	Lu et al., 2016	Singapore	Cross-sectional	Cohort	76 (59%)	68.4	Rockwood Frailty Index and Fried Frailty Scale	↑
	Hubbard et al., 2008	Australia	Cross-sectional	Continuing care	132 (40%)	75 to 95	Fried Frailty Phenotype	↓
MIP-1β	Hsu et al., 2019	Australia	Cross-sectional	Community-dwelling	901 (0%)	81.3	Fried Frailty Phenotype	↔
	Marzetti et al., 2019	Italy	Cross-sectional	Community-dwelling	200 (63%)	76.2	Physical Frailty & Sarcopenia	↓(F), ↓(M)
	Su et al., 2017	China	Cross-sectional	Community-dwelling	306 (60.1%)	70.5	Modified Fried Frailty Phenotype	↑
	Lu et al., 2016	Singapore	Cross-sectional	Cohort	76 (59%)	68.4	Rockwood Frailty Index and Fried Frailty Scale	↔
Eotaxin	Hsu et al., 2019	Australia	Cross-sectional	Community-dwelling	901 (0%)	81.3	Fried Frailty Phenotype	↔
	Marzetti et al., 2019	Italy	Cross-sectional	Community-dwelling	200 (63%)	76.2	Physical Frailty & Sarcopenia	↑ (F)
	Lu et al., 2016	Singapore	Cross-sectional	Cohort	76 (59%)	68.4	Rockwood Frailty Index and Fried Frailty Scale	↔
IL-17	Hsu et al., 2019	Australia	Cross-sectional	Community-dwelling	901 (0%)	81.3	Fried Frailty Phenotype	↔
	Marzetti et al., 2019	Italy	Cross-sectional	Community-dwelling	200 (63%)	76.2	Physical Frailty & Sarcopenia	↓(M)
	Lu et al., 2019	Singapore	Cross-sectional	Cohort	76 (59%)	68.4	Rockwood Frailty Index and Fried Frailty Scale	↔
MIP-1α	Hsu et al., 2019	Australia	Cross-sectional	Community-dwelling	901 (0%)	81.3	Fried Frailty Phenotype	↔
	Marzetti et al.,	Italy	Cross-	Community-	200 (63%)	76.2	Physical Frailty &	↓(F)

Cytokine	Authors	Location	Study type	Study details	N (% F)	Age (Mean unless stated otherwise)	Frailty assessment tool	Relation with frailty
	2019		sectional	dwelling			Sarcopenia	
	Lu et al., 2016	Singapore	Cross-sectional	Cohort	76 (59%)	68.4	Rockwood Frailty Index and Fried Frailty Scale	↔
IL-1α	Sathyan et al., 2020	USA	Cross-sectional	Community-dwelling	800 (54.8%)	75.4	Frailty Index	↑
	Nascimento et al., 2018	Brazil	Cross-sectional	Community-dwelling	347 (57%)	70.1	Modified Fried Frailty Phenotype	↑
	Lu et al., 2016	Singapore	Cross-sectional	Cohort	76 (59%)	68.4	Rockwood Frailty Index and Fried Frailty Scale	↔
CCL5	Hsu et al., 2019	Australia	Cross-sectional	Community-dwelling	901 (0%)	81.3	Fried Frailty Phenotype	↔
	Lu et al., 2016	Singapore	Cross-sectional	Cohort	76 (59%)	68.4	Rockwood Frailty Index	↑
CXCL10	Lu et al., 2016	Singapore	Cross-sectional	Cohort	76 (59%)	68.4	Rockwood Frailty Index and Fried Frailty Scale	↔
	Qu et al., 2009	USA	Cross-sectional	Community-dwelling	32 (88%)	83	Fried Frailty Phenotype	↑
IL-1ra	Hsu et al., 2019	Australia	Cross-sectional	Community-dwelling	901 (0%)	81.3	Fried Frailty Phenotype	↔
	Lu et al., 2016	Singapore	Cross-sectional	Cohort	76 (59%)	68.4	Rockwood Frailty Index and Fried Frailty Scale	↔
IL-4	Hsu et al., 2019	Australia	Cross-sectional	Community-dwelling	901 (0%)	81.3	Fried Frailty Phenotype	↔
	Lu et al., 2016	Singapore	Cross-sectional	Cohort	76 (59%)	68.4	Rockwood Frailty Index and Fried Frailty Scale	↔
IL-5	Hsu et al., 2019	Australia	Cross-sectional	Community-dwelling	901 (0%)	81.3	Fried Frailty Phenotype	↔
	Lu et al., 2016	Singapore	Cross-sectional	Cohort	76 (59%)	68.4	Rockwood Frailty Index and Fried Frailty Scale	↔
IL-7	Hsu et al., 2019	Australia	Cross-	Community-	901 (0%)	81.3	Fried Frailty Phenotype	↔

Cytokine	Authors	Location	Study type	Study details	N (% F)	Age (Mean unless stated otherwise)	Frailty assessment tool	Relation with frailty
			sectional	dwelling				
	Lu et al., 2016	Singapore	Cross-sectional	Cohort	76 (59%)	68.4	Rockwood Frailty Index and Fried Frailty Scale	↔
IL-9	Hsu et al., 2019	Australia	Cross-sectional	Community-dwelling	901 (0%)	81.3	Fried Frailty Phenotype	↔
	Lu et al., 2016	Singapore	Cross-sectional	Cohort	76 (59%)	68.4	Rockwood Frailty Index and Fried Frailty Scale	↔
IL-12p70	Compté et al., 2013	Belgium	Cross-sectional	General population and hospitalized	52 (67%)	81.4	Identification of Seniors at Risk	↓
	Lu et al., 2016	Singapore	Cross-sectional	Cohort	76 (59%)	68.4	Rockwood Frailty Index and Fried Frailty Scale	↔
IL-23	Lu et al., 2016	Singapore	Cross-sectional	Cohort	76 (59%)	68.4	Rockwood Frailty Index and Fried Frailty Scale	↔
	Compté et al., 2013	Belgium	Cross-sectional	General population and hospitalized	52 (67%)	81.4	Identification of Seniors at Risk	↓
G-CSF	Hsu et al., 2019	Australia	Cross-sectional	Community-dwelling	901 (0%)	81.3	Fried Frailty Phenotype	↔
	Lu et al., 2016	Singapore	Cross-sectional	Cohort	76 (59%)	68.4	Rockwood Frailty Index and Fried Frailty Scale	↔

Notes: CCL5 = C-C motif chemokine ligand 5; CXCL10 = C-X-C motif chemokine ligand 10; G-CSF = Granulocyte colony stimulating factor; IFN = interferon; IL = Interleukin; IL-1ra = Interleukin 1 receptor antagonist; MCP = Monocyte chemoattractant protein; MIP = Macrophage inflammatory protein; RANTES = regulated on activation, normal T cell expressed and secreted; TNF = Tumour necrosis factor. *denotes salivary measurement. Symbol meaning: ↑ = proportional relationship with frailty; ↓ = inversely proportional with frailty; ↔ = no relationship with frailty. M= Males; F = Females

Table 3.2 Lesser studied relationships between cytokines and frailty in humans. Cytokines with a significant relationship with frailty are bolded.

Cytokine	Study year	Authors	Location	Study type	Study details	N (% F)	Age (Mean unless stated otherwise)	Frailty assessment tool	Relation with frailty
CCL17	2016	Lu et al.	Singapore	Cross-sectional	Cohort	76 (59%)	68.4	Rockwood Frailty Index and Fried Frailty Scale	↔
CCL21	2016	Lu et al.	Singapore	Cross-sectional	Cohort	76 (59%)	68.4	Rockwood Frailty Index and Fried Frailty Scale	↔
CCL27	2016	Lu et al.	Singapore	Cross-sectional	Cohort	76 (59%)	68.4	Rockwood Frailty Index and Fried Frailty Scale	↔
CXCL5	2016	Lu et al.	Singapore	Cross-sectional	Cohort	76 (59%)	68.4	Rockwood Frailty Index and Fried Frailty Scale	↔
CXCL12	2016	Lu et al.	Singapore	Cross-sectional	Cohort	76 (59%)	68.4	Rockwood Frailty Index and Fried Frailty Scale	↔
CXCL13	2016	Lu et al.	Singapore	Cross-sectional	Cohort	76 (59%)	68.4	Rockwood Frailty Index and Fried Frailty Scale	↔
Eotaxin-2	2016	Lu et al.	Singapore	Cross-sectional	Cohort	76 (59%)	68.4	Rockwood Frailty Index and Fried Frailty Scale	↔
Eaotaxin-3	2016	Lu et al.	Singapore	Cross-sectional	Cohort	76 (59%)	68.4	Rockwood Frailty Index and Fried Frailty Scale	↔
Ftl-3l	2016	Lu et al.	Singapore	Cross-sectional	Cohort	76 (59%)	68.4	Rockwood Frailty Index and Fried Frailty Scale	↔

Cytokine	Study year	Authors	Location	Study type	Study details	N (% F)	Age (Mean unless stated otherwise)	Frailty assessment tool	Relation with frailty
GM-CSF	2016	Lu et al.	Singapore	Cross-sectional	Cohort	76 (59%)	68.4	Rockwood Frailty Index and Fried Frailty Scale	↔
GRO	2016	Lu et al.	Singapore	Cross-sectional	Cohort	76 (59%)	68.4	Rockwood Frailty Index and Fried Frailty Scale	↔
I-309	2016	Lu et al.	Singapore	Cross-sectional	Cohort	76 (59%)	68.4	Rockwood Frailty Index and Fried Frailty Scale	↑
IFNα2	2016	Lu et al.	Singapore	Cross-sectional	Cohort	76 (59%)	68.4	Rockwood Frailty Index and Fried Frailty Scale	↔
IL-12	2019	Hsu et al.	Australia	Cross-sectional	Community-dwelling	901 (0%)	81.3	Fried Frailty Phenotype	↔
IL-13	2019	Hsu et al.	Australia	Cross-sectional	Community-dwelling	901 (0%)	81.3	Fried Frailty Phenotype	↔
IL-15	2016	Lu et al.	Singapore	Cross-sectional	Cohort	76 (59%)	68.4	Rockwood Frailty Index and Fried Frailty Scale	↔
IL-16	2016	Lu et al.	Singapore	Cross-sectional	Cohort	76 (59%)	68.4	Rockwood Frailty Index and Fried Frailty Scale	↔
IL-20	2016	Lu et al.	Singapore	Cross-sectional	Cohort	76 (59%)	68.4	Rockwood Frailty Index and Fried Frailty Scale	↔
IL-28a	2016	Lu et al.	Singapore	Cross-sectional	Cohort	76 (59%)	68.4	Rockwood Frailty Index and Fried Frailty Scale	↔

Cytokine	Study year	Authors	Location	Study type	Study details	N (% F)	Age (Mean unless stated otherwise)	Frailty assessment tool	Relation with frailty
LIF	2016	Lu et al.	Singapore	Cross-sectional	Cohort	76 (59%)	68.4	Rockwood Frailty Index and Fried Frailty Scale	↔
MCP-2	2016	Lu et al.	Singapore	Cross-sectional	Cohort	76 (59%)	68.4	Rockwood Frailty Index and Fried Frailty Scale	↔
MCP-3	2017	Su et al.	China	Cross-sectional	Community-dwelling	306 (60.1%)	70.5	Modified Fried Frailty Phenotype	↔
	2016	Lu et al.	Singapore	Cross-sectional	Cohort	76 (59%)	68.4	Rockwood Frailty Index and Fried Frailty Scale	↔
MCP-4	2016	Lu et al.	Singapore	Cross-sectional	Cohort	76 (59%)	68.4	Rockwood Frailty Index and Fried Frailty Scale	↔
MDC	2016	Lu et al.	Singapore	Cross-sectional	Cohort	76 (59%)	68.4	Rockwood Frailty Index and Fried Frailty Scale	↔
MIP-1δ	2016	Lu et al.	Singapore	Cross-sectional	Cohort	76 (59%)	68.4	Rockwood Frailty Index and Fried Frailty Scale	↔
PDGF-AA	2016	Lu et al.	Singapore	Cross-sectional	Cohort	76 (59%)	68.4	Rockwood Frailty Index and Fried Frailty Scale	↔
PDGF-BB	2016	Lu et al.	Singapore	Cross-sectional	Cohort	76 (59%)	68.4	Rockwood Frailty Index and Fried Frailty Scale	↔
SCF	2016	Lu et al.	Singapore	Cross-sectional	Cohort	76 (59%)	68.4	Rockwood Frailty Index and Fried Frailty Scale	↔

Cytokine	Study year	Authors	Location	Study type	Study details	N (% F)	Age (Mean unless stated otherwise)	Frailty assessment tool	Relation with frailty
TGB-α	2016	Lu et al.	Singapore	Cross-sectional	Cohort	76 (59%)	68.4	Rockwood Frailty Index and Fried Frailty Scale	↔
TPO	2016	Lu et al.	Singapore	Cross-sectional	Cohort	76 (59%)	68.4	Rockwood Frailty Index and Fried Frailty Scale	↔
TRAIL	2016	Lu et al.	Singapore	Cross-sectional	Cohort	76 (59%)	68.4	Rockwood Frailty Index and Fried Frailty Scale	↔
TNF-β	2018	Nascimento et al.	Brazil	Cross-sectional	Community-dwelling	347 (57%)	70.1	Modified Fried Frailty Phenotype	↑

Table 3.3 Inferred relationships between cytokines/chemokines frailty in interventional studies using mice

Cytokine	Species / strain / model / intervention	Sex / age	Frailty assessment	Tissue / method	Relationship with frailty (Males)	Relationship with frailty (Females)	Reference
IL-1α	C57BL/6 mice	M & F; 17, 23 months	Clinical FI; FI-Lab; Combined (Clinical+Lab)	Serum (multiplex)	↔	↔	Kane et al., 2019
	C57BL/6 mice (Voluntary aerobic exercise)	M & F; 21-26 months	Clinical FI	Serum (multiplex)	↔	↔	Bisset et al., 2022
	C57BL/6J mice (alpha-ketoglutarate (AKG) treatment)	M & F; 24-31 months	Clinical FI	Plasma (multiplex)	↔	↔	Shahmirzadi et al., 2020
	C57BL/6 mice (ACE-inhibitor enalapril treatment)	M (13, 25 months) & F (13, 22 months)	Clinical FI	Serum (multiplex)	↔	↑ (22 months)	Keller et al., 2019
IL-1β	C57BL/6 mice	M & F; 17, 23 months	Clinical FI; FI-Lab; Combined (Clinical+Lab)	Serum (multiplex)	↔	↔	Kane et al., 2019
	C57BL/6 mice (Voluntary aerobic exercise)	M & F; 21-26 months	Clinical FI	Serum (multiplex)	↔	↔	Bisset et al., 2022
	C57BL/6J mice (alpha-ketoglutarate (AKG) treatment)	M & F; 24-31 months	Clinical FI	Plasma (multiplex)	↔	↔	Shahmirzadi et al., 2020
	C57BL/6 mice (ACE-inhibitor enalapril treatment)	M (13, 25 months) & F (13, 22 months)	Clinical FI	Serum (multiplex)	↔	↔	Keller et al., 2019
	C57BL/6 mice (sodium nitrite supplementation)	M; 20-26 months	Grip strength, locomotor activity, Rotarod (constant speed and accelerating)	Skeletal muscle (ELISA)	↑	N/A	Justice et al., 2015

Cytokine	Species / strain / model / intervention	Sex / age	Frailty assessment	Tissue / method	Relationship with frailty (Males)	Relationship with frailty (Females)	Reference
IL-2	C57BL/6 mice	M & F; 17, 23 months	Clinical FI; FI-Lab; Combined (Clinical+Lab)	Serum (multiplex)	↔	↔	Kane et al., 2019
	C57BL/6 mice (Voluntary aerobic exercise)	M & F; 21-26 months	Clinical FI	Serum (multiplex)	↔	↓	Bisset et al., 2022
	C57BL/6J mice (alpha-ketoglutarate (AKG) treatment)	M & F; 24-31 months	Clinical FI	Plasma (multiplex)	↔	↔	Shahmirzadi et al., 2020
	C57BL/6 mice (ACE-inhibitor enalapril treatment)	M (13, 25 months) & F (13, 22 months)	Clinical FI	Serum (multiplex)	↔	↔	Keller et al., 2019
IL-3	C57BL/6 mice	M & F; 17, 23 months	Clinical FI; FI-Lab; Combined (Clinical+Lab)	Serum (multiplex)	↔	↔	Kane et al., 2019
	C57BL/6 mice (Voluntary aerobic exercise)	M & F; 21-26 months	Clinical FI	Serum (multiplex)	↔	↓	Bisset et al., 2022
	C57BL/6 mice (ACE-inhibitor enalapril treatment)	M (13, 25 months) & F (13, 22 months)	Clinical FI	Serum (multiplex)	↔	↔	Keller et al., 2019
IL-4	C57BL/6 mice	M & F; 17, 23 months	Clinical FI; FI-Lab; Combined (Clinical+Lab)	Serum (multiplex)	↔	↔	Kane et al., 2019
	C57BL/6 mice (Voluntary aerobic exercise)	M & F; 21-26 months	Clinical FI	Serum (multiplex)	↔	↔	Bisset et al., 2022
	C57BL/6 mice (ACE-inhibitor enalapril treatment)	M (13, 25 months) & F (13, 22 months)	Clinical FI	Serum (multiplex)	↔	↔	Keller et al., 2019
IL-5	C57BL/6 mice	M & F; 17, 23	Clinical FI; FI-Lab;	Serum	↔	↔	Kane et al.,

Cytokine	Species / strain / model / intervention	Sex / age	Frailty assessment	Tissue / method	Relationship with frailty (Males)	Relationship with frailty (Females)	Reference
		months	Combined (Clinical+Lab)	(multiplex)			2019
	C57BL/6 mice (Voluntary aerobic exercise)	M & F; 21-26 months	Clinical FI	Serum (multiplex)	↔	↓	Bisset et al., 2022
	C57BL/6 mice (ACE-inhibitor enalapril treatment)	M (13, 25 months) & F (13, 22 months)	Clinical FI	Serum (multiplex)	↔	↔	Keller et al., 2019
IL-6	C57BL/6 mice	M & F; 17, 23 months	Clinical FI; FI-Lab; Combined (Clinical+Lab)	Serum (multiplex)	↔	↑ (FI-Lab and Combined)	Kane et al., 2019
	C57BL/6 mice (Voluntary aerobic exercise)	M & F; 21-26 months	Clinical FI	Serum (multiplex)	↔	↓	Bisset et al., 2022
	C57BL/6J mice (alpha-ketoglutarate (AKG) treatment)	M & F; 24-31 months	Clinical FI	Plasma (multiplex)	↔	↔	Shahmirzadi et al., 2020
	C57BL/6 mice (ACE-inhibitor enalapril treatment)	M (13, 25 months) & F (13, 22 months)	Clinical FI	Serum (multiplex)	↔	↔	Keller et al., 2019
	C57BL/6 mice (JAK inhibitor ruxolitinib treatment)	M; 24 months	Physical functional assessments (activity, grip strength, hanging time, Rotarod coordination test)	Serum (multiplex) and adipose (qPCR)	↑	N/A	Xu et al., 2015
	C57BL/6J mice (*H. Erinaceus* treatment)	M; 22-24 months	Locomotor FI (open, emergence tests; activity tracking – speed, resting times)	Brain (cerebellar) tissue (immuno-histochemistry)	↑	N/A	Roda et al., 2021
	C57BL/6 mice (sodium nitrite supplementation)	M; 20-26 months	Grip strength, locomotor activity, Rotarod (constant	Skeletal muscle (ELISA)	↔	N/A	Justice et al., 2015

Cytokine	Species / strain / model / intervention	Sex / age	Frailty assessment	Tissue / method	Relationship with frailty (Males)	Relationship with frailty (Females)	Reference
			speed and accelerating)				
	C57BL/6 mice (angiotensin II type I receptor blocker losartan treatment)	F; 18-22 months	Physical frailty (grip strength, body weight, treadmill endurance, activity)	Serum (ELISA)	N/A	↑	Lin et al., 2014
	C57BL/6 mice (Senescence induction (radiation and transplantation)	M; 5-7 months	Physical function assessment (speed, endurance, grip strength, activity, body weight), senescence evaluation	Skeletal muscle (qPCR)	↑	N/A	Xu et al., 2018
	C57BL/6 mice (Senolytic treatment with dasatinib & quercetin)	M & F; 24 months	Physical function assessment (speed, endurance, grip strength, activity, body weight), senescence evaluation	Visceral adipose tissue (qPCR)	↑	↑	Xu et al., 2018
IL-7	C57BL/6J mice (alpha-ketoglutarate (AKG) treatment)	M & F; 24-31 months	Clinical FI	Plasma (multiplex)	↔	↔	Shahmirzadi et al., 2020
IL-9	C57BL/6 mice	M & F; 17, 23 months	Clinical FI; FI-Lab; Combined (Clinical+Lab)	Serum (multiplex)	↔	↑ (FI-Lab and Combined)	Kane et al., 2019
	C57BL/6 mice (Voluntary aerobic exercise)	M & F; 21-26 months	Clinical FI	Serum (multiplex)	↔	↓	Bisset et al., 2022
	C57BL/6 mice (ACE-inhibitor enalapril treatment)	M (13, 25 months) & F (13, 22 months)	Clinical FI	Serum (multiplex)	↔	↔	Keller et al., 2019
IL-10	C57BL/6 mice	M & F; 17, 23 months	Clinical FI; FI-Lab; Combined	Serum (multiplex)	↔	↔	Kane et al., 2019

Cytokine	Species / strain / model / intervention	Sex / age	Frailty assessment	Tissue / method	Relationship with frailty (Males)	Relationship with frailty (Females)	Reference
			(Clinical+Lab)				
	C57BL/6 mice (Voluntary aerobic exercise)	M & F; 21-26 months	Clinical FI	Serum (multiplex)	↔	↔	Bisset et al., 2022
	C57BL/6J mice (alpha-ketoglutarate (AKG) treatment)	M & F; 24-31 months	Clinical FI	Spleen (Cell staining)	↔	↓	Shahmirzadi et al., 2020
	C57BL/6 mice (ACE-inhibitor enalapril treatment)	M (13, 25 months) & F (13, 22 months)	Clinical FI	Serum (multiplex)	↓ (25 months)	↔	Keller et al., 2019
IL-12 (p40)	C57BL/6 mice	M & F; 17, 23 months	Clinical FI; FI-Lab; Combined (Clinical+Lab)	Serum (multiplex)	↑ (Clinical FI and Combined)	↔	Kane et al., 2019
	C57BL/6 mice (Voluntary aerobic exercise)	M & F; 21-26 months	Clinical FI	Serum (multiplex)	↔	↔	Bisset et al., 2022
	C57BL/6J mice (alpha-ketoglutarate (AKG) treatment)	M & F; 24-31 months	Clinical FI	Plasma (multiplex)	↔	↔	Shahmirzadi et al., 2020
	C57BL/6 mice (ACE-inhibitor enalapril treatment)	M (13, 25 months) & F (13, 22 months)	Clinical FI	Serum (multiplex)	↔	↔	Keller et al., 2019
IL-12 (p70)	C57BL/6 mice	M & F; 17, 23 months	Clinical FI; FI-Lab; Combined (Clinical+Lab)	Serum (multiplex)	↔	↔	Kane et al., 2019
	C57BL/6 mice (Voluntary aerobic exercise)	M & F; 21-26 months	Clinical FI	Serum (multiplex)	↔	↔	Bisset et al., 2022
	C57BL/6J mice (alpha-ketoglutarate (AKG) treatment)	M & F; 24-31 months	Clinical FI	Plasma (multiplex)	↔	↑	Shahmirzadi et al., 2020

Cytokine	Species / strain / model / intervention	Sex / age	Frailty assessment	Tissue / method	Relationship with frailty (Males)	Relationship with frailty (Females)	Reference
	C57BL/6 mice (ACE-inhibitor enalapril treatment)	M (13, 25 months) & F (13, 22 months)	Clinical FI	Serum (multiplex)	↔	↔	Keller et al., 2019
IL-13	C57BL/6 mice	M & F; 17, 23 months	Clinical FI; FI-Lab; Combined (Clinical+Lab)	Serum (multiplex)	↔	↔	Kane et al., 2019
	C57BL/6 mice (Voluntary aerobic exercise)	M & F; 21-26 months	Clinical FI	Serum (multiplex)	↔	↔	Bisset et al., 2022
	C57BL/6 mice (ACE-inhibitor enalapril treatment)	M (13, 25 months) & F (13, 22 months)	Clinical FI	Serum (multiplex)	↔	↔	Keller et al., 2019
IL-15	C57BL/6J mice (alpha-ketoglutarate (AKG) treatment)	M & F; 24-31 months	Clinical FI	Plasma (multiplex)	↔	↔	Shahmirzadi et al., 2020
IL-17(a)	C57BL/6 mice	M & F; 17, 23 months	Clinical FI; FI-Lab; Combined (Clinical+Lab)	Serum (multiplex)	↔	↔	Kane et al., 2019
	C57BL/6 mice (Voluntary aerobic exercise)	M & F; 21-26 months	Clinical FI	Serum (multiplex)	↔	↔	Bisset et al., 2022
	C57BL/6J mice (alpha-ketoglutarate (AKG) treatment)	M & F; 24-31 months	Clinical FI	Plasma (multiplex)	↔	↑	Shahmirzadi et al., 2020
	C57BL/6 mice (ACE-inhibitor enalapril treatment)	M (13, 25 months) & F (13, 22 months)	Clinical FI	Serum (multiplex)	↔	↔	Keller et al., 2019
	C57BL/6 mice (JAK inhibitor ruxolitinib treatment)	M; 24 months	Physical functional assessments (activity, grip strength, hanging time, Rotarod coordination test)	Serum (multiplex)	↔	N/A	Xu et al., 2015

Cytokine	Species / strain / model / intervention	Sex / age	Frailty assessment	Tissue / method	Relationship with frailty (Males)	Relationship with frailty (Females)	Reference
CCL-11	C57BL/6 mice (JAK inhibitor ruxolitinib treatment)	M; 24 months	Physical functional assessments (activity, grip strength, hanging time, Rotarod coordination test)	Serum (multiplex)	↔	N/A	Xu et al., 2015
CXCL-1 (aka KC)	C57BL/6 mice	M & F; 17, 23 months	Clinical FI; FI-Lab; Combined (Clinical+Lab)	Serum (multiplex)	↔	↔	Kane et al., 2019
	C57BL/6 mice (Voluntary aerobic exercise)	M & F; 21-26 months	Clinical FI	Serum (multiplex)	↔	↓	Bisset et al., 2022
	C57BL/6 mice (ACE-inhibitor enalapril treatment)	M (13, 25 months) & F (13, 22 months)	Clinical FI	Serum (multiplex)	↔	↔	Keller et al., 2019
	C57BL/6J mice (alpha-ketoglutarate (AKG) treatment)	M & F; 24-31 months	Clinical FI	Plasma (multiplex)	↔	↔	Shahmirzadi et al., 2020
	C57BL/6 mice (JAK inhibitor ruxolitinib treatment)	M; 24 months	Physical functional assessments (activity, grip strength, hanging time, Rotarod coordination test)	Serum (multiplex)	↑	N/A	Xu et al., 2015
	C57BL/6 mice (Senolytic treatment with dasatinib & quercetin)	M & F; 24 months	Physical function assessment (speed, endurance, grip strength, activity, body weight), senescence evaluation	Visceral adipose tissue (qPCR)	↑	↑	Xu et al., 2018
CXCL-5	C57BL/6J mice (alpha-ketoglutarate (AKG) treatment)	M & F; 24-31 months	Clinical FI	Plasma (multiplex)	↔	↔	Shahmirzadi et al., 2020
CXCL-9	C57BL/6J mice (alpha-	M & F; 24-31	Clinical FI	Plasma	↔	↔	Shahmirzadi

Cytokine	Species / strain / model / intervention	Sex / age	Frailty assessment	Tissue / method	Relationship with frailty (Males)	Relationship with frailty (Females)	Reference
	ketoglutarate (AKG) treatment)	months		(multiplex)			et al., 2020
CXCL-10	C57BL/6J mice (alpha-ketoglutarate (AKG) treatment)	M & F; 24-31 months	Clinical FI	Plasma (multiplex)	↔	↔	Shahmirzadi et al., 2020
	C57BL/6 mice (JAK inhibitor ruxolitinib treatment)	M; 24 months	Physical functional assessments (activity, grip strength, hanging time, Rotarod coordination test)	Serum (multiplex)	↑	N/A	Xu et al., 2015
Eotaxin	C57BL/6 mice	M & F; 17, 23 months	Clinical FI; FI-Lab; Combined (Clinical+Lab)	Serum (multiplex)	↔	↔	Kane et al., 2019
	C57BL/6 mice (Voluntary aerobic exercise)	M & F; 21-26 months	Clinical FI	Serum (multiplex)	↔	↔	Bisset et al., 2022
	C57BL/6J mice (alpha-ketoglutarate (AKG) treatment)	M & F; 24-31 months	Clinical FI	Plasma (multiplex)	↔	↔	Shahmirzadi et al., 2020
	C57BL/6 mice (ACE-inhibitor enalapril treatment)	M (13, 25 months) & F (13, 22 months)	Clinical FI	Serum (multiplex)	↔	↔	Keller et al., 2019
G-CSF	C57BL/6 mice	M & F; 17, 23 months	Clinical FI; FI-Lab; Combined (Clinical+Lab)	Serum (multiplex)	↔	↔	Kane et al., 2019
	C57BL/6 mice (Voluntary aerobic exercise)	M & F; 21-26 months	Clinical FI	Serum (multiplex)	↔	↔	Bisset et al., 2022
	C57BL/6 mice (ACE-inhibitor enalapril treatment)	M (13, 25 months) & F (13, 22 months)	Clinical FI	Serum (multiplex)	↔	↔	Keller et al., 2019

Cytokine	Species / strain / model / intervention	Sex / age	Frailty assessment	Tissue / method	Relationship with frailty (Males)	Relationship with frailty (Females)	Reference
	C57BL/6 mice (JAK inhibitor ruxolitinib treatment)	M; 24 months	Physical functional assessments (activity, grip strength, hanging time, Rotarod coordination test)	Serum (multiplex)	↑	N/A	Xu et al., 2015
GM-CSF	C57BL/6 mice	M & F; 17, 23 months	Clinical FI; FI-Lab; Combined (Clinical+Lab)	Serum (multiplex)	↔	↔	Kane et al., 2019
	C57BL/6 mice (Voluntary aerobic exercise)	M & F; 21-26 months	Clinical FI	Serum (multiplex)	↔	↔	Bisset et al., 2022
	C57BL/6J mice (alpha-ketoglutarate (AKG) treatment)	M & F; 24-31 months	Clinical FI	Plasma (multiplex)	↔	↔	Shahmirzadi et al., 2020
	C57BL/6 mice (ACE-inhibitor enalapril treatment)	M (13, 25 months) & F (13, 22 months)	Clinical FI	Serum (multiplex)	↔	↔	Keller et al., 2019
	C57BL/6 mice (JAK inhibitor ruxolitinib treatment)	M; 24 months	Physical functional assessments (activity, grip strength, hanging time, Rotarod coordination test)	Serum (multiplex)	↑	N/A	Xu et al., 2015
IFN-γ	C57BL/6 mice	M & F; 17, 23 months	Clinical FI; FI-Lab; Combined (Clinical+Lab)	Serum (multiplex)	↔	↑	Kane et al., 2019
	C57BL/6 mice (Voluntary aerobic exercise)	M & F; 21-26 months	Clinical FI	Serum (multiplex)	↔	↓	Bisset et al., 2022
	C57BL/6J mice (alpha-ketoglutarate (AKG) treatment)	M & F; 24-31 months	Clinical FI	Plasma (multiplex)	↔	↔	Shahmirzadi et al., 2020
	C57BL/6 mice (ACE-	M (13, 25	Clinical FI	Serum	↔	↔	Keller et al.,

Cytokine	Species / strain / model / intervention	Sex / age	Frailty assessment	Tissue / method	Relationship with frailty (Males)	Relationship with frailty (Females)	Reference
	inhibitor enalapril treatment)	months) & F (13, 22 months)		(multiplex)			2019
	C57BL/6 mice (sodium nitrite supplementation)	M; 20-26 months	Grip strength, locomotor activity, Rotarod (constant speed and accelerating)	Skeletal muscle (ELISA)	↑	N/A	Justice et al., 2015
MCP-1	C57BL/6 mice	M & F; 17, 23 months	Clinical FI; FI-Lab; Combined (Clinical+Lab)	Serum (multiplex)	↔	↔	Kane et al., 2019
	C57BL/6 mice (Voluntary aerobic exercise)	M & F; 21-26 months	Clinical FI	Serum (multiplex)	↔	↔	Bisset et al., 2022
	C57BL/6J mice (alpha-ketoglutarate (AKG) treatment)	M & F; 24-31 months	Clinical FI	Plasma (multiplex)	↔	↑	Shahmirzadi et al., 2020
	C57BL/6 mice (ACE-inhibitor enalapril treatment)	M (13, 25 months) & F (13, 22 months)	Clinical FI	Serum (multiplex)	↔	↑ (22 months)	Keller et al., 2019
	C57BL/6 mice (Senolytic treatment with dasatinib & quercetin)	M & F; 24 months	Physical function assessment (speed, endurance, grip strength, activity, body weight), senescence evaluation	Visceral adipose tissue (qPCR)	↔	↔	Xu et al., 2018
M-CSF	C57BL/6J mice (alpha-ketoglutarate (AKG) treatment)	M & F; 24-31 months	Clinical FI	Plasma (multiplex)	↔	↔	Shahmirzadi et al., 2020
MIP-1α	C57BL/6 mice	M & F; 17, 23 months	Clinical FI; FI-Lab; Combined (Clinical+Lab)	Serum (multiplex)	↔	↔	Kane et al., 2019
	C57BL/6 mice	M & F; 21-26	Clinical FI	Serum	↔	↔	Bisset et al.,

Cytokine	Species / strain / model / intervention	Sex / age	Frailty assessment	Tissue / method	Relationship with frailty (Males)	Relationship with frailty (Females)	Reference
	(Voluntary aerobic exercise)	months		(multiplex)			2022
	C57BL/6J mice (alpha-ketoglutarate (AKG) treatment)	M & F; 24-31 months	Clinical FI	Plasma (multiplex)	↔	↔	Shahmirzadi et al., 2020
	C57BL/6 mice (ACE-inhibitor enalapril treatment)	M (13, 25 months) & F (13, 22 months)	Clinical FI	Serum (multiplex)	↔	↑ (22 months)	Keller et al., 2019
MIP-1β	C57BL/6 mice	M & F; 17, 23 months	Clinical FI; FI-Lab; Combined (Clinical+Lab)	Serum (multiplex)	↔	↔	Kane et al., 2019
	C57BL/6 mice (Voluntary aerobic exercise)	M & F; 21-26 months	Clinical FI	Serum (multiplex)	↔	↔	Bisset et al., 2022
	C57BL/6J mice (alpha-ketoglutarate (AKG) treatment)	M & F; 24-31 months	Clinical FI	Plasma (multiplex)	↔	↔	Shahmirzadi et al., 2020
	C57BL/6 mice (ACE-inhibitor enalapril treatment)	M (13, 25 months) & F (13, 22 months)	Clinical FI	Serum (multiplex)	↔	↔	Keller et al., 2019
	C57BL/6 mice (JAK inhibitor ruxolitinib treatment)	M; 24 months	Physical functional assessments (activity, grip strength, hanging time, Rotarod coordination test)	Serum (multiplex)	↑	N/A	Xu et al., 2015
MIP-2	C57BL/6J mice (alpha-ketoglutarate (AKG) treatment)	M & F; 24-31 months	Clinical FI	Plasma (multiplex)	↔	↔	Shahmirzadi et al., 2020
RANTES	C57BL/6 mice	M & F; 17, 23 months	Clinical FI; FI-Lab; Combined (Clinical+Lab)	Serum (multiplex)	↔	↔	Kane et al., 2019

Cytokine	Species / strain / model / intervention	Sex / age	Frailty assessment	Tissue / method	Relationship with frailty (Males)	Relationship with frailty (Females)	Reference
	C57BL/6 mice (Voluntary aerobic exercise)	M & F; 21-26 months	Clinical FI	Serum (multiplex)	↔	↔	Bisset et al., 2022
	C57BL/6J mice (alpha-ketoglutarate (AKG) treatment)	M & F; 24-31 months	Clinical FI	Plasma (multiplex)	↔	↔	Shahmirzadi et al., 2020
	C57BL/6 mice (ACE-inhibitor enalapril treatment)	M (13, 25 months) & F (13, 22 months)	Clinical FI	Serum (multiplex)	↔	↔	Keller et al., 2019
TNF-α	C57BL/6 mice	M & F; 17, 23 months	Clinical FI; FI-Lab; Combined (Clinical+Lab)	Serum (multiplex)	↔	↔	Kane et al., 2019
	C57BL/6 mice (Voluntary aerobic exercise)	M & F; 21-26 months	Clinical FI	Serum (multiplex)	↔	↔	Bisset et al., 2022
	C57BL/6J mice (alpha-ketoglutarate (AKG) treatment)	M & F; 24-31 months	Clinical FI	Plasma (multiplex)	↔	↔	Shahmirzadi et al., 2020
	C57BL/6 mice (ACE-inhibitor enalapril treatment)	M (13, 25 months) & F (13, 22 months)	Clinical FI	Serum (multiplex)	↔	↔	Keller et al., 2019
	C57BL/6 mice (JAK inhibitor ruxolitinib treatment)	M; 24 months	Physical functional assessments (activity, grip strength, hanging time, Rotarod coordination test)	Adipose (qPCR)	↑	N/A	Xu et al., 2015
	C57BL/6 mice (sodium nitrite supplementation)	M; 20-26 months	Grip strength, locomotor activity, Rotarod (constant speed and accelerating)	Skeletal muscle (ELISA)	↑	N/A	Justice et al., 2015
	C57BL/6 mice	M; 5-7 months	Physical function	Skeletal muscle	↑	N/A	Xu et al., 2018

Cytokine	Species / strain / model / intervention	Sex / age	Frailty assessment	Tissue / method	Relationship with frailty (Males)	Relationship with frailty (Females)	Reference
	(Senescence induction (radiation and transplantation)		assessment (speed, endurance, grip strength, activity, body weight), senescence evaluation	(qPCR)			

Notes: ACE = angiotensin converting enzyme; CCL = C-C motif chemokine ligand; CXCL = C-X-C motif chemokine ligand; G-CSF = Granulocyte colony stimulating factor; GM-CSF = Granulocyte-macrophage colony-stimulating factor; IFN = interferon; IL = Interleukin; KC = Keratinocyte-derived chemokine; MCP = Monocyte chemoattractant protein; MIP = Macrophage inflammatory protein; RANTES = regulated on activation, normal T cell expressed and secreted; TNF = Tumour necrosis factor. Symbol meaning: ↑ = proportional relationship with frailty; ↓ = inversely proportional with frailty; ↔ = no relationship with frailty. M= Males; F = Females

Table 3.4 Phenotypic Characteristics of the Interleukin-10 knockout (IL-10tm/tm) mouse frailty model

Tissue / system	Age (months)	Sex	Frailty / Functional Assessment	Major Findings - Phenotype	Reference
Plasma / serum	2 vs. 12	F	Grip strength & activity levels.	• IL-6 levels ↑ with age in IL-10$^{tm/tm}$ mice when compared to age-matched WT controls and to younger IL-10$^{tm/tm}$ mice. • Grip strength & activity levels ↓ with age in IL-10$^{tm/tm}$ mice compared to WT controls.	Walston et al., 2008
	3 vs. 5	F & M	Not assessed.	• ↑ in circulating levels of IFN- γ, decrease in body weight and bone mass relative to WT littermates at both ages. • Similar results seen in both sexes.	Pini et al., 2009
	21 months	F & M	Mortality.	• Serum levels of pro-inflammatory cytokines (IL-6, IL-1β, TNF-α, IFN-γ) and the chemokine KC ↑ in aged IL-10$^{tm/tm}$ mice compared to WT with ↔ in IL-12p70 (pro-inflammatory). • Mortality is ↑ in IL-10$^{tm/tm}$ mice. • Similar results seen in both sexes.	Ko et al., 2012
	3, 10 & 20	F	Activity and VO_2 levels.	• Adiponectin, leptin, fat mass and metabolic rate ↓ with age in IL-10$^{tm/tm}$ mice relative to WT controls, while IL-6 ↑. • Insulin sensitivity and glucose homeostasis is ↔ in older WT and IL-10$^{tm/tm}$ mice. • Activity ↔ in WT & IL-10$^{tm/tm}$ mice at all ages • VO_2 ↓ with age in IL-10$^{tm/tm}$ mice.	Westbrook et al., 2017
	3 vs 21	F & M	Not assessed.	• Cold stress (4°C, 5 hrs) ↑ corticosterone (1° mouse corticosteroid; anti-inflammatory hormone), ↓ TNFR1 and does not affect IL-6. • Effect on TNFR1 exacerbated in IL-10$^{tm/tm}$ mice. • No effect of age, but corticosterone is ↑ in males relative to females.	Ge et al., 2020
	1.5-5.5	M	FI, rotarod, grip strength, endurance, and frailty index.	• Serum myostatin ↓ in IL-10$^{tm/tm}$ mice treated with rapamycin (1.5 mg/kg/day in water), maraviroc (300 mg/L in water) or rapamycin plus maraviroc to reduce expression of chemokine receptor type 5. Serum IL-1β, IL-6, or TNF-α were not affected by treatment. • No effect of treatment on frailty or function.	Pérez-Martínez et al., 2020
	18-23	F & M	Treadmill running, falls and mortality.	• Deletion of anti-inflammatory IL-10 and pro-inflammatory IL-6 (IL-10$^{tm/tm}$/IL-6$^{tm/tm}$ mouse) increased levels of protective mitochondrial-	Ma et al., 2021

Tissue / system	Age (months)	Sex	Frailty / Functional Assessment	Major Findings - Phenotype	Reference
				associated lipid metabolites. (lysophosphatidylcholine compounds) when compared to WT and IL-10$^{tm/tm}$ mice. • IL-6 deletion in IL-10$^{tm/tm}$ mice ↓ falls, ↑ running distance and ↑ mortality. • Sex differences unclear.	
Skeletal muscle	2 vs. 12	F	Grip strength & activity levels.	• Differential expression of genes related to mitochondria and apoptosis in skeletal muscle between older IL-10$^{tm/tm}$ mice and age-matched WT controls. • No expression differences between young and old IL-10$^{tm/tm}$ mice. • Grip strength & activity levels ↓ with age in IL-10$^{tm/tm}$ mice compared to WT controls.	Walston et al., 2008
	21	M	Not assessed.	• Skeletal muscle energy metabolism is ↓ in IL-10$^{tm/tm}$ mice, with ↓ ATP synthesis, ↓ high-energy phosphate levels and ↓ energy release by ATP hydrolysis.	Akki et al., 2014
	3-5 vs. 22-24	F	Not assessed.	• Advanced age, especially in IL-10$^{tm/tm}$ mice, ↑ mitochondrial damage and alters mitophagy in muscle.	Ko et al., 2016
	1.5 vs. 3	F	Not assessed.	• Muscle atrophy, inflammasome formation and increased NF-κB signalling are ↓ in IL-10$^{tm/tm}$ mice treated 0.1% grape seed extract (antioxidant/anti-inflammatory compound).	Wang et al., 2014
	5-7 vs. 21-26	M	Not assessed.	• MIF (anti-apoptotic cytokine) and Nix (pro-apoptotic mitochondrial protein) are co-localized in muscle from aged IL-10$^{tm/tm}$ mice. • MIF and Nix crosstalk may promote myopathy.	Abadir et al., 2017
	1.5-5.5	M	FI, rotarod, grip strength, endurance and frailty index.	• Muscle myostatin ↓ in IL-10$^{tm/tm}$ mice treated with rapamycin (1.5 mg/kg/day in drinking water) or rapamycin plus maraviroc (300 mg/L in water) to ↓ expression of CCR5. • IL-1β and CCR5 levels ↓ in muscle with treatment. • No effect of treatment on frailty or function.	Pérez-Martínez et al., 2020
Heart	3-4 vs. >9	Unclear	Echocardio-graphy	• Hearts are larger (↑ mass, dilation) in IL-10$^{tm/tm}$ mice compared to WT mice. • Evidence of systolic and diastolic impairment, especially in older mice.	Sikka et al., 2013
	18-23	F & M	Treadmill running, falls, and mortality.	• IL-10$^{tm/tm}$ mice have impaired myocardial oxidative phosphorylation. • Myocardial mitochondrial metabolism is normalized to levels seen in WT	Ma et al., 2021

Tissue / system	Age (months)	Sex	Frailty / Functional Assessment	Major Findings - Phenotype	Reference
				controls in the IL-$10^{tm/tm}$/IL-$6^{tm/tm}$ double knockout mice. • IL-6 deletion in IL-$10^{tm/tm}$ mice ↓ falls, ↑ running distance and ↑ mortality. • Sex differences unclear.	
Vasculature	3-4 vs. >9	Unclear	Blood pressure, pulse wave velocity	• Blood pressure and pulse wave velocity (indicates stiffness) is ↑ in IL-$10^{tm/tm}$ mice compared to WT controls. • Vascular endothelial-mediated relaxation is impaired in aortas from IL-$10^{tm/tm}$ mice.	Sikka et al., 2013
Visceral adipose tissue	8-9	F & M, pooled	Not assessed in IL-$10^{tm/tm}$ mice.	• IL-$10^{tm/tm}$ mice have more senescent cells than WT controls.	Xu et al., 2018

Abbreviations: Chemokine (C-X-C motif) ligand 1 (KC); chemokine receptor type 5 (CCR5); frailty Index (FI); interferon gamma (IFN-γ); interleukin 1 beta (IL-1β); interleukin-10 knockout (IL-$10^{tm/tm}$); interleukin 6 (IL-6); interleukin-6 knockout (IL-$6^{tm/tm}$); macrophage migration inhibitory factor (MIF); NIP3-like protein X (Nix); nuclear factor kappa B (NF-κB); tumor necrosis factor-alpha (TNF-α); tumor necrosis factor receptor 1 (TNFR1); wildtype (WT). M= Males; F = Females

3.8 Figures

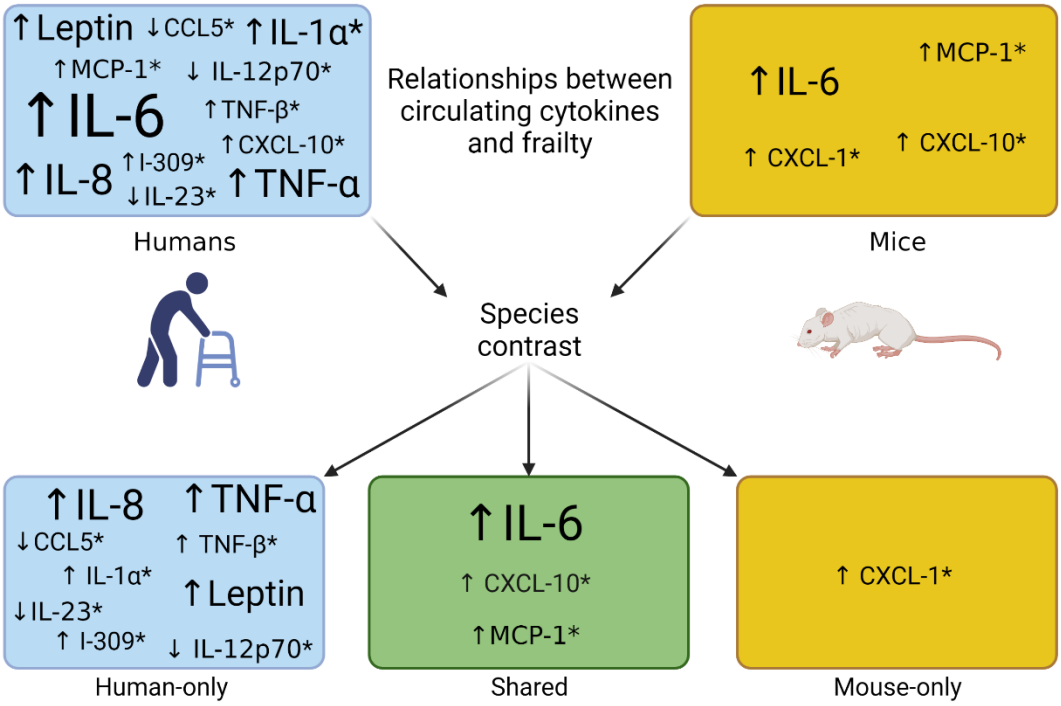


Figure 3.1 Relationships between frailty and circulating cytokines. Analytes were included if ≥50% of reviewed studies reported the same directional relationship. Font sizes denote relative supporting evidence. *Denotes analytes either for which 50% of studies showed the same directional relationship with frailty or with promising but limited evidence supporting the link. Created in BioRender.com.

Chapter 4 Testosterone's impact on frailty in intact or gonadectomized older male mice

Disclaimer: Some of the results and text presented in this chapter have been published previously in Heinze-Milne, Banga, Godin et al., 2022. As per the Journal Author Rights of the publisher, Elsevier, I retain and exercise the rights to include published content in a dissertation not published commercially (see Appendix C). See Appendix A for author contributions.

4.1 Introduction

Chapter 3 describes the putative molecular mechanisms of frailty. That chapter also highlights two major mechanisms of frailty that relate to such molecular changes: 1) sarcopenic signs like muscle weakness and wasting and 2) elevated, chronic low-grade systemic inflammation. Low circulating testosterone levels in men relate to detrimental changes in both entities, suggesting that low testosterone can contribute to frailty. Existing clinical studies that observationally investigate the relationship between frailty and circulating testosterone levels in humans lack a mechanistic focus. Further, androgen deprivation studies that investigate frailty (or its determinants) in older men are confounded by the primary indication of deprivation, like prostate cancer. Thus, preclinical models enable the study of how chronically low testosterone levels impact frailty progression in older but otherwise healthy animals. In this chapter, we use a preclinical model to investigate how gonadectomy (removal of testicles), or a natural age-related decline in testosterone, relates to frailty over a 6-month study using older male C57BL/6 mice.

4.2 Low testosterone, disease risk, and frailty

There is evidence that circulating testosterone levels in men can decline at about 0.4% per year between 45-80 years of age, as reported from the Massachusetts Male Aging Study which included 2769 observations from 1532 men (median age=58 years; Travison et al., 2007). However, this is not always the case, as a report from the Health in Men study reported no age-related difference in circulating testosterone levels in 4165 men with a mean age of 77 years (range=65-83 years; Yeap et al., 2007). A study

combining results from several databases of healthy men (n=10098 males aged 3-88 years) suggested that circulating testosterone levels on average rise rapidly between the second and third decade of life before remaining somewhat stable into old age (Kelsey et al., 2014). However, an observation of that dataset reveals that the *risk* of having lower testosterone increases with age (*i.e.*, mean levels are not different over time, although higher percentages of older healthy men have low testosterone). A reconciliation between Kelsey et al.'s study and the Massachusetts Male Aging Study (Travison et al., 2007) is that disease state and testosterone are inversely related. Indeed, the Massachusetts Male Aging Study released a report in 1991 stratifying men as "healthy" (*i.e.*, non-obese, non-excessive alcohol intake, no chronic disease) or "less healthy" (*i.e.*, having at least one of those conditions). The "healthy" group had 14% higher serum testosterone levels than the "less-healthy" group, which was not solely accounted for by a small but significant difference in age (Gray et al., 1991). The finding that age-related disease states are linked with lower testosterone levels has been replicated more recently, including in diabetes mellitus (Dhindsa et al., 2004), metabolic syndrome (Blouin et al., 2005), and obesity (Svartberg et al., 2004), and can even be predictive of heart failure (Schäfer et al., 2021). Thus, age-related declines in testosterone appear minimal in healthy populations, but testosterone levels tend to be lower in metabolic and other disease states and relate with the presence of disease.

For these reasons, there is considerable interest in the role low testosterone plays in frailty progression in aging men. Low testosterone has been theoretically linked with frailty in part due to its association with age-related adverse health outcomes, including excess adiposity, poor physical function, impaired cognition, and heart problems (Yeap et al., 2012; Blava et al., 2017; Yabluchanskiy & Tsitouras, 2019; Kloner et al., 2016; Kirby et al., 2019). It is therefore possible that age- or disease-related declines in physiologic levels of testosterone may promote frailty progression (Yabluchanskiy & Tsitouras, 2019; Saad et al., 2017; and O'Connell et al., 2011). There is evidence in clinical literature that frailty and testosterone indeed relate to one another. In a report from the European Male Aging Study, 3219 men aged 40-79 years (mean=59.7) had their frailty levels assessed using a 43-item FI and fasting serum testosterone measured by gas chromatography in tandem with mass spectrometry (Tajar et al., 2011). After adjusting for age and three

other confounders, higher serum testosterone levels (total and free levels) were significantly associated with lower frailty scores (Tajar et al., 2011). For reference, "free" testosterone levels denote any testosterone not bound to blood borne proteins (*e.g.*, sex hormone binding globulin or albumin; Keevil & Adaway, 2019).

Longitudinal data in a report from the Concord Health and Ageing in Men Project, a cohort study from Australia, also provides illuminating data. Men aged 70 years or older (mean=77 years) participated in this study (n=1645), which investigated how serum testosterone levels related to two modified versions of the FP (Travison et al., 2011). Their age-adjusted analysis showed that higher total and free testosterone levels were related to lower frailty levels (Travison et al., 2011). Notably, they also demonstrated that the decreases in testosterone during a repeated measures follow-up assessment (~2 years later) significantly related to increased frailty scores (Travison et al., 2011). This suggested that frailty and testosterone correlate longitudinally, strengthening the suggestion of causality. A report from the Toledo Study for Health Aging contained similar findings, where 552 men (mean age=74 years) had their serum testosterone compared with their frailty score, assessed via Fried's FP (Carcaillon et al., 2012). Lower testosterone levels were related to higher frailty scores, even after adjusting for age and several sociodemographic characteristics and comorbidities (Carcaillon et al., 2012). This study and others were meta-analyzed in a systematic review relating endogenous serum testosterone to the risk of being frail (Peng et al., 2022). Four of the eight studies included in the meta-analysis reported a significant odds ratio between the presence of physical frailty and lower testosterone levels (Peng et al., 2022). The weight of these studies in the analysis was 59.45%. Overall, the meta-analysis reported that lower testosterone levels did relate to higher physical frailty.

However, as may be gleaned from the relative weight of studies that report an association between low testosterone and frailty, not all studies agree. An analysis of data from the Massachusetts Male Aging Study reported that lower testosterone levels did not relate to frailty, assessed using a modified FP approach (Mohr et al., 2007). That study included 646 men whose mean age was 68 years. Eichholzer et al. (2012) also reported no relationship between FP score and serum testosterone in a sub-population of 461 men in

the Third National Health and Nutrition Evaluation Survey aged 60 years or older. Thus, there is evidence that frailty and testosterone do not always correlate cross-sectionally in older men. A longitudinal follow-up study from the European Male Ageing Study (the same study group from Tajar et al., 2011) investigated whether baseline serum testosterone levels predicted a change in frailty scores using both the FI and FP approaches (Swiecicka et al., 2018). After adjusting for age, lower baseline testosterone levels significantly predicted an increase in FI score but not the FP score. This was interesting, given the relationship between lower testosterone and poorer physical function. However, the relationship between testosterone and change in FI score held after adjusting for smoking but disappeared after adjusting for body mass index.[6] Thus, several clinical studies did not find an independent relationship between serum testosterone and frailty levels. There is also little evidence in preclinical models supporting a connection between frailty and low testosterone. One possible link is that mice with low testosterone tend to have lower overall muscle mass and higher body fat, although frailty was not measured in these mice (Pan et al., 2016). There is therefore still no consensus regarding whether low levels of testosterone contribute to frailty progression either clinically or preclinically and the evidence has been called weak, at best (Hsu et al., 2018). On balance, however, there arguably is a relationship. Whether this relationship is causal remains unknown and may in part be answered by the rigorous experimental control offered by preclinical research.

The possible relationship between low testosterone and higher frailty levels likely relate to two mechanisms of frailty: sarcopenia and chronic inflammation. Lower levels of testosterone are related to higher (detrimental) levels of both mechanisms. These two mechanisms of frailty and their relationship with endogenous testosterone are reviewed

[6] For critical thought purposes, it is worthwhile to reflect on the value of adjusting for body mass index here. Is the effect of testosterone on frailty diluted by this adjustment? Body mass index indubitably relates to blood borne testosterone (*e.g.*, Eriksson et al., 2017). Epidemiologically speaking, higher body mass indices = lower serum testosterone. Further, the relationship between testosterone and obesity, a key determinant of body mass index, is bidirectional (Kelly & Jones, 2015), with low testosterone contributing to higher body mass indices. Now, consider the fact that higher body mass indices relate to higher frailty scores (Hubbard et al., 2010). By adjusting for body mass index when predicting frailty from testosterone, we risk diluting the true theoretical relationship between testosterone and frailty. I.e., if higher body mass indices are partially caused by low testosterone, are we not losing valuable, causal predictive power? This possible issue can otherwise be referred to as "overadjustment", which can be a critical logical error in inferring causality (Schistermann et al., 2009).

below, before presenting a study rationale and hypothesis regarding an investigation into how low testosterone relates to frailty in a preclinical mouse model.

4.2.1 Mechanism: Low testosterone and sarcopenia and sarcopenic symptoms

There is good evidence that having low levels of circulating testosterone can predispose poor physical fitness and muscle wasting, which can lead to frailty. For example, serum testosterone levels were inversely related to aerobic fitness levels in the Cooper Center Longitudinal Study (DeFina et al., 2018). This was found without any age-related declines in testosterone in their study population of 3974 men aged 50-79 years (mean=58 years). In addition to aerobic fitness, muscular strength and functional abilities also negatively related to testosterone (DeFina et al., 2018). A study from the Concord Health and Ageing in Men study reported that lower testosterone levels related to increased functional decline (measured as an activity of daily living questionnaire) and reduced handgrip strength (Hsu et al., 2014). A longitudinal study from Amsterdam involving 596 men aged 65-88 years reported that lower testosterone levels related to reduced handgrip strength physical performance (a test battery of activities of daily living) and self-reported functional limitations (Schaap et al., 2005). The relationship between testosterone and physical performance and functional limitations disappeared when adjusting for six confounding variables, although strength remained significant (Schaap et al., 2005). Similar results have been reported from a study completed in Japan, which reported that older men with higher testosterone levels had stronger grip strengths and a faster walking speed during a walking balance test (Auyeung et al., 2011). However, a study involving 1183 older men (mean age=72 years) reported that lower testosterone levels were not predictive of impaired physical performance using a walking balance test, leg power, or change in 6-minute walking speed, although they were related to better sit-to-stand time (LeBlanc et al., 2011). Similarly, Araujo et al. (2008) reported no correlation between testosterone and three different physical performance measures in 810 men aged 30-79 years (mean=46), casting doubt on the relationships. Thus, lower testosterone levels into old age can accompany age-related declines in muscular strength and physical fitness and performance, although this is not always found.

Besides muscular strength, the other aspect of sarcopenia is muscle loss. Lower

levels of testosterone parallel declines in muscle mass too, as they do with muscular strength. This aligns with the correlative relationship described between muscular strength and size presented in Chapter 3. For instance, analyses from the Health and Ageing in Men study mentioned in the previous paragraph found that lower testosterone levels related to lower muscle mass in arms and legs (Hsu et al., 2014). This was after adjusting for age and body mass index. Auyeung et al. (2011) also reported a strong independent relationship between lower testosterone levels and lower appendicular (*i.e.*, arm and leg) skeletal muscle mass (although this did not translate to improved physical performance). On the other hand, not all studies report significant relationships between total lean mass and testosterone. In a study of 3014 Swedish men with a mean age of 75 years testosterone actually correlated negatively with lean mass but became be non-significant once adjusting for estradiol (Vanderput et al., 2010). These results followed an earlier study by the same study group, which evaluated 2587 American men (mean age=73 years) and reported no relationship between serum testosterone levels and lean body mass (Orwoll et al., 2006). Overall, low testosterone levels in older men tend to relate to impairments in physical frailty, such as muscle mass, muscular strength and walking speed to a certain degree (Kaufman & Lapauw, 2020).

The relationship between low testosterone and reduced muscle mass and strength has also been shown in a mouse model. Male C57BL/6 mice that were castrated between 6-9-months of age showed a significant reduction in body weight within two months, which was accompanied by lowered skeletal muscle mass of six muscles, and reduced grip strength (Pan et al., 2016). There is therefore evidence suggesting that lower testosterone levels negatively relate to lower appendicular lean mass and muscular strength in older humans and in a preclinical mouse model.

4.2.2 Mechanism: Low testosterone and systemic inflammation

Chronically elevated circulating inflammatory markers are linked to many non-infectious diseases and are linked with frailty as a potential mechanism. This was reviewed in Chapter 3. Interestingly, there is evidence that low circulating levels of testosterone may enable this low-grade pro-inflammatory state. Specifically, low levels of testosterone relate to increased pro-inflammatory cytokines and reduced levels of anti-

inflammatory cytokines that relate to frailty in humans (Mohamad et al., 2019). Such associations between low testosterone levels and higher circulating pro-inflammatory markers have been reported not merely in older men (*e.g.*, Malkin et al., 2004) but also slightly younger men (*e.g.*, Bobjer et al., 2013). The mean ages of the participants within these studies were 62 and 37 years, respectively, which suggests to a certain degree there is an age-independent relationship.

Higher levels of the pro-inflammatory cytokine IL-6 have been most often related to increased frailty in humans (Table 3.1), which makes it an important analyte to consider. In a study of men aged 18-79 years (mean=39) lower serum levels of free testosterone were related to higher serum IL-6 concentrations (Grandys et al., 2021). Levels of total testosterone were not as closely related, although the relationship was still negative (r=-0.11). Further, lower serum testosterone levels were significantly related to elevated soluble IL-6 receptors (a major receptor for IL-6 throughout the body (Mihara et al., 2012)) in the blood of older Italian men with a mean age of 75 years (Maggio et al., 2006). Interestingly, a trial investigating the effects of acute testosterone withdrawal on circulating IL-6 levels suggests an important causal link. Fourteen men with idiopathic hypogonadotropic hypogonadism (*i.e.*, insufficient pituitary stimulation of the testicles to create testosterone) underwent a two-week deprivation of testosterone-raising treatment (Yialamas et al., 2007). Circulating levels of IL-6 rose significantly over the two-weeks of androgen deprivation, which strengthens the likelihood of a causal correlation between the two (Yialamas et al., 2007). A strong negative relationship between circulating testosterone levels and IL-6 has been reported in Type II diabetes patients too (Kapoor et al., 2007). These results are notable, given the very strong associations between IL-6 and frailty. However, IL-6 does not always correlate with testosterone and circulating IL-6 such as in the study by Maggio et al. (2006) mentioned earlier.

Other inflammatory markers associated with frailty have been linked to low testosterone levels. Bobjer et al. (2013) reported an inverse relationship between testosterone and TNF-α, which has been related to frailty in humans (Table 3.1). Higher levels of IL-1β have also been associated with lower testosterone levels, as reported in a study of coronary artery disease patients stratified by circulating testosterone (Nettleship

et al., 2007). However, they reported no relationship between testosterone and IL-6 or TNF-α (Nettleship et al., 2007). Further, TNF-α levels were unrelated to testosterone levels in the study by Maggio et al. (2006), muddying the estimated relationships between circulating inflammatory markers and testosterone levels. Testosterone may influence IL-10 expression as well, which is a potent anti-inflammatory cytokine. In a Brazilian study that focused on differences in IL-10 levels between untrained and endurance trained middle-aged men (mean=51 years), they reported that higher testosterone levels were related to higher IL-10 levels (Gutierrez et al., 2021). Although this study has the modifying effect of fitness, it does suggest that putative strategies to manage frailty (*i.e.*, exercise) can improve circulating IL-10 levels concomitantly with testosterone. However, it must be noted that IL-10 levels were often unrelated to frailty in humans (Table 3.1), which suggests that any impact of testosterone on IL-10 expression may have negligible relationships to frailty. Moderating impacts of testosterone on IL-10 expression and subsequent inflammation are speculative but possible.

There is also preclinical evidence suggesting that low testosterone levels relate to higher levels of chronic inflammation. Serum IL-6 levels were higher after gonadectomy in C57Bl/6 mice 3- and 28-days post-surgery compared to sham operated controls (Zhang et al., 1998). A similar study using 3-month-old gonadectomized male Sprague-Dawley rats also reported higher serum IL-6 levels 8-weeks post-surgery compared to sham operated rats, although no effect of gonadectomy on IL-1α, IL-1β, or TNF-α was noted (Chin & Ima-Nirwana, 2016). On the other hand, treatment with testosterone led to lower circulating IL-6 levels in genetically feminized mice (Kelly et al., 2012). Suppression of chronic inflammation in gonadectomized male rodents is not always found, however, as Kelly et al. (2012) reported no change in IL-1β, IL-10, MCP-1, and TNF-α, and another study reported no change in IL-1α, IL-1β, IL-6, and TNF-α in gonadectomized Sprague-Dawley rats after treatment with testosterone enanthate for 8-weeks (Chin & Ima-Nirwana, 2016). In summary, circulating pro-inflammatory mediators that associate with frailty in humans sometimes relate to low circulating androgen levels in human males. The implication of low testosterone on these relationships and subsequent frailty is unknown, but low testosterone may play a role in promoting chronic low-grade inflammation to a degree. In preclinical models, similar trends emerge, whereby low

testosterone levels sometimes relate to higher circulating inflammatory markers.

4.3 Rationale and hypothesis

Sarcopenia and chronic circulating inflammatory markers are putative mechanisms of frailty that relate to low testosterone levels. Artificial suppression of testosterone levels in healthy humans is uncommon, which leaves important mechanistic questions about how low testosterone affects frailty unanswered. Animal models provide a unique opportunity to investigate controlled low testosterone levels and frailty. The goal of this study was to investigate the relationship between serum testosterone levels and frailty during a longitudinal analysis using older male mice with or without lifelong testosterone deficiency. Older mice were chosen in order to most accurately model the portion of the human male lifespan when low testosterone may be a concern regarding frailty, as testosterone deficiency is rare during early adulthood (Kelsey et al., 2014). The primary objective was to determine the relationship between serum testosterone levels and frailty in mice using an FI. Secondary objectives were to examine the impact of lifelong low testosterone levels on estimated healthspan and lifespan using the Frailty Inferred Geriatric Health Timeline (FRIGHT) and Analysis of Frailty and Death (AFRAID) clocks (Schultz et al., 2020), which use the FI scores to infer biologic age and remaining lifespan. Mechanisms of frailty including lean mass loss and circulating inflammatory markers were also investigated, because of their association with testosterone levels. It was theorized that lifelong or any age-related testosterone deficiency would exacerbate frailty progression compared to mice with regular testosterone levels. Here we tested the null hypothesis that there would be no differences in frailty progression between mice with low or normal levels of testosterone.

4.4 Methods

4.4.1 Mice: Study protocols were approved by the Dalhousie University Committee on Laboratory Animals and were performed within the guidelines of the Canadian Council on Animal Care. C57Bl/6 mice were purchased from Charles River (St. Constant, Quebec) and were assessed longitudinally after undergoing either a sham surgery or a gonadectomy (GDX) at four-weeks of age. This early insult to physiologic levels of testosterone was done to ensure a substantial difference in physiologic levels of

testosterone to maximize the intervention's impact. During sham surgeries, mice were anaesthetized and surgically prepared identically to the GDX mice, except their testes were not removed.

Mice were housed together (≤5 mice/cage) on a 12-hour day and night cycle (21ºC, 35% humidity) in Dalhousie University's animal care facilities. Ten sham and 11 GDX mice were investigated longitudinally from 18- to 24-months of age. This timeframe was chosen because it enabled longitudinal analysis of a significant portion of the mouse's older adult lifespan. However, mice were co-enrolled in a different study that ended at 24-months for tissue collection, making 6-months the maximum duration of the study (Banga et al., 2021). Mice were euthanized with an overdose of sodium pentobarbital (200 mg/kg i.p.) *ad hoc* based on humane endpoints (*e.g.*, uncontrolled tumor growth) or at study completion. Causes for euthanasia are listed in Table 4.1.

4.4.2 Frailty scoring: A 31-item non-invasive and validated mouse clinical FI was used. A full description of the tool can be found here (Whitehead et al., 2014). In brief, the FI assessed several bodily systems (*e.g.*, integument, musculoskeletal systems) as well as the body weight and body surface temperature. Raters were not blinded for the frailty assessments. Of note, blinding would be difficult, given that GDX mice were often visually different than sham mice (*i.e.*, their coats were darker and sleeker). All items except body weight and temperature were scored as: 0=no deficit, 0.5=mild, and 1=severe. Weight and temperature were scored based on how many standard deviations the measure was from the group mean within a timepoint. The sum of all deficits was divided by the number of items assessed (*i.e.*, 31 for the non-invasive FI used herein) to yield an FI score between 0 and 1. The FRIGHT and AFRAID clocks were used to estimate biologic age and estimated life expectancy, respectively (Schultz et al., 2020). Briefly, the FRIGHT clock uses 21 items from the FI described above that best correlate with age and predicts the chronologic age of the mice. The AFRAID clock uses 32 items, also derived from the FI, to predict lifespan. Both clocks are sensitive to healthspan/lifespan-increasing interventions, as shown previously (Schultz et al., 2020) and were thus employed here to estimate differences between sham and GDX mice.

4.4.3 Testosterone measurement: To collect blood samples, mice were first

anaesthetized using 2-3% isoflurane. Then, blood from the facial vein was collected in a microtube following a puncture by a 23-gauge needle. Blood was left undisturbed for 30 minutes then centrifuged at 1500g for 10 minutes. Serum was stored at -20ºC. Enzyme-linked immunosorbent assays were used to measure total serum testosterone levels as per the manufacturer's instructions (Crystal Chem, IL, USA). Absorbance was read at 450 nm with a reference reading of 630 nm and a standard curve was created using a 4-parameter logistic curve from eight standards, from which sample concentrations were extracted. Testosterone values for mice used in this study have been reported in supplemental data in a previous publication (Banga et al., 2021), where they were used to investigate separate outcomes (*e.g.,* echocardiography measures).

4.4.4 Body composition

Mice had their body composition measured using DEXA scans while still anaesthetized following the blood draw (Chapter 4.3.3). Mice were positioned prone on a machine specifically designed to perform dual-energy x-ray absorptiometry on small animals (General Electric Lunar PIXImus, WI, USA). Throughout the recording mice were kept warm by a lamp and anesthesia was maintained using 1-2% isoflurane. The skull was removed from the image analysis to focus on measuring fat and lean mass of the torso and limbs. Percent fat mass and percent lean mass were calculated relative to body mass by the Lunar PIXImus software.

4.4.5 Circulating cytokine measurements: Serum cytokine levels were quantified using a Luminex sandwich immunoassay that multiplexed 23 analytes using terminal cheek vein blood samples. Assays were performed as per the manufacturer's instructions and read using a MagPix multiplex system (BioRad). A 1:4 dilution was performed with sample diluent provided and 15µL of serum was used per mouse per well. Standard curves were created using a 4-paramater logistic curve from which sample concentrations were interpolated. A blank well was measured and subtracted from all readings to account for background noise. Samples were run in duplicate and averaged before analysis or curve fitting.

4.4.6 Statistics: Hierarchical linear regression was performed to test overall between-group differences with the statistical software *R* (version 4.0.1 Vienna, Austria) using the

'nlme' package. Nested analysis during hierarchical regression was at the individual mouse level. ß-coefficients and 95% confidence limits are provided for predictor variables in regression models. This statistical approach can evaluate effects on frailty without completely removing mice that died during the 6-months of the study. Cross-sectional analysis between sham and GDX mice were performed using unpaired t-tests with Holm-Bonferroni adjustments using α=0.05 and Cohen's *d* to describe effect size with Microsoft Excel (Microsoft Office, 2019; Redmond, WA) for FI scores and frailty clocks. Longitudinal analyses for body composition were performed using a restricted maximum likelihood model and cross-sectional analyses were Fisher's least significant difference tests performed using GraphPad Prism 8.4.2 (La Jolla, CA). Prism was also used to produce all graphs. Values are mean ± 95% confidence intervals unless otherwise specified.

4.5 Results

4.5.1 Artificially lowered testosterone did not potentiate frailty

Frailty: Many deficit differences emerged over time within the FI. Figure 4.1 depicts several elevated deficits in sham mice compared to GDX mice between 18- and 24-months of age. Notably, across timepoints there are statistically significant differences for eight individual deficits spanning four of the seven bodily systems investigated. When predicting overall frailty scores, sham mice in general were frailer than GDX mice according to hierarchical linear regression predicting FI from age and group, nested on individual mice (p=0.004; Figure 4.2A). Cross-sectional analyses revealed the older GDX mice actually had lower FI scores compared to sham animals at 18-, 20- and 23-months of age (Figure 4.2A; Table 4.2). Thus, early-life castration did not exacerbate frailty compared to intact animals and may have slowed its progression.

Testosterone's relationship with frailty: Serum testosterone levels were higher in sham mice at 18-months (p=4.34E-4; Figure 4.2B) and 20-months of age (p=0.013). However, testosterone levels in the sham mice began to decline at 20-months relative to 18-months. By 22-months, mean serum testosterone concentrations became similar to GDX mice (p=0.147) and remained so at 24-months (p=0.380). Hierarchical linear regression showed testosterone alone had little influence on frailty progression alone or after

adjustment for age (Table 4.3). Because serum testosterone levels declined dramatically in sham mice, group denomination (*i.e.*, sham and GDX) was added to the model. This analysis showed that age positively and strongly predicted frailty. By contrast, testosterone levels still did not relate to frailty (Table 4.3).

Frailty clocks: We then used another frailty-related measure, the FRIGHT clock, to investigate how testosterone deficiency affected estimated biologic age (Figure 4.2C). Estimated biological age (FRIGHT scores) increased over time ($p=0.002$) and estimated life expectancy (AFRAID scores) decreased over time ($p<0.0001$). There were no overall between-group differences between sham and GDX mice in group effects for the FRIGHT scores ($p=0.142$). The AFRAID clock was used to estimate how testosterone deficiency affected estimated lifespan (Figure 4.2D). One cross-sectional difference was noted in the AFRAID scores at 23-months, where GDX mice had a longer life expectancy than sham mice (Figure 2D; Table 4.3). However, there were no overall group differences in AFRAID scores between sham and GDX mice ($p=0.485$). Taken together, these findings suggest long-term testosterone deficiency did not substantially affect estimated biological age or estimated lifespan.

We then sought to investigate the relationship between testosterone and frailty, independent of gonadectomy. This was to test whether low testosterone predicted frailty in a natural state, without artificially lowering testosterone. To do this we compared frailty and testosterone within the 18- to 24-month-old intact mice only (Figure 4.3A). A hierarchical linear regression analysis concluded that age, not testosterone, contributed to frailty progression in intact mice (sham only model in Table 4.4). Similarly, there were no associations between serum testosterone levels and FRIGHT or AFRAID scores (Figures 4.3B & 4.3C). These results indicate that serum testosterone concentrations in aging sham and testosterone-deficient mice were not a strong predictor of frailty.

4.5.2 Body composition and frailty

Because decreases in lean mass and increases in fat mass are related to increased frailty status, we analysed the relationship between both measures and frailty in GDX and sham mice. Firstly, total lean mass was significantly lower in GDX mice compared to sham mice at 20-months ($p=0.017$), 22-months ($p=0.024$), and 24-months ($p=0.009$), as

shown in Figure 4.4A. On the other hand, differences in total fat mass were not significantly different between the groups (Figure 4.4B). These cross-sectional analyses were then converted to relative amounts to see if the GDX mice had proportionally different amounts of lean mass or fat mass. This was done by dividing the total mass of lean or fat mass by the total body mass, to create a percentage of lean mass or percentage of fat mass, respectively. No statistically significant cross-sectional differences were noted in percent lean mass and percent fat mass. Although, GDX mice arguably have lower percent lean mass and higher percent fat mass, but these differences were undetectable given the current statistical power (Figure 4.4C & D). To explore the association between body composition measures and frailty, all four parameters were regressed with frailty at the 20-month timepoint. This timepoint was chosen because it had the largest sample size and clear differences in testosterone, which otherwise diminished at 22- and 24-months (*e.g.*, Figure 4.2B). Of the four, only lean mass associated with frailty, with higher lean mass relating to higher frailty scores (Figure 4.4E-H).

To investigate the relationship between lean mass and frailty over time, sham and GDX mouse groups were pooled, and a longitudinal analysis was performed. A hierarchical regression analysis was chosen so all available data could be incorporated and increase statistical power above the cross-sectional analyses presented in Figure 4.4. The FI scores were predicted from body composition measures from all three body composition measurements nested at the individual mouse level. Regression coefficients for whole-number percentage points (*i.e.*, 10% = 10) are presented in Table 4.5 and confirms the relationship between lean mass and frailty in this sample, where higher lean masses related to higher frailty (denoted by the β coefficient, which is the slope value for the parameter). This relationship held true after adjusting for age (Table 4.5). On the other hand, fat mass was not as strongly related to frailty in this sample and was diminished after adjusting for age. Interestingly, higher percent lean mass was significantly related to frailty (but became non-significant after adjusting for age) and percent fat mass negatively related to frailty scores. Even after adjusting for age, lower percent fat masses were related to higher frailty scores (Table 4.5). In summary, this analysis suggests that older gonadectomized male mice have lower lean mass compared to intact controls, but

this lower lean mass related to more favorable frailty scores, as did higher relative fat mass.

4.5.3 Relationships between pro-inflammatory cytokines, testosterone, and frailty

A mechanism of frailty is low-grade chronic circulating inflammation. As such, we investigated the relationships between frailty scores and cytokines. Cytokines were measured from terminal blood draws for a sub-set of 17 mice (9 sham, 8 GDX) for which samples were available, which were compared with FI values at the closest timepoint to the blood draws. Arguably the most important cytokine of interest was IL-6, which associates with frailty in humans and mice (as discussed in Chapter 3). However, no relationship between frailty and IL-6 was observed (Table 4.6). No other cytokines were associated with frailty, either. Because low testosterone can predispose towards chronic circulating inflammation, the relationships between circulating pro-inflammatory cytokines and testosterone was also examined. No significant relationships were found between circulating testosterone levels and serum cytokines (Table 4.7).

In addition, the relationship between circulating cytokines and body composition was explored, because higher chronic inflammation has links to unfavorable body composition and muscle wasting (Tuttle et al., 2020). As shown in Figure 4.5 and reported in Table 4.8, the majority of cytokines related to whole-body lean mass, but not fat mass. If an association was present, it was always such that higher circulating cytokines related to lower lean body mass. However, IL-6 was one of the few cytokines that did not relate to lean mass ($p=0.573$). Further, there were no between-group differences in cytokine levels, suggesting that GDX did not alter cytokines in male mice of this age (Table 4.9). The directionality between cytokine concentration and lean mass held within both sham and GDX mice alone, although the significance was often lost (not shown), likely due to reduced sample size.

4.6 Discussion

Our results showed that frailty increased with age in both sham and GDX mice. However, frailty scores were actually lower in GDX mice compared to sham controls at several timepoints and overall, according to hierarchical linear regression. Interestingly, there was no relationship between serum testosterone levels and frailty alone or after

adjustments for age or GDX denomination. This was also true when we examined this relationship in older intact mice only. Taken together, these results demonstrate that serum testosterone concentrations did not relate to frailty scores in aging male C57BL/6 mice with and without lifelong testosterone deficiency. This finding leads us to reject our hypothesis that lifelong testosterone deficiency would exacerbate frailty progression compared to mice with regular testosterone levels.

That said, there is an association between low testosterone levels and increased risk for several health problems that contribute to frailty in humans (Blaya et al., 2017; Yeap et al., 2012), although teasing out differences between age *per se* or low testosterone has been challenging. Our results showed that age, but not testosterone, related to frailty in aging mice. In 2018, Hsu, Cumming, & Handelsman concluded there was "weak evidence suggesting any independent relationship between low serum [testosterone] levels and frailty in older men" (Hsu et al., 2018). This was because crude relationships between testosterone and frailty were often absent or disappeared when adjusting for covariates such as age. Furthermore, Gordon & Hubbard's review (2020) found little evidence that supplementing with testosterone or the precursor hormone dehydroepiandrosterone improves aspects of frailty. Our study provides preclinical evidence that this is indeed the case when testosterone is lowered artificially via gonadectomy, albeit from a very early age.

Alongside the observation that serum testosterone did not associate with frailty scores in our study, the FRIGHT scores, which estimated biologic age and are based upon a machine-learned algorithm, similarly found that GDX mice were no 'biologically' older than the sham operated mice. We also used the AFRAID scores to predict life expectancy and found these to be equal between groups from 19- to 22-months of age. Interestingly, the GDX mice had a higher life expectancy by this metric at 23-months. Because this metric occurred 1-month prior to study completion, whether the GDX mice would live longer than sham operated mice is unknown. The difference at 23-months in estimated lifespan according to the AFRAID clock is likely due to the cross-sectional difference in frailty, from which the AFRAID score is calculated. Much larger sample sizes are needed to elucidate differences in survival, and it is worth noting that clinical data suggest lower

testosterone levels increase the risk of mortality in humans (Yeap et al., 2014).

The fact that GDX mice had lower FI scores than sham mice overall and at several timepoints is difficult to explain. There is little available evidence that low testosterone levels are beneficial to health relative to normal levels. If anything, clinical literature suggests low serum testosterone levels are detrimental to overall health (Saad et al., 2017). However, Yeap et al. (2014) showed a U-shaped relationship between testosterone and all-cause mortality in older men, suggesting that high and low levels can be detrimental. We have no reason to believe, however, that all the sham mice in this study displayed 'high' testosterone levels. Levels within the sham group were demonstrably variable, as some sham mice had testosterone levels a few times greater than the highest GDX, while other sham mice had overlapping testosterone levels with the GDX group.

Consequently, mechanisms underlying why GDX mice had lower FI scores than sham mice overall, with three cross-sectional differences, are unclear. There is evidence that higher testosterone levels associate with shorter telomere lengths in young males (Drury et al., 2014). Because telomere shortening is a proposed mechanism of frailty (Bisset & Howlett, 2019), this is a potential contributor. However, Drury et al.' findings (2014) were in youth, and whether this relationship holds true and contributes to frailty in old age is not known. It is important to note that the GDX mice used in this study had lifelong testosterone deficiency that may lead to differences in frailty progression compared to natural testosterone deficiency occurring in older males. Still, we saw no relationship between frailty and testosterone when we examined this in older intact mice with naturally declining testosterone levels.

However, it is worth noting that the early life gonadectomy model used in this study causes testosterone deficiency differently than how age-related testosterone deficiency occurs in humans. Human males can live several decades with normal testosterone levels before experiencing testosterone deficiency. It is possible that a relatively sudden drop in androgens after years of normal androgen levels may affect how low testosterone impacts frailty progression. As demonstrated by Yialamas et al. (2007), a sudden drop in testosterone can increase levels of IL-6, which is related to frailty progression, but it is difficult to infer how drops in testosterone due to disease relate to

frailty. The decline in testosterone levels into older age in humans differs from our GDX model, which is practically lifelong. Nonetheless, we did investigate intact mice only, who did experience a sudden drop in testosterone around 22-months of age and found no acute relationship between testosterone and frailty. Extrapolation to humans who are experiencing a sudden drop in testosterone later in life suggests that having low testosterone alone may not potently induce frailty.

To better understand the impact of gonadectomy and testosterone levels on frailty progression, mechanisms of frailty in the form of chronic inflammation and lean mass wasting were also explored. While it has been reported that testosterone-deficient mice have elevated levels of pro-inflammatory cytokines like TNF-α and IL-6 (Kelly et al., 2012), we did not find a difference between sham and GDX mice. This may be because our mice were significantly older than the 8-week-old mice used in the paper by Kelly et al. (2012) and the ages in our study during which most cytokines were assessed were when sham and GDX mice had very similar testosterone levels. Future studies involving either testosterone supplementation in C57BL/6 mice, or a mouse strain with higher testosterone levels, like CD-1 mice (Brouillette et al., 2005) may be illuminating. Although no cytokines related to frailty levels, the heterogeneity in findings across studies in mice and indeed the lack of relationships between frailty and many cytokines in mouse models of aging suggests this may be common (Table 3.3). This does not mean necessarily that mice never demonstrate such relationships, as there are studies that have implicated elevated pro-inflammatory cytokines like IL-6 in frailty progression in mice (*e.g.*, Kane et al., 2019; Lin et al., 2014). Rather, the relationships may be highly variable.

The same might be said for lean mass, which arguably related to frailty in an opposite manner than expected. However, there was no evidence of lean mass wasting in these mice. In humans, higher levels of lean mass tend to associate with lower levels of frailty, while here the reverse was reported. The main reason for this is likely due to differences in measuring lean mass between humans and the mice used in this study. In humans, appendicular lean mass is typically used, as it targets the assessment of functional skeletal muscle (*e.g.*, Nguyen et al., 2022). Here, whole-body lean mass is reported and likely reduces the relative signal of the assay by including all non-fatty and

non-bone tissue as “lean mass”. Further, large organs like the liver tend to get heavier into old age in mice (Marino, 2012). Because the mechanism of frailty regarding lean mass directly relates to the size of skeletal muscle, a more sensitive method for measuring muscle weight may be needed to accurately model the effects on frailty. This casts doubt on the statistically significant relationship between lean mass and frailty in these mice.

Even though none of the cytokines investigated here related to frailty scores, many inversely related to lean mass. This is similar to relationships in humans, where elevated levels of IL-6, IL-8, MCP-1, and TNF-α significantly relate to low muscle mass (Tuttle et al., 2020). The murine homologue of IL-8, KC, and TNF-α both significantly inversely related to muscle mass in this study, along with several other pro-inflammatory cytokines. In human sepsis survivors, chronic inflammation is related both to muscle wasting and organ dysfunction (Hawkins et al., 2018). It could be fruitful to investigate the interplay between chronic inflammation-related losses in lean mass, organ dysfunction, and frailty. However, this information is also interesting to contrast with results relating frailty with lean mass or even testosterone, where no relationships were found. This suggests that in this aging mouse model, canonical clinical relationships between pro-inflammatory cytokines and lean mass tend to hold true, but their impacts on frailty in this model are negligible. These findings provide more evidence that lean mass alone does not relate strongly to frailty scores, especially when evaluated using a multidimensional frailty tool. The same can be said for the relationship between testosterone and frailty in a preclinical sense.

Finally, it is worth considering too that free testosterone related to frailty levels in some studies, where total testosterone did not (Travison et al., 2011; Hyde et al., 2010). Thus, a limitation in the present study is that only total testosterone was measured. Additionally, concerns have been raised regarding overestimating samples with low testosterone concentrations using immunoassays (Taieb et al., 2003). However, we previously used this assay to show that GDX reduces testosterone compared to young intact males (Ayaz et al., 2019) and that testosterone levels decline with age in males and are much lower in females (Banga et al., 2021, Ghimire et al., 2019). This suggests that changes in serum testosterone can be detected with this experimental approach, although

liquid chromatography with tandem mass spectrometry may be more effective. Interestingly, two of the four studies included in a meta-analysis that found a statistically significant relationship between frailty and blood-borne testosterone used some form of chromatography in tandem with mass spectrometry (Peng et al., 2022). The other two used immunoassays. This was contrasted by the three[7] studies that reported no relationship, which all used immunoassays. This suggests that more precise measurements of testosterone can improve the statistical power when relating testosterone measurements to frailty. Lastly, attrition rates in this study likely reduced statistical power to find differences between groups in FI scores, FRIGHT/AFRAID clocks, and testosterone, especially at 24-months of age.

4.7 Chapter summary

Our study shows that early life gonadectomy did not exacerbate frailty, but rather lowered frailty scores overall in 18- to 24-month-old male mice. This work helps elucidate the role testosterone plays in frailty development in a preclinical setting, which is important to unraveling how sex influences frailty (Kane & Howlett, 2021). Interestingly, testosterone levels did not relate to frailty in an unadjusted analysis or after adjusting for group and/or age. This agrees with several observational clinical studies and adds to the literature by demonstrating that inducing low testosterone in otherwise healthy animals did not increase frailty. Our results support the idea that low testosterone may simply be a natural marker of frailty or frailty risk in aging males, rather than a potent driver, which has been suggested previously (O'Connell et al., 2011).

[7] One study not included in this count which found no relationship between frailty and testosterone claimed they used "validated commercial kits", which if interpreted as immunoassays, brings this number up to four.

4.8 Tables

Table 4.1 Causes for euthanasia based on humane endpoints of sham and gonadectomized mice between 18- and 24-months of age

Cause	Number of Sham mice	Number of gonadectomized mice
Dermatitis	0	1
Died under anaesthetic	1	0
Tumour(s) present	0	3
Excessive weight loss	1	2
Unknown	4	0
Vestibulocochlear issues	1	0

Note. "Unknown" denotes the mouse died from an unknown cause. Examples include dying overnight of undetermined natural causes or being euthanized based on severe grimace with an undetermined reason.

Table 4.2 Frailty index scores were generally higher in Sham mice compared to gonadectomized (GDX) mice.

Age (months)	*p*-value between groups	Adjusted *p*-value between groups	Cohen's *d*
18	0.004	0.024	-1.45
19	0.131	0.555	-0.79
20	0.003	0.021	-1.80
21	0.111	0.555	-1.02
22	0.932	1.00	0.05
23	0.001	0.008	-3.02
24	0.537	1.00	-0.51

Note. Adjusted *p*-values were calculated using the Holm-Bonferroni method and effect sizes are presented as Cohen's *d*. Groups sizes (sham, GDX) were: 18-months=(10,11); 20-months=(8,8); 22-months=(6,6); and 24-months=(3,5).

Table 4.3 Unadjusted and age- and group-adjusted models using hierarchical linear regression predicting frailty index scores from serum testosterone.

Model DV = Frailty	Predictor variable: Testosterone		Age		Group (*i.e.*, a sham mouse)	
	β (±95%CI)	p-value	β (±95%CI)	p-value	β (±95%CI)	p-value
Unadjusted	-0.285 (-0.334, 0.236)	0.281				
Age adjusted	0.007 (-0.022, 0.037)	0.626	**0.014 (0.012, 0.016)**	**≤5E-5**		
Age + Group adjusted	-0.019 (-0.059, 0.021)	0.357	**0.013 (0.011, 0.015)**	**≤5E-5**	0.035 (-0.002, 0.073)	0.077
Sham only	-0.007 (-0.048, 0.034)	0.730	**0.014 (0.004, 0.024)**	**0.013**		

Note. DV = Dependent Variable; CI = confidence interval.

Table 4.4 FRIGHT and AFRAID clock scores were similar between groups across most timepoints.

Age (months)	*p*-value between groups	Holm-Bonferroni corrected *p*-value	Cohen's *d*
FRIGHT (estimated biologic age)			
19	0.611	1.000	0.25
20	0.556	1.000	0.27
21	0.362	1.000	-0.55
22	0.668	1.000	-0.30
23	0.158	0.948	-1.02
24	0.265	1.000	-0.88
AFRAID (estimated life expectancy)			
19	0.862	1.00	0.09
20	0.777	1.00	-0.14
21	0.738	1.00	-0.19
22	0.417	1.00	-0.49
23	<0.001	0.006	4.26
24	0.110	0.550	1.94

Note. Adjusted *p*-values were calculated using the Holm-Bonferroni method and effect sizes are presented as Cohen's *d*. Groups sizes (sham, GDX) were: 19-months=(9,8); 20-months=(8,8); 21-months=(6,8); 22-months=(6,6); 23-months=(6,5); and 24-months=(3,5).

Table 4.5 Unadjusted and age-adjusted models predicting frailty from body composition scores

Model	**Coefficients and significance**	
DV = Lean mass	β (SE)	*p*-value
Unadjusted	**0.010 (0.002)**	**0.002**
Age adjusted	**0.018 (0.005)**	**0.0035**
DV = Fat mass	β (±95%CI)	*p*-value
Unadjusted	-0.002 (0.001)	0.053
Age adjusted	-0.001 (0.001)	0.172
DV = % Lean mass	β (±95%CI)	*p*-value
Unadjusted	**0.002 (0.001)**	**0.027**
Age adjusted	0.001 (0.001)	0.0529
DV = % Fat mass	β (±95%CI)	*p*-value
Unadjusted	**-0.022 (0.001)**	**0.007**
Age adjusted	**-0.002 (0.001)**	**0.027**

Note. DV = Dependent Variable; SE = Standard error of the mean.

Table 4.6 Spearman correlations between cytokines and frailty. n=14 comparisons.

Cytokine	*ρ*	p-value
L-1α	-0.337	0.2372
IL-1β	-0.282	0.3262
IL-2	-0.394	0.163
IL-3	-0.143	0.6232
IL-4	-0.176	0.5439
IL-5	-0.332	0.2439
IL-6	-0.421	0.1348
IL-9	-0.249	0.3879
IL-10	-0.394	0.1630
IL-12(p40)	0.148	0.6124
IL-12(p70)	-0.28	0.3302
IL-13	-0.33	0.2469
IL-17A	-0.368	0.1950
Eotaxin	0.137	0.6395
G-CSF	-0.427	0.1282
GM-CSF	-0.306	0.2849
IFN-γ	-0.284	0.3223
KC	-0.253	0.3793
MCP-1	-0.396	0.1604
MIP-1α	-0.253	0.3793
MIP-1β	-0.337	0.2359
RANTES	0.139	0.6341
TNF-α	-0.251	0.3835

Table 4.7 Spearman correlations between cytokines and serum testosterone. n=14 comparisons.

Cytokine	*ρ*	p-value
L-1α	-0.0286	0.9276
IL-1β	0.213	0.4636
IL-2	0.169	0.5629
IL-3	0.143	0.6266
IL-4	0.0549	0.8557
IL-5	0.143	0.6233
IL-6	-0.0505	0.8676
IL-9	0.27	0.3492
IL-10	0.218	0.4542
IL-12(p40)	-0.332	0.2464
IL-12(p70)	0.0681	0.8201
IL-13	0.134	0.6485
IL-17A	0.152	0.6051
Eotaxin	-0.323	0.2597
G-CSF	-0.0242	0.9396
GM-CSF	0.191	0.5121
IFN-γ	0.156	0.5944
KC	-0.007	0.9879
MCP-1	0.204	0.4827
MIP-1α	0.248	0.3911
MIP-1β	0.18	0.5344
RANTES	-0.068	0.8201
TNF-α	0.182	0.5321

Table 4.8 Spearman correlations between body composition measures and cytokines

	Lean mass		Fat mass		% Lean mass		% Fat mass	
Cytokine	***ρ***	**p-value**	***ρ***	**p-value**	***ρ***	**p-value**	***ρ***	**p-value**
L-1α	**-0.65**	**0.0257**	0.34	0.2781	-0.427	0.1689	0.427	0.1689
IL-1β	**-0.825**	**0.0016**	0.231	0.4661	-0.371	0.2367	0.371	0.2367
IL-2	**-0.657**	**0.0238**	0.308	0.3267	-0.413	0.1845	0.413	0.1845
IL-3	**-0.657**	**0.0238**	0.182	0.5679	-0.35	0.2662	0.35	0.2662
IL-4	**-0.72**	**0.0106**	0.298	0.3447	-0.392	0.2097	0.392	0.2097
IL-5	**-0.862**	**0.0006**	0.305	0.331	-0.438	0.1555	0.438	0.1555
IL-6	-0.182	0.5731	0.375	0.229	-0.329	0.2973	0.329	0.2973
IL-9	**-0.797**	**0.0029**	0.165	0.607	-0.308	0.331	0.308	0.331
IL-10	**-0.636**	**0.0299**	0.322	0.3043	-0.378	0.2276	0.378	0.2276
IL-12(p40)	-0.503	0.0989	0.389	0.211	-0.371	0.2367	0.371	0.2367
IL-12(p70)	**-0.888**	**0.0003**	0.347	0.2677	-0.497	0.1041	0.497	0.1041
IL-13	**-0.769**	**0.0049**	0.277	0.3811	-0.399	0.201	0.399	0.201
IL-17A	**-0.755**	**0.0062**	0.308	0.3267	-0.455	0.1404	0.455	0.1404
Eotaxin	-0.168	0.6039	-0.291	0.3565	0.231	0.4708	-0.231	0.4708
G-CSF	-0.345	0.2993	0.417	0.1779	-0.378	0.2276	0.378	0.2276
GM-CSF	**-0.853**	**0.0008**	0.294	0.35	-0.427	0.1689	0.427	0.1689
IFN-γ	**-0.839**	**0.0011**	0.266	0.3995	-0.413	0.1845	0.413	0.1845
KC	**-0.601**	**0.0428**	0.308	0.3267	-0.385	0.2183	0.385	0.2183
MCP-1	-0.538	0.0750	0.266	0.3995	-0.315	0.3194	0.315	0.3194
MIP-1α	**-0.804**	**0.0025**	0.179	0.5761	-0.315	0.3194	0.315	0.3194
MIP-1β	**-0.722**	**0.0102**	0.239	0.4507	-0.35	0.2624	0.35	0.2624
RANTES	-0.329	0.2973	-0.0736	0.8214	0.035	0.921	-0.035	0.921
TNF-α	**-0.839**	**0.0011**	0.249	0.4327	-0.385	0.2183	0.385	0.2183

Table 4.9 Results from independent t-tests on log-transformed cytokine values calculated using cytokine value from 9 sham mice and 8 GDX mice between 20-24 months of age. Cohen's *d* was used to estimated effect size, with the direction proportional to the change in gonadectomized mice versus sham ($\bar{X}_{GDX} - \bar{X}_{Sham}$).

Cytokine	*d*	p-value
IL-1α	0.099	0.842
IL-1β	0.449	0.375
IL-2	0.249	0.621
IL-3	0.417	0.413
IL-4	0.394	0.437
IL-5	0.648	0.211
IL-6	0.057	0.909
IL-9	0.475	0.353
IL-10	0.465	0.365
IL-12(p40)	0.916	0.078
IL-12(p70)	0.646	0.213
IL-13	0.491	0.333
IL-17A	0.380	0.454
Eotaxin	0.189	0.708
G-CSF	-0.400	0.433
GM-CSF	0.536	0.299
IFN-γ	0.511	0.323
KC	0.195	0.697
MCP-1	-0.030	0.952
MIP-1α	0.335	0.506
MIP-1β	0.187	0.707
RANTES	0.523	0.305
TNF-α	0.487	0.339

4.9 Figures

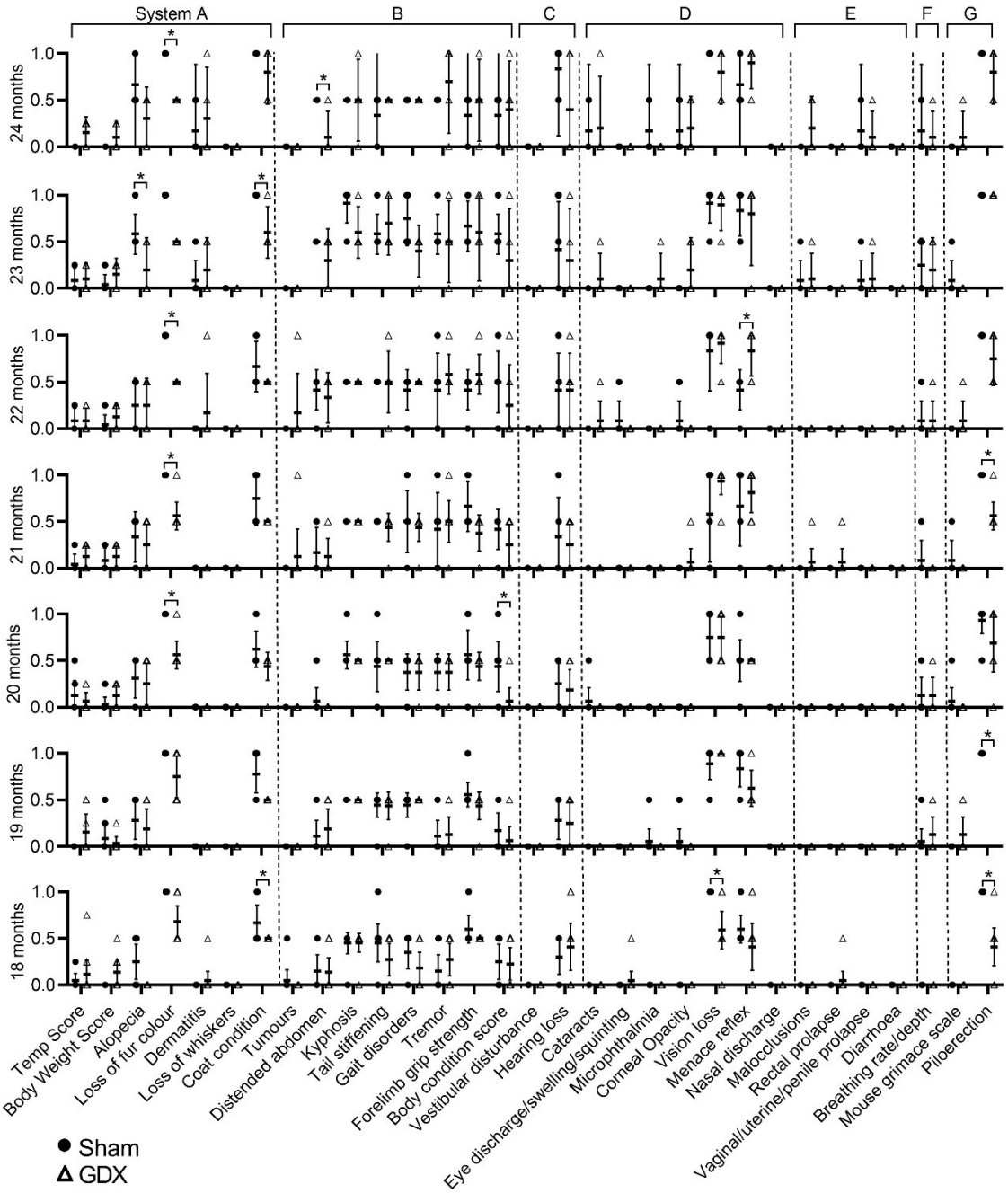


Figure 4.1 Caption on next page

Figure 4.1 Individual deficits were lower in gonadectomized (GDX) mice over several timepoints. Individual deficit scores (mean ± 95% confidence interval) are plotted across different timepoints and compared between sham and GDX mice across different ages. Group sizes (sham, GDX) were: 18-months=(10,11); 19-months=(9,8); 20-months=(8,8); 21-months=(6,8); 22-months=(6,6); 23-months=(6,5); and 24-months=(3,5). Mann-Whitney U test statistics yielding $p \leq 0.05$ are denoted by * symbols. Vertical lines separate individual systems as: A = integument, B = musculoskeletal system, C = vestibulocochlear/auditory systems, D = ocular/nasal systems, E = digestive / urogenital systems, F = respiratory system, and G = general signs of discomfort.

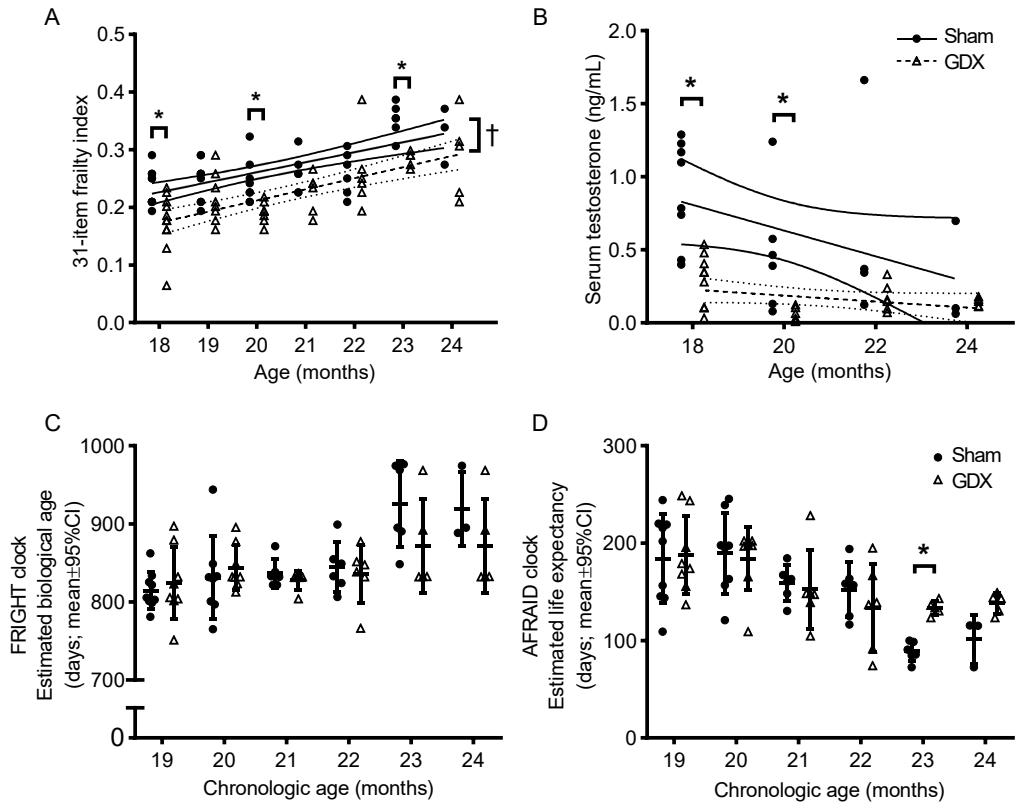


Figure 4.2 Frailty scores were notably lower overall and at several timepoints for gonadectomized (GDX) mice between 18- and 24-months. There were no differences between sham and gonadectomized (GDX) mice in estimated biologic age and one difference in estimated life expectancy. A) Frailty index scores for sham and GDX mice are presented as individual values overlapping simple regression lines ± 95% confidence intervals. B) Serum testosterone levels were higher in sham versus GDX mice and are presented as individual values overlapping simple regression lines ± 95% confidence intervals. C) Estimated biologic age using the Frailty Inferred Geriatric Health Timeline (FRIGHT) clock (18) were similar. D) Estimated life expectancy from the Analysis of Frailty and Death (AFRAID) clock (18) was longer in GDX mice only at 23-months with no overall group effect. Group sizes (sham, GDX) were: 18-months=(10,11); 19-months=(9,8); 20-months=(8,8); 21-months=(6,8); 22-months=(6,6); 23-months=(6,5); and 24-months=(3,5). The † symbol denotes an overall between-group (*e.g.*, sham vs. GDX) effect for frailty according to hierarchical linear regression ($p = 0.004$). Cross-sectional effect sizes and *p*-values for both clocks are in Supplemental Table 3. *p*-values for overall effects were non-significant and are within the Results section. Cross-sectional analysis using unpaired t-tests that had $p \leq 0.05$ are denoted by * symbols. Effect sizes and *p*-values for cross-sectional analyses are in Supplemental Table 2.

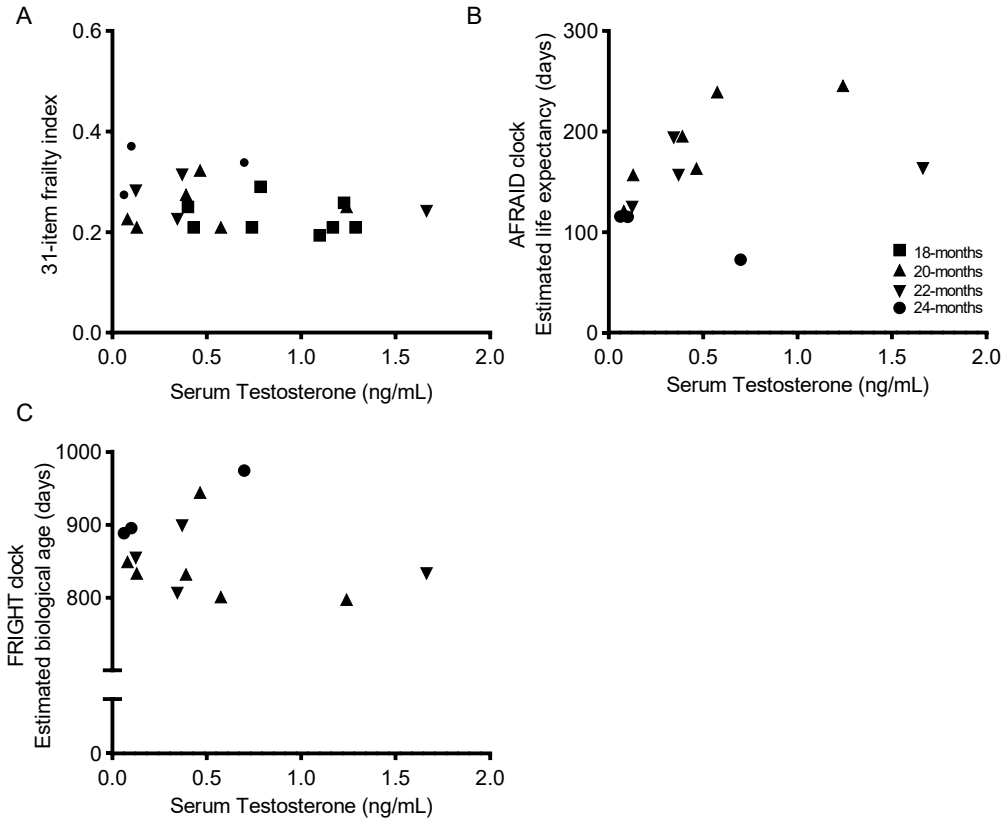
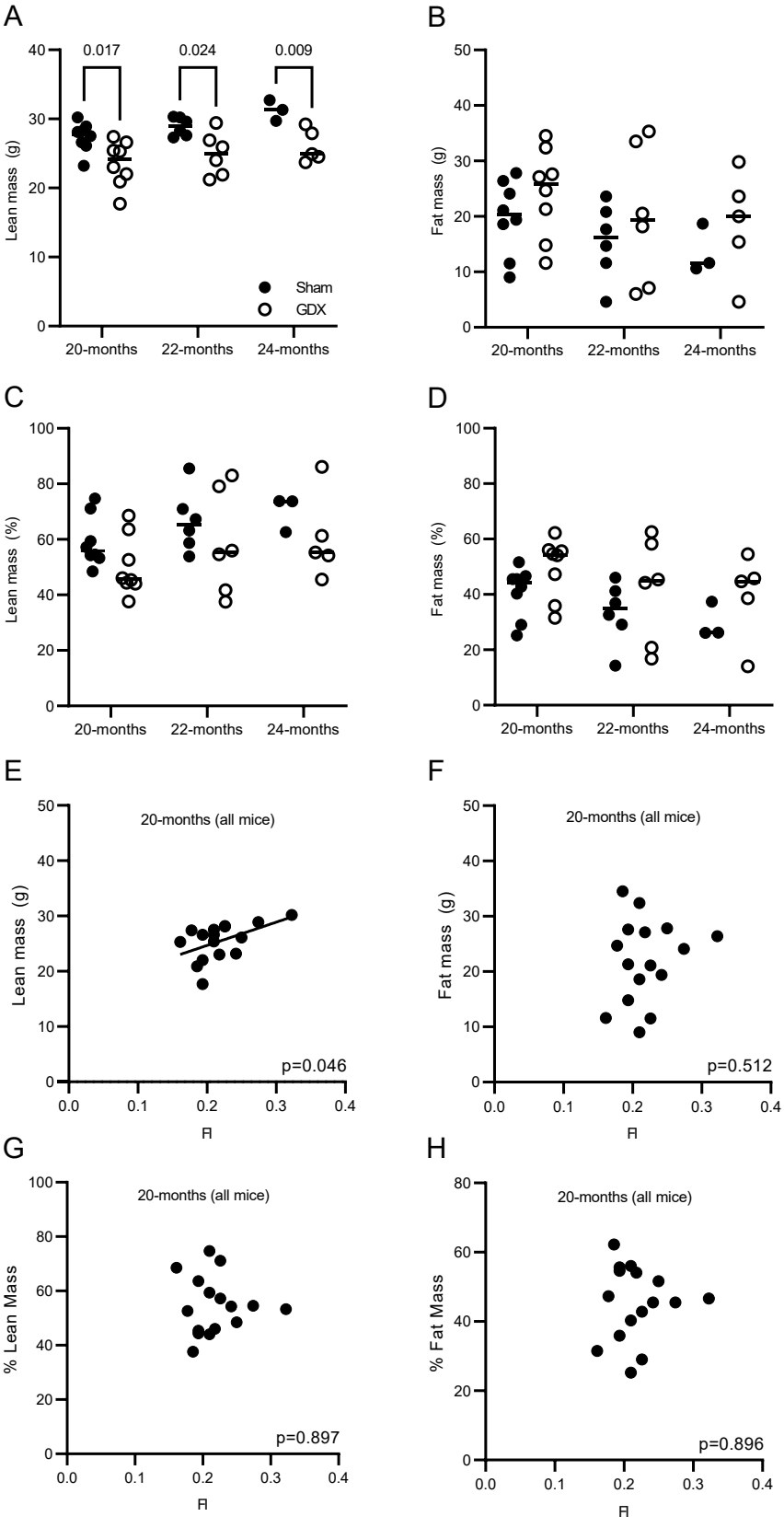


Figure 4.3 There was no relationship between intact male serum testosterone levels and frailty index (FI) scores, Analysis of Frailty and Death (AFRAID) scores, or Frailty Inferred Geriatric Health Timeline (FRIGHT) clock scores. A) 31-item FI scores versus serum testosterone. B) AFRAID scores versus serum testosterone. C) FRIGHT scores versus serum testosterone. Group sizes were: 18-months=10; 20-months=8; 22-months=6; 24-months=3.



(Figure 4.4 Caption on next page)

Figure 4.4 Gonadectomized mice had lower lean mass that related to frailty scores. A) Total estimated lean mass measured using dual-energy X-ray absorptiometry was significantly lower in gonadectomized mice versus sham mice. B) Significant differences in total estimated fat mass were not observed. C) Percent lean mass was not statistically different between groups, nor percent fat mass (D). The relationship between lean mass and frailty (frailty index) was investigated by comparing the two variables from 20-month-old animals. Only 20-month-old animals were assessed to avoid repeated-measures in a linear regression analysis. On the other hand, frailty did not relate cross-sectionally at 20-months with fat mass (F), % lean mass (G), or % fat mass (H).

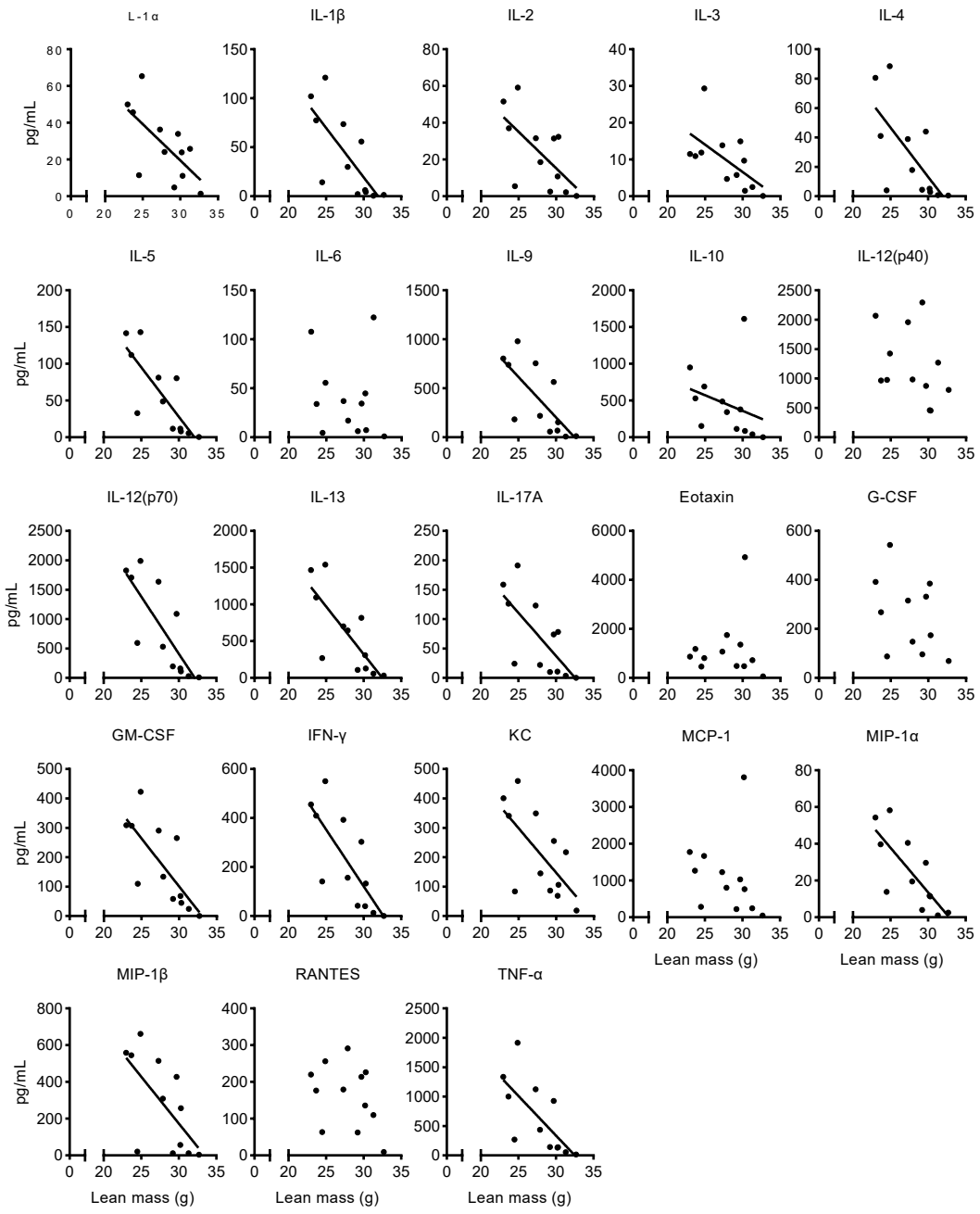


Figure 4.5 Correlations between whole-body lean mass, measured by dual-energy X-ray absorptiometry, and circulating cytokines in older C57BL/6 mice (aged 22-24 months). Associated p-values are in Table 4.8.

Chapter 5 The impact of a selective androgen receptor modulator on frailty and frailty mechanisms

5.1 Introduction

The relationship between higher frailty, poor health outcomes, and low circulating testosterone sparked interventional trials aimed at treating older, frail people with testosterone. Although these trials have primarily involved older men, some involved women, and it is important to recognize that testosterone levels play a role in maintaining muscle and function in older women and can also decline in older age (Glasier & Dimitrakakis, 2013). The AR is present in women across several bodily systems, including skeletal muscles and the heart (Bianchi et al., 2021). Unfortunately, the results of trials have been equivocal, suggesting that testosterone treatment may not effectively treat frailty in all people. This may relate to effective dosing, where higher doses of testosterone are more effective but have health risks when above physiologic ranges (*i.e.*, supraphysiologic). There is therefore room for improvement in the treatment of frailty with androgen-like drugs.

This opens the door to investigate an exciting new drug class, SARMs, which also act on the AR (Figure 5.1) but are more selective to improve the health of organs associated with frailty like muscle and bone. As reviewed later, their tissue selectivity relates to how the AR functions as a nuclear transcription factor. Table 5.1 summarizes current clinical evidence using SARMs, which are demonstrably very anabolic and can help improve some aspects of physical function in older adults. They may also be safer than testosterone, offering an advantage, although this is not yet proven. Thus, SARMs offer an opportunity to selectively target mechanisms of frailty where testosterone supplementation may fall short.

5.2 The impact of androgen therapies on frailty and related systems

5.2.1 Testosterone treatment and frailty

The association between low testosterone and frailty has motivated clinical trials into the beneficial effects of testosterone supplementation on overall health. One such study investigated the impact of three different testosterone preparations on frailty

progression in patients with clinically diagnosed low testosterone (Strollo et al., 2013). Sixty-four men with documented low testosterone were randomized to receive testosterone replacement therapy via an oral preparation (80 mg testosterone undecanoate/day; n=15), transmucosally (60 mg/day; n=18), or transdermally (30 mg/day; n=15) for 6-months, which were compared to a control untreated group (n=16). Only the transmucosal and transdermal preparations led to a significant increase in serum testosterone levels compared to the untreated control, although the oral administration did marginally increase levels within the group (Strollo et al., 2013). Notably, Fried FP scores were significantly reduced in all three testosterone groups, while the control group remained unchanged. Both the transmucosal group and transdermal groups went from being classified on average as frail (mean frailty scores between 3.6-3.9 out of a maximum of 5) to being 'fit' (mean frailty scores of <1), while the oral treatment group became 'pre-frail'. These results suggest that testosterone supplementation in men with *bona fide* low testosterone levels can significantly improve frailty, as measured using an FP (Strollo et al., 2013).

However, testosterone may not help those with low testosterone if the dose is not adequate. A different study randomized 130 men (mean age of 77 years) with confirmed low testosterone levels into groups receiving either transdermal testosterone gel (5 mg/day) or placebo for 12-months and found no change in Fried FP frailty scores (Kenny et al., 2010). These doses were notably lower than those in Strollo et al. (2013), where frailty improved, suggesting a minimum effective dose to treat frailty was not reached.

Evidence is not as clear in more heterogenous groups that do not have low testosterone levels. Theou et al. (2016) investigated the impact of a nutritional supplement (high-calorie) along with testosterone supplementation (oral testosterone undecanoate; 160 mg/day for men, 40 mg/day for women) on FI-Lab scores and a self-reported FI compared to a control group with a low-calorie supplement. Undernourished men and women with a mean age of 77 years (n=53, 36% women) were randomized into either group and completed the study. Overall, frailty scores did not change significantly between groups, despite the participants in the testosterone group having a 4.8-times higher likelihood of improving their frailty scores (Theou et al., 2016). Thus, evidence

suggests that supplementing with testosterone can benefit frailty in people with low testosterone, although these effects are not as robust at lower doses or in a general, albeit frail population.

Besides frailty, there are generally positive effects of testosterone supplementation on muscle mass and strength, which relate to frailty. One study evaluated the effects of 50 mg/day transdermal testosterone on body composition, muscle strength, and physical function in 262 men (mean age=74 years; Srinivas-Shankar et al., 2010). Although they measured frailty using a FP, it was not investigated as an outcome measure, but rather as a covariate. After 6-months of treatment there were significant increases in lean mass, knee joint strength, 6-minute walk test scores, and physical performance test scores. Further, there was a significant decrease in fat mass in the testosterone group (Srinivas-Shankar et al., 2010). Further evidence can be gleaned from the Testosterone's Effect on Atherosclerosis Progression in Aging Men trial, which included 256 men (mean age=67 years) with low-to-normal testosterone levels undergoing testosterone therapy (75 mg/day transdermally) for three-years (Storer et al., 2017). Total lean body mass increased significantly, as well as upper body strength and lower body strength and power, although upper body power did not improve. Notably, testosterone levels were within the upper portions of the reference range for adult males. This provides valuable evidence that testosterone alone can improve physical function and body composition into older age (Storer et al., 2017). On the other hand, Kenny et al. (2010), who used a daily transdermal dose that was 7% of the dose used in Storer et al. (2017), reported no significant changes in seven physical function tests, although beneficial changes in lean mass and fat mass were noted. Similarly, a different study found no change in handgrip strength, leg extension strength, or timed up-and-go tests in men with a low testosterone (mean age=67 years) after receiving 80 mg testosterone undecanoate per day or placebo for 6-months, despite seeing positive effects in lean mass. On balance, testosterone regularly improves lean mass and decreases fat mass in older men, especially those with low testosterone, but functional improvements only sometimes occur and are related to higher doses.

Lastly, some investigations using older women have tested the impacts of testosterone on lean mass and physical function. A study involving 71 post-menopausal

women (mean age=53 years) randomized to receive a placebo or escalating doses of testosterone (transdermal, 3-25 mg/week) noted a dose-dependent increase in lean mass, chest press, and loaded stair climb power (Huang et al., 2014). A dose-dependent increase in trunk muscle mass has also been shown in 24 hysterectomized women (mean age=52 years) receiving between 3-25 mg of testosterone per week (Tapper et al., 2019). These results suggest that androgens may help older women improve muscle mass and physical fitness too. Overall, there is evidence in older men that testosterone therapy can improve physical function, although not all studies agree, which may be related to differential dosing and baseline testosterone levels. Evidence in older women with low testosterone is also promising, suggesting that testosterone therapy can improve muscle mass and fitness.

Supplementing with testosterone may also benefit chronic inflammation (Bianchi, 2018). Twenty-seven males with low testosterone with a mean age of 62 years were given a testosterone preparation (100 mg of a long-lasting formula every 14-days) to examine the effects on circulating inflammation (Malkin et al., 2004). Levels of TNF-α and IL-1β were reduced following treatment, which did not change in the age-matched control group, although levels of IL-6 remained unchanged after treatment (Malkin et al., 2014). Additional evidence comes from a trial involving 184 men with low testosterone and metabolic syndrome (mean age=52 years), who were randomized to receive testosterone (1000 mg of a long-acting testosterone parenterally once every 6-weeks for 30-weeks) or placebo (Kalinchenko et al., 2010). The testosterone treated group had significantly lower IL-1β, and TNF-α compared to placebo, although IL-6 was not reduced significantly (Kalinchenko et al., 2010). On the other hand, androgen deprivation therapy in prostate cancer patients has led to significant rises in IL-6 levels (Hoogland et al., 2021). Evidence supporting the role of testosterone in suppressing chronic inflammation is supported by work in animal studies (Mohamad et al., 2019). For example, testosterone supplementation has reduced circulating IL-6 levels in genetically feminized mice (Kelly et al., 2012). Overall, there is evidence that supplementing with testosterone can suppress circulating inflammatory markers that relate to frailty like TNF-α, although effects on IL-6 are less convincing.

There is also evidence that testosterone supplementation can improve bone health.

A study randomized 211 men with low testosterone (mean age=72 years) to either receive testosterone gel to raise levels to within the normal range, or a placebo for 12-months (Snyder et al., 2017). BMD of the spine and hip were increased significantly with testosterone treatment (Snyder et al., 2017). Additionally, testosterone supplementation has been investigated with regards to cardiac function in disease states, particularly heart failure. Clinical evidence suggests that low testosterone levels are detrimental to left ventricular function and are common in heart failure patients (Di Lodovico et al., 2022; Gheorghe et al., 2021). Preclinical evidence also suggests that low testosterone levels are detrimental to cardiac function (Banga et al., 2021). However, supplementing with testosterone in states of low testosterone did not help improve heart failure class or EF, although there are other benefits like improved insulin sensitivity and exercise capacity (Cannarella et al., 2021). Taken together, testosterone therapy has led to equivocal improvements in frailty in studies involving mostly men, although there have been beneficial effects on frailty-related systems (*e.g.*, musculoskeletal) in both men and women. Further, there may be beneficial effects of testosterone therapy on other aspects of frailty like chronic inflammation and bone health, but perhaps not in the heart, despite low testosterone levels relating to worse cardiac function.

5.2.2 Concerns about testosterone therapy

Although there are many potential benefits of testosterone therapy, there are some risks. One retrospective study reported a significantly elevated risk of myocardial infarction, strokes, or death in patients on testosterone replacement therapy compared to those not on replacement therapy (Vigen et al., 2013). A cohort study corroborated this by reporting that testosterone therapy increased the risk of myocardial infarction in older men or younger men with cardiovascular disease (Finkle et al., 2014). However, others do not agree, with one study finding a reduction in overall major adverse cardiovascular event risk (Anderson et al., 2016). Two systematic reviews including meta-analyses of randomized controlled trials concluded that testosterone therapy did not increase the risk of any cardiovascular event, and even reduced the risk when supplemented to a physiologic range or used in obese patients (Corona et al., 2018; Corona et al., 2014). Some explanations have been given regarding the disparity between studies, including the use of cardiovascular risk-lowering drugs in control populations (Dupree et al., 2014).

Overall, it has been proposed that increased cardiovascular risk related to testosterone therapy may occur at supraphysiologic doses (Cardona Attard & Fava, 2019), limiting therapeutic use beyond such levels.

There is also a concern that testosterone therapy is hepatotoxic, especially at supraphysiologic doses. Long-term testosterone therapy in physiologic ranges do not seem to harm liver function in older men (Al-Qudimat et al., 2021), but supraphysiologic doses have been associated with liver damage, which can be inferred from elevated activity levels of liver enzymes like alanine aminotransferase (ALT) and aspartate aminotransferase (AST) in blood samples (Niedfeldt, 2018). Another concern with testosterone therapy regards prostate health. While the risk of prostate cancer due to testosterone therapy is virtually nil (Lenfant et al., 2020), its use in metastatic prostate cancers is contraindicated due to its enabling effect on the disease, even in castration-resistant prostate cancer (Cornford et al., 2021). Other concerns relating to testosterone therapy include reduced high-density lipoprotein levels (Fernández-Balsells et al., 2010), sleep apnea (Hoyos et al., 2012), and infertility (Samplaski et al., 2014). While these risks do not contraindicate testosterone therapy for many people (Cardona Attard & Fava, 2019), they do present an opportunity to consider alternative drugs.

5.2.3 Summary of testosterone treatment and frailty

Although there is some evidence that testosterone treatment can positively affect frailty levels, the effects are strongest in populations with low levels of testosterone and when doses are higher. However, there are several benefits to testosterone therapy on various aspects of health related to frailty, such as improved muscle mass, some functional fitness gains, improved BMD, and reduced chronic inflammation. Despite there being an association between low testosterone levels and cardiac dysfunction, a condition intimately linked with frailty (Pandey et al., 2019), treatment with testosterone does not seem to ameliorate this, but not much is known in this regard. Importantly, testosterone therapy offers dose-dependent beneficial effects, but treatment at supraphysiologic levels can be toxic to the liver and lead to increased cardiovascular health risks. It is therefore unknown if higher doses would lead to a reduction in frailty, as the beneficial effects on many bodily systems suggest, although drug-related toxicity

contraindicates such trials. Thus, there is a need to search for drugs that can impart the same beneficial effects of higher doses of testosterone while mitigating the adverse effects of such doses. A targeted therapeutic approach is likely needed to achieve this.

5.3 Selective androgen receptor modulators and frailty

5.3.1 Origins and current state of selective androgen receptor modulators

The development of SARMs is an advancement in targeted hormonal therapies.[8] The first breakthrough in the discovery of SARMs was in 1998, where three non-steroidal ligands were reported to have AR-mediated transcriptional properties similar to the potent testosterone derivative dihydrotestosterone (Dalton et al., 1998). Subsequent animal experiments reported the strong hypertrophic effect of SARMs on androgen-sensitive muscles such as the levator ani, while not having nearly as potent effects on prostate hypertrophy, as is normally seen from supraphysiologic endogenous androgen treatment (Gao et al., 2005). Further, beneficial effects on preserving bone structure were reported in ovariectomized rats (Kearby et al., 2007). SARMs also have some anti-cancer effects, including those of the breast and prostate (Narayanan et al., 2010; Peters et al., 2008). These observations have led to several clinical trials investigating their use to improve lean body mass and physical function (Neil et al., 2018; Papanicolau et al., 2013; Dalton et al., 2011), prostate cancer androgen deficiency (Pencina et al., 2021), triple-negative breast cancer (Yuan et al., 2021), and metastatic breast cancer (LoRusso et al., 2022). The future of this class of drugs likely involves the treatment of muscle wasting, osteoporosis, cancers, and stress urinary incontinence, although much work is needed to elucidate their true benefit (Solomon et al., 2019; Narayanan et al., 2018).

5.3.2 Tissue-selective mechanism of selective androgen receptor modulators

While androgens such as testosterone are steroidal ligands of the AR, most SARMs are non-steroidal (*i.e.*, they lack the four carbon rings that define steroid

[8] This type of therapeutic approach can be traced as early as the 1930s. Targeted modulating hormonal therapies were proposed for breast cancer as early as 1936, as can be seen in A. Lacassagne's address before the American Association for Cancer Research in Boston, April 7th that year: "*If one accepts the consideration of adenocarcinoma of the breast as the consequence of a special hereditary sensibility to the proliferative action of oestrone, one is led to imagine a therapeutic preventive for subjects predisposed by their heredity to this cancer. It would consist-perhaps in the very near future when the knowledge and use of hormones will be better understood-in the suitable use of a hormone, antagonistic or excretory, to prevent the stagnation of oestrone in the ducts of the breasts.*"

molecules) but still act on the AR (Figure 5.1A & B; Narayanan et al., 2018). The AR is a 110 kDa protein that functions as a nuclear transcription factor and contains two activation function (AF) domains (Ferraldeschi et al., 2015). AF-1 is responsible for constitutive activity in the absence of ligand binding, while AF-2 is more adjacent to the ligand binding domain and responds to ligand binding (Ferraldeschi et al., 2015). Upon binding of a ligand, the AR sheds associated heat shock proteins and dimerizes with another activated AR before translocating to the nucleus to bind to androgen response elements to upregulate gene transcription (Figure 5.1A & B; Davey & Grossmann, 2016). Importantly, signalling from constitutive activity and ligand binding is heavily regulated by coregulators that either suppress or promote transcription via the AR on these elements (Chen & Dehm, 2014). There are over 280 proteins that act as coregulators for the AR, although most evidence comes from *in vitro* work (van de Wijngaart et al., 2012). Nonetheless, the multitude of coregulators helps to specify and diversify the genes upregulated in different tissues in the body (van de Wijngaart et al., 2012). Deciphering and harnessing this complexity would benefit diseases related to AR signalling.

Differential coregulator recruitment is one such mechanism by which SARMs convey a tissue-selective approach beyond endogenous ligands (Narayanan et al., 2018). This was demonstrated simply in a set of experiments evaluating the relative transactivation by the AR in prostate cancer cells and skeletal muscle cells in the presence of an activating coregulator, steroid receptor coactivator-1. The endogenous androgen dihydrotestosterone was much more potent in prostate cancer cells than muscle cells when compared to a SARM, while in skeletal muscle the SARM had significantly more efficacy (Narayanan et al., 2018). Further evidence of differential cofactor recruitment was found when evaluating cofactor recruitment in prostate cancer cells by immunoprecipitation, whereby dihydrotestosterone increased the recruitment of the coactivator, steroid receptor coactivator-1 to the androgen response element but did not recruit the corepressor, nuclear receptor corepressor protein, while a SARM recruited both (Narayanan et al., 2018). This may explain in part why SARMs have potential to treat prostate cancers, which are usually sensitive to AR signalling (Nyquist et al., 2021). In a similar vein, SARMs also possess tissue selectivity beyond endogenous ligands via operating through different pathways. When compared to dihydrotestosterone,

downstream signalling after SARM treatment in prostate cancer cells occurred via different mechanisms, which altered the recruitment of further cofactors (Narayanan et al., 2008). Thus, differential coregulator recruitment, either directly or by modifying recruitment, is one way SARMs are selective.

Another important consideration underlying tissue selectivity of SARMs relates to their differences to the endogenous ligand testosterone and its more potent reduced form, dihydrotestosterone. The enzyme that converts testosterone to dihydrotestosterone, 5α-reductase is much more active in the prostate than in muscle cells (Gao & Dalton, 2007). Thus, when testosterone (the primary circulating androgen versus dihydrotestosterone; Yeap et al., 2012) enters the prostate its signal becomes amplified, which does not happen in muscle cells (Gao & Dalton, 2007). SARMs that do not undergo metabolism within tissues therefore have no enzyme-dependent amplification and thus are not subject to tissue-dependent levels of 5α-reductase when causing an effect. Other potential mechanisms that may lead to tissue selectivity include different conformational changes in the receptor leading to altered signalling, which some *in silico* studies have alluded to (Zierau et al., 2019; Narayanan et al., 2018). Therefore, although mechanisms underlying the tissue selectivity of SARMs are not yet fully understood, there is evidence that tissue selectivity is related to the alteration in signalling mechanisms related to nuclear transcription factors like differential coregulator recruitment.

5.3.3 Selective androgen receptor modulators to treat systems related to frailty

It is proposed that SARMs can be a treatment of choice for muscle and bone wasting conditions (Narayanan et al., 2018), which includes age and thus makes them an attractive candidate to help manage frailty. Further, SARMs have the potential to impact a diverse set of age-related conditions due to the prevalence of the AR across multiple tissue types (Christiansen et al., 2020). Only one trial has evaluated the effect of a SARM on frailty thus far. Dobs et al. (2013) used the Fried FP criteria to calculate frailty and determined that SARM treatment (1 or 3 mg per day of the SARM GTx-024 (Enobosarm) for approximately 16 weeks) had no impact on frailty levels in adult cancer patients or placebo (n=32-54 per group; 33-50% women per group). Human trials investigating the effects of a SARM on frailty are therefore lacking, as are preclinical investigations.

However, multiple investigations regarding bodily systems involved in frailty are available. These studies are reviewed here to describe the potential of SARMs to manage frailty through their beneficial effects on musculoskeletal health, as well as any potential effects on chronic inflammation and other systems related to frailty. A summary of the information presented below can be found in Table 5.1.

5.3.3.1 Lean mass and strength

Because of their putative actions on the AR in skeletal muscle, SARMs have the potential to be function-promoting therapies during treatments for diseases like cancer, heart failure, and osteoporosis, as well as generally into older age (Bhasin & Jasuja, 2009). They may achieve this by facilitating anabolism in skeletal muscles and bones to either maintain or increase physical function. Six clinical trials (all phase I or II) have evaluated the effects of SARMs on lean mass and strength, including participants that are healthy older men and women or cancer patients (Table 5.1). Five different SARMs were investigated across all studies. All six studies reported an increase in lean body mass following SARM treatments, and dose-dependent effects were noted (*e.g.*, Pencina et al., 2021). Importantly, appendicular lean mass increased in all four studies that measured it. One study investigated thigh muscle volume and reported that a SARM increased this measure in both men and women (Neil et al., 2018). Three studies reported no change in fat mass due to SARM therapy (Pencina et al., 2021; Basaria et al., 2013; Dobs et al., 2013), although one study did report a decrease (Dalton et al., 2011). A decrease in percent fat mass was also reported in Pencina et al., 2021. Thus, SARMs in general are anabolic drugs that primarily affect body composition by increasing total and appendicular lean mass and may play a lesser role in fat loss.

With SARM treatment leading to increased muscle sizes and overall lean mass, it is reasonable to speculate that people will be stronger after taking SARMs, given the relationship between muscle cross-sectional area and strength (Goodpaster et al., 2008). However, this was not typically the case. Three studies evaluated the impact of SARMs on muscular strength using a leg press and all reported no significant change in strength (Pencina et al., 2021; Papanicolaou et al., 2013; Basaria et al., 2013). Dobs et al. (2013) also reported that SARM treatment did not affect grip strength in cancer patients aged

≥45 years. Further, improvements in physical function tests were typically non-existent, including 6-minute walk tests (Pencina et al., 2021; Basaria et al., 2013; Dobs et al., 2013), stair climb speed (Pencina et al., 2021; Basaria et al., 2013; Dalton et al., 2011), stair climb power (Pencina et al., 2021), or the physical movement component of the Activity Measure for Post Acute Care measure (Papanicolaou et al., 2013). On the other hand, benefits were reported in some of the studies. Increased leg power during stair climbing was observed in two studies (Dobs et al., 2013; Dalton et al., 2011), with one reporting a significant reduction in time spent climbing stairs during the test, which indicates better function (Dobs et al., 2013). Thus, SARMs have strong anabolic properties overall that only sometimes relate to improved physical performance.

5.3.3.2 SARMs and inflammation

One clinical trial investigated the relationship between SARM therapy and circulating inflammatory markers. Dobs et al. (2013) evaluated the impact of the SARM GTx-024 on circulating levels of IL-6 and TNF-α. Confusingly, levels of TNF-α were significantly lower in the 1 mg group, compared to the placebo group and the 3 mg group, which were both similar. Levels of IL-6 were also lower in the 1 mg group but not the 3 mg group, although these differences did not reach statistical significance (Dobs et al. 2013). For reference, other statistically significant and favorable results in the 1 mg group were lower prostate specific antigen, an improved functional assessment of anorexia/cachexia therapy, and improved scores for the Functional Assessment of Chronic Illness Therapy: Fatigue Scale (Dobs et al., 2013). These results were not found in the 3 mg group, which actually had approximately six-times the circulating levels of TNF-α, or in the placebo group. Thus, the true effect of this SARM or others are on circulating inflammatory markers is not clear.

5.4 Rationale and hypothesis

Available evidence suggests that SARMs are highly anabolic drugs that lead to lean mass gains in adult men and women. Their impacts on physical function are less clear, but some benefit exists. These drugs have the potential to help manage certain aspects of frailty like muscle wasting and loss of physical function, although their effects directly on frailty are poorly studied, as are their impacts on chronic inflammation.

Androgen therapies like testosterone supplementation are promising, particularly for people with low testosterone levels. However, some concerns exist about testosterone therapy, cardiovascular risk, cancer risk for metastatic prostate cancer, and liver toxicity at supra-physiologic doses. It is arguable that the dose-dependent benefits of testosterone are thus limited by the latter point and that finding other drugs to enhance anabolism may convey further benefits without toxicity.

Further, very little is known about their effects on heart function, which has important relevance to frailty as outlined in Chapter 3. Thus, the purpose of this study was to evaluate the impact of a SARM on frailty progression in older male and female mice, as well as evaluate bodily systems related to frailty including chronic inflammation, musculoskeletal health, and cardiac function. Frailty was assessed using both a non-invasive FI and an FI-Lab, of which the latter has shown promise in humans but has not yet been used extensively in preclinical models (Sapp et al., unpublished (see Chapter 2.3)). The non-steroidal SARM chosen was RAD140 (Testolone), because of its demonstrated anabolic properties in muscle but not the prostate at low doses (Miller et al., 2011). A mouse model of aging was chosen to create a highly controlled study and to gain access to samples otherwise difficult to obtain (*e.g.*, heart tissue samples). It was theorized that SARM treatment would attenuate frailty progression and beneficially impact chronic inflammation, musculoskeletal health, and cardiac function. We tested the null hypothesis that there would be no difference in frailty progression, chronic inflammation, musculoskeletal health, or cardiac function between SARM and vehicle treated mice.

5.5 Methods

5.5.1 Study design

Mice were obtained and housed according to the methods described in Chapter 4. Mice aged 22-24 months (mean age=23.0 months; n=21 males; 15 females) were split into two groups: Vehicle-treated or SARM-treated (see Figure 5.1 for a timeline). Mice in the SARM group were hand-fed 100 μL of a cherry syrup mixture daily for 6-weeks, including 0.1% dimethyl sulfoxide (DMSO) as a vehicle for RAD140 dosed at 5 mg/kg/day. Vehicle mice were fed the same volume of syrup with 0.1% DMSO but

without RAD140. Body composition was measured at baseline and after 6-weeks of treatment. Echocardiography was completed after the 6-weeks of treatment, and mice were then euthanized (200 mg/kg sodium pentobarbital plus heparin *i.p.*). Upon full anaesthesia, the chest cavity was opened, and blood was harvested by snipping the aorta and collecting blood directly from the heart. Some blood was used immediately for a clinical chemistry analysis machine (see Chapter 5.5.4), while the rest was left at room temperature for 15-minutes before centrifuging at 1500 g for 15-minutes. Supernatant serum was pipetted and stored at -80ºC for later use. Ventricular tissue was separated into two ventricular sections containing approximately equal parts right and left ventricle. The quadriceps femoris and soleus skeletal muscles were taken, alongside ventricular tissue. Harvested tissue samples were frozen in liquid nitrogen and transferred to a -80ºC freezer for later use.

5.5.2 Body composition

Body composition was measured using DEXA at baseline and 6-weeks as described in Chapter 4.

5.5.3 Cytokines

Serum was collected upon study completion from a terminal blood draw after euthanasia. Cytokines were analyzed using 15 μL of this serum according to the methods reviewed in Chapter 4.

5.5.4 Frailty and FI-Lab

Frailty was assessed weekly using a 31-item non-invasive FI reviewed in Chapter 4, beginning at Week 0, and continuing until study completion (6-weeks later). Raters were blinded to the treatment allocation. A 34-item FI-Lab was constructed using the items in Table 5.2. These values included a 23-plex cytokine assay and 11 clinical chemistry measures obtained from terminal blood draws using an iSTAT portable diagnostic analyzer (Abbott Diagnostics) using CHEM8+ chips. Reference values for the iSTAT analyzer were obtained from two publications, referenced in the table. Cytokine references were created from serum obtained from 12-week-old C57BL/6 mice (n=5 males; n=15 females) from our lab group, housed in the same conditions. As with a previous mouse-based FI-Lab (Kane et al., 2019), each variable was coded as a “1” if the

mouse's value was beyond ±1.5 standard deviations of the mean (or median, in the case of Otto et al., 2016). The FI-Lab was then calculated as a normal FI, whereby scores are summed and divided by the total number of variables measured for each mouse.

5.5.5 Grip strength

Mice were held by their tail and allowed to grip a grate, angled at 45º downward, with their forepaws. Then, mice were smoothly but somewhat swiftly pulled posteriorly to assess maximal voluntary grip strength. This was done three times in the span of approximately 30 seconds per mouse. The grate is attached to a piezoelectric force transducer (Bioseb) and gives a maximal readout. This is measured in grams (converted from Newtons) to maximize resolution and was recorded as the mouse's grip strength.

5.5.6 Alanine aminotransferase and aspartate aminotransferase

Serum levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were quantified as a surrogate measure for liver health, since higher amounts of these enzymes relate to possible liver damage (Gianni et al., 2005). Elevated levels of ALT and AST have been found in clinical trials investigating the safety of SARMs (see Table 5.1) as well as from anabolic steroid use (Urhausen et al., 2003). Higher levels of ALT and AST from anabolic steroid use have also been demonstrated in mice (Kahal et al., 2020). Thus, both enzymes were investigated as a marker of drug-induced liver toxicity. To assess levels of both enzymes (measured separately), a sandwich ELISA was used according to the manufacturer's instructions (Abcam; ref# ab263882 and ab282882). A 1:400 dilution factor was used for each assay and the absorbance was read at 450 nm. Sample values were extrapolated from a 4-parameter logistic curve from known standards and corrected for dilutions before analysis.

5.5.7 Echocardiography

Mice were anaesthetized using 3% isoflurane infused into oxygen gas exhausting at 0.8L/min and maintained at 1-2% isoflurane during echocardiography. Mice were positioned on a heated platform containing four electrodes that monitored the animal's heart rate (HR) during imaging. The heated platform enabled mice to be kept at physiologic temperature (approximately 36.5ºC measured via rectal probe). Mice were positioned supine and attached to the platform using surgical tape by their paws, which

were laid over the electrodes. A depilating cream was then used to bare the animal's chest prior to transmission gel application and imaging. Mice were kept supine and their hearts were imaged transthoracically using a 30 MHz ultrasound probe using a Vevo 2100 electrocardiography machine (Fujifilm VisualSonics Inc.). Brightness mode (B-mode) was used to correctly position the probe in the short-axis view of the heart, followed by Motion mode (M-mode) that provided the images for later analysis. Long axis views were also recorded in B-mode for future longitudinal strain analysis. Strain analysis and estimations of cardiac structure and function were calculated using the default settings on the Vevo Lab software (version 5.6.1).

5.5.8 RNA isolation and quantification

RNA was isolated using a column-based fatty and fibrous RNA isolation kit (Bio-Rad; ref# 732-6830). In brief, frozen tissue samples were transferred to 1.5 mL tubes and disrupted using a ceramic bead homogenizer machine (Omni International) in 1 mL of PureZOL RNA isolation reagent (Bio-Rad, ref# 1708891). After a 5-minute incubation 0.2 mL chloroform was mixed followed by another 5-minute incubation time. Samples were then spun at 12000 g for 15-minutes at 4ºC and the aqueous phase was removed and placed into a fresh tube. The same volume of 70% ethanol as the aqueous phase was mixed into this tube and driven through an RNA-binding column by spinning the tube at 10000 g for 30-seconds. The column was washed with a low-stringency wash solution (added and spun at 10000 g for 30-second) before a DNase I solution was added for 15-minutes to break down genomic DNA. One high-stringency wash solution was followed by a low-stringency wash, spun in the same manner, before RNA was eluted and quantified using a NanoDrop Lite (Thermo Scientific).

5.5.9 Real-time quantitative polymerase chain reaction (RT-qPCR)

Complementary DNA (cDNA) was synthesized using a cDNA kit (Bio-Rad; ref# 17326880). One ng of RNA template was added to a super-mix containing reverse transcriptase, repeating deoxythymidine oligonucleotide primers, and random hexamer primers. Samples were then set to prime for 5-minutes at 25ºC and reverse-transcribe for 20-mintues at 45ºC, before undergoing an inactivating step (1-minte at 95ºC). Samples were stored at -20ºC for later use in real-time quantitative polymerase chain reaction (RT-

qPCR) assays.

RT-qPCR was performed using SYBR green (Bio-Rad; ref# 1725271). The SYBR and samples were thawed on ice and master-mixes were made to allow for 10 µL SYBR, 2 µL primers (forward + reverse), 2 µL cDNA template, and 6 µL nuclease-free water per well (20 µL total reaction size). Each well was covered using a transparent film and thermocycled using a CFX96 thermocycler (Bio-Rad). The first step in the cycle was a polymerase activation and DNA denaturation step (95ºC for 5-minutes), followed by 40-cycles of 10-seconds at 95ºC and 20-seconds at 60ºC. Six reference genes were checked and those chosen as reference genes had the highest stability (*i.e.*, the lowest M-values <0.5) between other reference genes. The genes *gapdh* and *hprt* were not statistically different between treatment groups in ventricular and skeletal muscle samples and were chosen as reference genes. Non-template controls were run alongside reference genes to confirm the samples were not contaminated with DNA. The $2^{-\Delta\Delta Ct}$ method was used to analyze relative changes in mRNA expression in the SARM-treated mice normalized to vehicle-treated mice separated by sex (Livak & Schmittgen, 2001). Primer sequences for each gene of interest can be found in Appendix E, Table 1.

5.5.10 Statistics

Between-treatment group tests: Longitudinal frailty assessments were analyzed using a restricted maximum likelihood method with three fixed effects (time, treatment, and time × treatment). Body composition, ALT, AST, and echocardiographic measures were all assessed using a 2-way ANOVA (time × treatment), or (time × sex) for the 6-week frailty assessments. Šídák post hoc tests were used to examine subsets within the ANOVAs and the restricted maximum likelihood method.

Cytokines: Cytokine values were non-normally distributed and were log-transformed prior to analysis (but back-transformed for graphing purposes). Data were removed case-wise if they were left-skewing outliers (defined as being above the third quartile by 1.5x the interquartile range) after log-transformation. When deemed normal according to visual inspection of quantile-quantile plots, parametric statistics were used (*e.g.*, t-tests and Pearson's *r*). If datasets were non-normal after removing outliers, between-group tests within male and female groups were assessed using the non-parametric Mann-

Whitney *U*-test, performed using a modified approach to accommodate tied values with outliers included to maximize power.[9] This approach essentially determined the exact probability by which the data would achieve a *U*-score (a test statistic) of equal or lesser value, which is the p-value presented. Any cytokines below the detection limit were substituted by the limit divided by two, which imparts a smaller bias than just using the limit alone (Hewett & Ganser, 2007). Kendall's τ-b assessed the relationship between dependent variables and cytokine values for cytokines with many missing datapoints (>20%) to help manage non-linear yet monotonic cytokine data, because it can effectively handle tied ranks within datasets with small sample sizes (Field, 2018). Specific tests are indicated where appropriate.

All statistical tests were two-tailed and considered significant at $\alpha \leq 0.05$.

5.6 Results

5.6.1 Effects of RAD140 on frailty

Non-invasive FI: Frailty scores from the non-invasive FI increased significantly over the 6-week period in both the SARM treated and vehicle treated mice ($p<0.0001$; Figure 5.3A). This was reported alongside a significant interaction term (time × treatment; $p=0.046$), although this was likely due to fluctuations within-groups rather than a linear difference in frailty progression due to SARM treatment. When mice were separated by sex, SARM treatment affected frailty progression differently over time in males (time × treatment; $p=0.034$). Frailty scores were significantly higher than baseline in the vehicle treated mice at weeks 4, 5, and 6 ($p<0.05$), which was not observed in the SARM treated mice, although there were no between-group differences (Figure 5.3B). On the other hand, frailty scores increased over time in female mice similarly between SARM treated and control mice (Figure 5.3C). SARM and vehicle treated females had higher FI scores compared to baseline in later weeks as well ($p<0.05$).

FI-Lab: A 34-item FI-Lab was constructed using the items in Table 5.2 to investigate the effect of RAD140 treatment on frailty at the 6-week timepoint. For reference, non-invasive FI scores are provided in Figure 5.4A and did not differ by treatment ($p=0.891$)

[9] Full description of the modified approach can be found here: https://www.graphpad.com/support/faq/for-a-mann-whitney-u-test-how-does-prism-handle-ties/

or by sex (p=0.858). Further, the effects of RAD140 on FI-Lab scores did not differ between sexes (p=0.665) or after pooling males and females (p=0.578). However, females had significantly higher FI-Lab scores compared to males (p<0.0001; Figure 5.4B). The same result was found when FI-Lab scores and the non-invasive FI were combined to make a 65-item FI-Combined. This did not differ by treatment group (p=0.578), but females were frailer than males (p<0.0001; Figure 5.4C).

FRIGHT and AFRAID clocks: FRIGHT and AFRAID clocks were calculated using the non-invasive 31-item FI scores for each mouse. Neither estimated biologic age (FRIGHT) or estimated time until death (AFRAID) differed overall, or when separated by sex, between RAD140 treated and vehicle treated mice (Figure 5.5A-F).

5.6.2 Effects of RAD140 on body composition

Body mass: There was a significant decline in body weight between baseline and terminal measures in both the vehicle and SARM treated mice (p<0.0001; Figure 5.6A). This was also true when separating mice by sex (Figure 5.6B & C). No effect of treatment over time was noted in pooled analysis (p=0.378) or in males (p=0.419) or females (p=0.555).

Lean mass: Because SARMs are anabolic, the impact on lean mass was a particular variable of interest in the setting of aging. SARM treatment significantly affected how lean mass changed over time, with control animals losing lean mass while RAD140 treated mice gaining it (p=0.024; Figure 5.7A). Interestingly, this result was driven by effects on males. Vehicle treated male mice significantly lost lean mass over the 6-week period (p=0.036) and RAD140 treated males maintained lean mass (p=0.878; Figure 5.7B), whereas female mice did not differ in lean mass by treatment group (interaction p=0.448). This was not the case for relative (%) lean mass, as both vehicle and RAD140 treated mice increased over time in a pooled analysis (p=0.008; Figure 5.8A). There was a non-significant increase in percent lean mass after pooling the treatment and vehicle groups in males (p=0.070; Figure 5.8B) and a significant increase in females (p<0.0001; Figure 5.8C). None of the changes in percent lean mass were moderated by SARM treatment (p>0.23 for all treatment × time interactions). Overall, RAD140 seemed to have more effect on preserving lean mass in males than females.

Fat mass: The inverse relationship between adiposity and testosterone, and the

association between testosterone treatment and fat loss suggested the measurement of fat as well. In a pooled analysis fat mass decreased over time in both groups ($p<0.0001$; Figure 5.9A). This was true also when only evaluating males ($p=0.001$; Figure 5.9B) or females ($p<0.0001$; Figure 5.9C). The same results were found when evaluating relative fat mass overall ($p<0.0001$; Figure 5.10A), in males $p=0.04$; Figure 5.10B) or females ($p<0.0001$; Figure 5.10C). No time × treatment interactions were significant (all $p>0.1$), which suggests that RAD140 acted more potently on lean mass than fat mass in these mice.

Bone mineral density: Treatment with RAD140 significantly attenuated the decline in BMD over time otherwise observed in the control mice in a pooled analysis ($p=0.001$; Figure 5.11A). In a *post hoc* analysis, BMD declined in vehicle-treated mice ($p=0.012$) but not in the SARM treated mice ($p=0.471$). When separated by sex, the time × treatment effect held in males ($p=0.004$; Figure 5.11B), as only control mice significantly lost BMD ($p=0.006$). The SARM treated males did not experience BMD loss ($p=0.446$). These findings however were not observed in females, as SARM treatment did not affect changes in BMD over time ($p=0.170$; Figure 5.11C). The direction of effects suggests an effect may be present in females, although perhaps not as robust as in males. Thus, treatment with the SARM RAD140 significantly attenuated BMD loss in older age for male mice but not female mice.

5.6.3 Effects of RAD140 on grip strength

The anabolic potential for SARMs suggested they may be able to influence skeletal muscle strength. Thus, the maximal voluntary grip strength was measured on a weekly basis to determine the effects of SARM treatment. In a pooled analysis and those separated by sex, grip strength declined over time ($p<0.0003$ for all; Figure 5.12A). The same was true when grip strength normalized to body weight was investigated ($p<0.0007$ for all; Figure 5.12B). Treatment with RAD140 had no effect on the change in grip strength over time in the pooled or sex-specific analysis for raw or normalized strength ($p>0.3$ for all six interaction terms). Thus, RAD140 treatment did not affect grip strength in these older male and female mice over the 6-week treatment perior.

5.6.4 Effects of RAD140 on systemic inflammatory markers

Because testosterone levels can suppress chronic inflammatory markers, circulating inflammatory cytokines were measured in this study to determine if RAD140 impacted their levels. Cross-sectional analyses for each sex can be found in Table 5.3. Interestingly, the RAD140 treated males had significantly lower IL-6 levels compared to their control group ($p=0.043$; Figure 5.13). Levels of TNF-α, IL-17a and KC trended downward but were not statistically significant. On the other hand, females treated with RAD140 had higher levels MIP-1α, MIP-1β, and RANTES compared to vehicle treated females ($p<0.05$ for all), and KC trended upward. The cytokines MCP-1 and GM-CSF were not different between treatment groups but are included in Figure 5.13 as a graphical reference because they are relevant to later correlational analyses. Overall, RAD140 treated males had lower levels of the potent pro-inflammatory cytokine IL-6, whereas treated females had higher levels of three different pro-inflammatory cytokines, suggesting a sex-dependent influence of RAD140 on circulating inflammatory markers.

Correlations with frailty: Because SARM treatment related to changes in circulating cytokines and due to the link between higher levels of chronic inflammation and frailty (Ferrucci & Fabbri, 2018), circulating cytokines were correlated with non-invasive FI scores (Table 5.4). Higher levels of MCP-1 were related to higher frailty scores in males ($p=0.008$). In females, higher levels of IL-5, IL-6, GM-CSF, and RANTES were related to higher frailty scores ($p<0.05$ for all). It is worth remembering that SARM treatment was related to higher RANTES levels in females. Lower levels of IL-1α were also related to higher frailty scores in females. Thus, RAD140 treatment related to higher levels of the cytokine RANTES, which proportionally related to frailty in females. Whereas in males, RAD140 treatment was not related to any changes in cytokines relating to frailty.

Correlations with bone mineral density: Because declines in BMD are associated with chronic inflammation, especially to higher IL-17a and TNF-α (Weitzmann, 2017), circulating cytokines were correlated with BMD (Table 5.5). In male mice, higher levels of IL-17a related to lower BMD levels ($p=0.001$). Notably, circulating levels of IL-17a were not significantly related to SARM treatment, although there was trend for treated male mice to have lower levels (Figure 5.13). Further, SARM treated male mice

maintained BMD while vehicle treated males lost it (Figure 5.10), suggesting a link. TNF-α did not relate to BMD in males (p=0.544). Higher levels of MCP-1 and IL-4 related to lower BMD in females, although there were no overt differences between SARM treated and control females in levels of these cytokines. Overall, this suggests that RAD140 may in part help prevent bone loss by suppressing chronic inflammation in males, while no strong connection was identified in females.

5.6.5 Effects of RAD140 on circulating liver enzymes

The possibility for drug-related liver toxicity during SARM treatment warranted the monitoring of liver health. Serum concentrations of the metabolic enzymes ALT and AST, which are found in high concentrations in the liver, were measured as a surrogate for liver damage (Figure 5.14). ALT levels were not elevated in RAD140 treated mice versus vehicle controls in males (p=0.267) or females (p=0.564). AST levels on the other hand trended upward in male mice but did not reach statistical significance (p=0.075). Measurements were available for only four females due to oversaturation of the assay, although similar numbers of females were excluded for this reason from both treatment groups (4 vehicle and 5 SARM treated). Thus, RAD140 at 5 mg/kg/day for 6-weeks was not related to higher AST levels compared to vehicle treated males, and ALT levels were not elevated. Similarly, ALT levels were not elevated in females, but the effects on AST levels were inconclusive.

5.6.6 Effects of RAD140 on mRNA in skeletal muscle

Because SARMs act by altering gene transcription, mechanisms of hypertrophy and genes that can be affected by androgens were investigated using RT-qPCR and are displayed alphabetically in Figure 5.15. Specifically, mRNA levels of the hypertrophy-related genes insulin growth factor-1 (*IGF-1*), mammalian target of rapamycin (*mTOR*), myostatin (*mstn*), and protein kinase B (*Akt*) were measured, given their relevance to testosterone mediated muscle growth (White et al., 2013; Braga et al., 2012). L-type calcium channels (*cacna1c*) and myosin heavy chain (*myh*) isoforms 1, 2, 4, 6, and 7 were also investigated, to evaluate the impact of SARM treatment on aspects of calcium handling and contractile proteins, which are mediated by testosterone (Sakakibara et al., 2021; Anttila et al., 2008). No overt changes were observed in normalized gene

expression for the 12 genes evaluated (Figure 5.15), although myostatin was non-significantly elevated by RAD140 treatment in a pooled analysis of both sexes (p=0.051).

5.6.7 Effects of RAD140 on left ventricular structure and function

Because circulating testosterone has related to cardiac function in humans (*e.g.*, Gheorghe et al., 2021) and in mice (Banga et al., 2021), the effects of RAD140 treatment on cardiac structure and function were evaluated using echocardiography. Firstly, there were no clear differences in left ventricular structure between RAD140 treated males or females compared to vehicle treated controls (Figure 5.16A-G). This included analyses of estimated mass and anterior and posterior wall thicknesses during end systole and end diastole.

Systolic function: However, there were clear differences between RAD140 treated mice and vehicle controls in measures of systolic function (Figure 5.17). Stroke volume was significantly higher in RAD140 treated mice in a pooled analysis (p=0.013), as was cardiac output (p=0.021), despite no changes in HR (p=0.874; Figure 5.17A-C). These changes were underpinned by a higher fractional shortening and ejection fraction for SARM treated mice in a pooled analysis (p=0.016; Figure 5.17E & F), which had a significant *post hoc* test for the males (p=0.050 and 0.042, respectively). Isovolumic contraction time (IVCT) was measured via blood flow using pulse-wave doppler (PWD on Figure 5.17) or tissue movement at the mitral annulus using the pulse-wave tissue doppler analysis (PWT). The PWD recording revealed that sex impacted how SARM treated mice related to their respective controls (sex × treatment). Male mice treated with RAD140 took slightly longer to transition from relaxation to contraction compared to male controls but was shortened in females using PWD (p=0.022; Figure 5.17F). The trend was similar for the PWT recording, but non-significant (p=0.100; Figure 5.17G).

Global longitudinal strain (GLS) was also measured, which denotes the relative movement of the left ventricle in the longitudinal axis each beat. This strain is in negative units because it denotes the shortening of the heart muscle. Male SARM treated mice tended to have increased strain (*e.g.*, more negative) versus controls, whereas the opposite was observed in females, but this did not reach significance (p=0.07 Figure 5.17H). However, the difference in global circumferential strain was significantly affected by

treatment and sex (sex × treatment p=0.050; Figure 5.17I), with male SARM treated mice having increased strain compared to controls (p=0.042). Circumferential strain tracks how much the left ventricle twists while beating, which is normal, and more 'negative' numbers mean more movement. Thus, RAD140 treated mice had increased systolic function relative to vehicle treated controls, especially in stroke volume and ejection fraction, and males in particular had higher global myocardial strains.

Myocardial strain was then separated into epicardial and endocardial sections for a deeper analysis. Further, time-to-peak (T2P) and the magnitude of tissue velocity, strain, and strain rate were assessed in three myocardial axes (longitudinal, radial, and circumferential). Radial strain denotes the thickening of tissue during each beat, which happens as the muscle contracts, while the other two strains are described above. In the longitudinal axis, males and females differed in their relationships between SARM and vehicle treated mice (Figure 5.18H & L). Male SARM treated mice had greater epicardial longitudinal strain and strain rates versus controls, whereas the opposite was seen in females (sex × treatment p=0.024 and 0.020, respectively). On the other hand, SARM treatment was associated with a faster T2P longitudinal strain rate in the endocardium (p=0.040; Figure 5.18I). Similarly, SARM treated males had increased tissue velocity and strain in the endocardium, and strain in the epicardium, in the radial axis versus control mice ($p<0.05$ for all; Figure 5.19B, F, & H). There were no significant differences between treated and control female mice in the radial axis. Lastly, endocardial circumferential tissue velocity, strain, and strain rate was again greater in SARM treated males versus controls ($p<0.05$ for all; Figure 5.20B, F, & J), but no differences were observed in females. Thus, myocardial strain was in general greater (better) in RAD140 treated males versus controls, but these differences were not seen in females.

Myocardial strain and circulating cytokines: Because impaired myocardial strain can relate to higher levels of chronic inflammation (Dessì et al., 2011), global circumferential strain was correlated with circulating cytokines. As a reminder, more 'negative' values denote more circumferential movement, and a positive correlation would therefore mean as one cytokine increases in concentration, heart movement declines. In male mice, higher levels of IL-1α, IL-6, and MCP-1 related to worse (lower) circumferential

myocardial strain ($p<0.05$ for all; Table 5.6). This was notable, given the anti-inflammatory relationship on IL-6 in the RAD140 treated males. On the other hand, no relationships between any cytokine and global circumferential strain were found in female mice.

Diastolic function: Diastolic function was assessed by evaluating the flow of blood into the left ventricle during the early (E) and atrial (A) filling stages along with their ratio (Figure 5.21A, B, & C). An impaired heart may have difficulty filling, leading to a reduced E wave and subsequent lower E/A ratio. No significant differences were observed in these parameters ($p>0.05$). Further, the ratio between E and E' was evaluated (E' is the velocity of the mitral valve annulus, which moves slower when the heart has difficulty relaxing). No effect of treatment was noted in E/E' ($p=0.861$; Figure 5.21D). However, the time spent transitioning from contraction into relaxation was shorter in RAD140 treated males but not females (Figures 5.21E & F). The isovolumic relaxation time (IVRT) was significantly lower in males treated with RAD140 compared to vehicle controls when evaluated using PWT ($p=0.014$), which was the opposite for females (interaction term $p=0.012$; Figure 5.21F). Thus, SARM treatment was associated with one metric of improved diastolic function in males and not females, although other measures were unaffected. This and other significant cardiac changes are graphically summarized in Figure 5.22.

5.6.8 Effects of RAD140 on mRNA expression in ventricular tissue

Androgens are known to influence calcium handling and the expression of different myosin heavy chains in the heart (Ayaz & Howlett, 2015). Given the changes in contractile function seen in RAD140 treated mice, genes encoding proteins relating to calcium handling were measured from ventricular samples. These included the sarcoendoplasmic reticulum calcium ATPase-2 (*atp2a2*), L-type calcium channel (*cacna1c*), calsequestrin (*casq2*), phospholamban (*pln*), and the ryanodine receptor type-2 (*ryr2*). mRNA levels of the cardiac signalling proteins β_1-adrenergic receptor (*adrb1*) and calcium/calmodulin dependent protein kinase kinase 2 (*camkk2*) were also measured, alongside genes for the AR (*ar*), two types of collagen (*col1a1* and *col3a1*), and two types of myosin heavy chains common in the heart (*myh6* and *myh7*). Only the expression

levels of mRNA encoding for the AR differed after SARM treatment, with treated mice having lower expression compared to vehicle treated mice ($p=0.015$; Figure 5.23B). The other genes were not significantly affected ($p>0.05$).

5.7 Discussion

The purpose of this study was to investigate the impact of treating older male and female mice with a SARM on frailty and bodily systems related to frailty. Frailty scores as measured by both a non-invasive FI and an FI-Lab were not changed after 6-weeks of treatment with RAD140 at 5 mg/kg/day in either sex. Frailty scores in males increased more in the vehicle treated group than in the RAD140 treated group, although no between-group differences were significant. Further, no between-group differences were observed in FRIGHT or AFRAID scores. Although this result does not clearly support the hypothesis that a SARM would prevent frailty progression, it cannot be concluded that treatment with RAD140 will not impact frailty. Pharmacologic interventions in mice tend to require several months to impact frailty scores when older, healthy C57BL/6 mice are investigated (*e.g.*, Asadi Shahmirzadi et al., 2020; Keller et al., 2019). Thus, it is possible that treatment with RAD140 for more than 1.5 months is needed to see a meaningful change in FI scores in older mice. This idea is supported by the positive differences observed in several health parameters associated with frailty in the RAD140 treated mice, especially males, compared to vehicle treated controls.

5.7.1 RAD140 positively affected cardiac function in males and females

An interesting finding from this study was the association between RAD140 treatment and improved cardiac function in older but otherwise healthy mice. Frailty in humans has been related to worse myocardial strain and lower EF in both men and women (Tan et al., 2021). Both female and male RAD140 treated mice had higher EFs relative to their vehicle controls in this study. Further, treated male mice had much higher (better) myocardial strains compared to the vehicle controls. The favorable quickening in IVRT in treated males was also notable. Lower testosterone levels have been shown to associate with longer IVRTs in older male mice with low testosterone (Banga et al., 2021). This suggests that RAD140 can act to reverse this change, and this may be mediated by the activation of the AR. It may therefore be worthwhile to further

investigate the effect of SARMs on cardiac function in the setting of aging or even disease.

The relationship between higher circulating cytokines in males, especially IL-6, and reduced myocardial strain was interesting and provides more evidence linking myocardial dysfunction to circulating cytokines and mechanisms of frailty. Higher IL-6 levels are linked with worse myocardial systolic function in healthy middle-aged men and women (Dessì et al., 2011; Yan et al., 2010). Higher IL-6 levels have also associated with poorer myocardial strain in children with multisystem inflammatory syndrome (Chang et al., 2021). In men with chronic heart failure, higher testosterone levels were associated with more favorable cardiac resynchronization therapy, which was paralleled by decreases in IL-6 levels (Enina et al., 2018). Further, reduction of intracardiac IL-6 levels by raloxifene in a pressure-overload model of heart failure helped preserve ejection fraction by inhibiting hypertrophy (Huo et al., 2021). Whether RAD140 would have similar effects is not known, although the results from the present study suggest some linkage. If causality were proven, there may be a role for SARM treatment to help mitigate detrimental pro-inflammatory effects on the heart and possibly improve function, at least in males.

The increase in EF and cardiac output for females treated with RAD140 is difficult to explain with the available evidence. Left ventricular EF is determined by both myocardial strain and wall thickness (MacIver et al., 2015), but neither were substantially different in female mice treated with RAD140 versus vehicle treated mice. It is likely that small differences in systolic wall thicknesses (anterior and posterior) and ventricular diameters, with little change in diastolic thicknesses or diameter, led to the difference in CO. This is because SV is directly determined by these parameters and is elevated in female mice on RAD140 versus vehicle treated controls, while HR was similar. Nonetheless, more samples are needed in future trials to adequately power a study to elucidate the effects of RAD140 on female cardiac function.

5.7.3 RAD140 positively affected bone mineral density

Although the clinical evidence in humans that SARMs can improve BMD is lacking, preclinical evidence is promising. The results reported here suggested that

RAD140 prevented the loss of BMD in older male mice, but not females. Other SARMs have demonstrated efficacy in promoting BMD in males, however. The SARM LGD2226 was effective at increasing BMD in younger (7-week-old) male orchiectomized Sprague-Dawley rats to levels of sham controls (Miner et al., 2007). However, another SARM, Ostarine did not increase BMD in male orchiectomized Sprague-Dawley rats, although testosterone treatment did (Komrakova et al., 2020). This suggests that not all SARMs are equal at promoting BMD in males, but that RAD140 may be one that helps. It is notable that the mice in this study were not orchiectomized and yet saw improvements in bone health. This may be explained, however, by the age-related decline in testosterone around this age for this strain of mouse (Banga et al., 2021).

The effects of RAD140 on BMD in females were non-significant. Unlike males, the female controls did not lose BMD over the 6-weeks. Thus, although there was a slight increase in BMD for treated females, there was no overall treatment effect. Other studies evaluating the impact of SARMs on BMD in females generally conclude that they help increase BMD. Contrary to the effects in males, the SARM Ostarine was able to reverse ovariectomy-induced losses in BMD in Sprague-Dawley rats (Hoffmann et al., 2019). Similarly, the SARM S-4 was able to increase BMD above the levels of ovariectomized rats, but not quite to baseline levels (Kearbey et al., 2007). Given the slight upward trend of the BMD in the treated female group, RAD140 may have the potential to prevent BMD loss in similar models, although this needs further testing over a longer timeframe. Sex differences in the effects on BMD would also be beneficial to elucidate which SARM works best for each sex. Nonetheless, interest in SARMs as a potential therapy for osteoporosis is growing, and although preclinical work like that presented here is promising, clinical trials are also needed to further the rationale (Xie et al., 2022).

5.7.4 RAD140 positively affected lean mass into older age

In this study, the male vehicle treated mice lost lean mass over the 6-week period, whereas SARM treated mice did not. This effect was not observed in females, where there was no change in the vehicle groups and a small non-significant increase in the treated group. The preservation of lean mass by RAD140 in the males was a salient finding, particularly in the setting of aging. Discovering drugs that prevent muscle

wasting is important, because there is an unmet need to develop effective and safe anabolic compounds for clinical use (Da Fonseca et al., 2020). The results of whole-body lean mass must be interpreted with caution, however. Testosterone administration in males can lead to hypertrophy of parenchymal organs, which count as lean mass in DEXA imaging alongside skeletal muscle, bones, and connective tissue (Prado & Heymsfield, 2014). In men receiving 50-600 mg testosterone enanthate per week, liver volume increases in a dose-dependent fashion alongside lean mass, although effects on the kidneys only trended upward and spleens were unchanged (Gagliano-Jucá et al., 2017). In Wistar rats, anabolic steroids have led to increased liver weight too (Viera et al., 2008; Friedel et al., 2006). Increases in organ volume could therefore have contributed to the increases in lean mass. However, higher testosterone levels relate to heavier skeletal muscles in C57BL/6 mice (Davidyan et al., 2021), suggesting that increases in skeletal muscle likely also contribute to increases in lean mass after SARM treatment. Thus, the results presented here suggest that RAD140 may be an effective drug for increasing or maintaining lean mass, particularly in males, while more work would need to be done with females. Determining the relative effects on skeletal muscle versus parenchymal organs would be useful in understanding the therapeutic anabolic potential of these drugs.

There was also an upward trend in myostatin expression in SARM treated males and females. Myostatin is a negative modulator of skeletal muscle growth and increased expression would suggest impaired anabolism (Aiello et al., 2018). In humans treated with testosterone, myostatin expression decreased significantly in skeletal muscle (Ghanim et al., 2019). However, the opposite has been demonstrated in rats, who showed significantly reduced myostatin gene expression after testosterone treatment (although myostatin protein levels were much higher; Dablo et al., 2017). Further, treatment with the SARM Enobosarm led to significantly elevated myostatin levels in levator ani muscles in 14-week-old mice, despite marked hypertrophy in the muscle (Dubois et al., 2015). One mediating effect of myostatin is follistatin (*fst*), which halts the inhibition on muscle growth that myostatin exerts (Amthor et al., 2004). The fold-change in *mstn* was lower than *fst*, so it is possible that this contributed to the anabolic response observed, particularly in males, but this is not certain from the evidence available in this study. Further, long-term testosterone therapy does not seem to upregulate myostatin levels

(Kruse et al., 2020). Thus, skeletal muscle hypertrophy is clearly possible in the presence of increased myostatin levels during SARM treatment. Further research investigating the increase in myostatin levels after SARM treatment and other signalling mechanisms like *fst* would help elucidate whether the upregulation is overpowered other anabolic signals, and if so, how.

Despite preservation of lean mass due to SARM treatment, there was no effect on grip strength after SARM treatment. This lack of strength gain observed in these trials is similar to results from clinical studies reviewed in Table 5.1. Although there were no appendicular assessments made here, it is likely that mice increased skeletal muscle size in their limbs. This relationship seems paradoxical when reflecting on the relationship between muscle size and strength, which is usually proportional. However, this is not always the case, and highlights an important challenge for SARMs and other anabolic therapies used on their own to improve physical function (Dalton, 2017). If paired with a resistance training program the strength gains would likely be found, and possibly augmented, by treatment with a SARM, as is seen with testosterone (Bhasin et al., 1999). Until drugs are developed that lead to strength gains, resistance training will likely remain necessary to see fitness gains in this regard. In fact, strength training interventions can lead to significant increases in strength without noticeable hypertrophy, even in older adults (Strasser et al., 2018). Thus, it is not surprising that these older mice did not exhibit an increase in grip strength. SARMs may play a crucial role in promoting lean mass preservation or gains in times of catabolism (*e.g.*, age or disease). However, SARM treatment with a concurrent exercise regimen may be necessary to translate this anabolism into physical function (Bhasin, 2015). Future studies investigating this notion would be illuminating.

5.7.5 RAD140 differentially affected chronic inflammation in males and females

Because elevated circulating levels of pro-inflammatory cytokines contribute to frailty progression, the fact that RAD140 treated males had lower levels of IL-6 was notable. As reviewed in Chapter 3, higher circulating levels of IL-6 are associated with components of physical frailty (Xu et al., 2015) and can play a major role in frailty progression in mice (Jergović et al., 2021) and humans (McKechnie et al., 2021). Further,

low testosterone levels relate to higher IL-6 levels in men (Tremellen et al., 2017; Maggio et al., 2006) and IL-6 levels increase during androgen deprivation (Hoogland et al., 2021). It is possible that RAD140 acted to suppress IL-6 production. However, in clinical trials testosterone treatment has led to either no change (Mohler et al., 2018; Kalinchenko et al., 2010) or a slight increase in serum IL-6 levels (Basaria et al., 2013). Further, testosterone administration increased levels of IL-6 in the prostate of rats (Abo-Youssef et al., 2020) but prevented IL-6 production in the aorta of mice with aortic aneurysms (Son et al., 2019). It is therefore difficult to understand why RAD140 treated mice had lower IL-6 levels in this study based on testosterone studies, and no mechanistic evidence from SARMs on inflammation is available yet.

It is also worth noting that testosterone replacement in men led to the reduction of other important pro-inflammatory cytokines IL-1β and TNF-α in some (Dhinda et al., 2016; Kalinchenko et al., 2010; Malkin et al., 2004) but not all human trials (et al., 2014; Kapoor et al., 2007). Overall, the relationship between testosterone and chronic inflammation is not fully understood, and the effects of SARMs are not well characterized. The finding that IL-6 levels were lower in RAD140 treated mice is undoubtedly interesting from a frailty perspective, but it must be noted that IL-6 levels in males were highly variable in this study and more mechanistic studies are needed to confirm that RAD140 caused this difference. IL-6 levels also did not relate to frailty scores in male mice in the present study, which casts doubt on short-term implications. However, it is possible that the long-term impact of reduced IL-6 levels could lead to improved frailty scores, given the proportional relationship between IL-6 and frailty (McKechnie et al., 2021; Jergović et al., 2021). It is important overall to confirm and identify how RAD140 may lead to lower circulating levels of IL-6.

In this study higher circulating levels of IL-5, IL-6, GM-CSF, and RANTES related to frailty levels in female mice, while only higher levels of MCP-1 related to higher frailty scores in males. Interestingly, this is not the first time that higher circulating cytokines related to higher FI scores in primarily in female mice but not males. A study by Kane et al. (2019) reported that IL-6, IL-9, and IFN-γ positively correlated with FI scores in older female mice. This adds to the evidence suggesting that IL-6 is a common

mechanism of frailty between humans and mice, specifically females (Heinze-Milne et al., 2022). Why lower levels of IL-1α related to higher frailty scores in females is uncertain. Keller et al. (2019) reported IL-1α levels were lower in a group of female mice treated with the ACE inhibitor enalapril, which had lower frailty scores compared to mice eating normal chow. Their result suggested that higher IL-1α might relate to higher frailty scores, but the present study suggests the opposite.

In addition, the relationship between elevated MCP-1 levels and higher frailty scores in male mice is consistent with many findings in mixed-gender human studies (Table 3.1), but not in mice (Table 3.3). Two studies using mice reported that levels of MCP-1 lowered in response to an intervention to help with frailty (Asadi Shahmirzadi et al., 2020; Keller et al., 2019), although these findings were in females. None of the four studies identified in Table 3.3 reported a relationship between circulating MCP-1 levels and frailty in males. Thus, the relationship between elevated MCP-1 levels and higher frailty scores in males is uncommon in male mice, but sometimes present in humans. Lastly, it is important to note that RAD140 treated female mice had higher levels of RANTES, MIP-1α, and MIP-1β, suggesting a pro-inflammatory response. This may be because testosterone can act differently between men and women, being anti-inflammatory in men but pro-inflammatory in women (Di Stasi et al., 2022). Because higher RANTES levels associated with higher FI scores, this result is potentially concerning. This suggests that SARMs may promote elements of chronic inflammation in females. Regarding the overall impact of RAD140 treatment on circulating cytokines, the beneficial difference of IL-6 in males and the pro-inflammatory responses related to SARM treatment in females are two opposite and intriguing findings, although their significance in frailty is uncertain.

5.7.6 Safety concerns of RAD140 treatment

Circulating levels of AST trended upward in RAD140 treated males, although the meaningfulness of this is questionable. While elevated AST levels can be related to drug-induced liver injury in mice, they may not be robust predictors of toxicity in humans (Dirven et al., 2021). Further, clinical practice guidelines suggest that AST levels need to be at least 3-times higher than the upper normal limit (along with other tests) to warrant

concern for liver toxicity (Avigan et al., 2014). Because ALT levels in males were not elevated, the AST:ALT ratio increased after RAD140 treatment. An increased yet non-pathologic AST:ALT ratio is related to higher testosterone levels and reduced liver steatosis risk in men, suggesting a beneficial correlation between androgens, this ratio, and liver health (Yang et al., 2018). Thus, the safety of this dose of RAD140 does not seem concerning, at least after evaluating serum ALT and AST levels. This agrees with the clinical trials in Table 5.1. Although elevated ALT levels in clinical trials were observed, they were typically of small relative incidence (*e.g.*, 6% for the treatment group versus 1% in the placebo group in Papanicolaou et al., 2013) and resolved themselves while still on drug treatment (*e.g.*, Dalton et al., 2011). Further, although dose-dependent effects have been observed, the majority of the increases remained within normal ranges for AST and ALT (Pencina et al., 2013). Thus, although increases in circulating liver enzymes was a relatively consistent finding, the clinical relevance is not fully known but is likely insignificant. Future studies investigating its significance preclinically and clinically would be helpful, especially with regards to polypharmacy.

Lastly, it is important to note other safety concerns relating to RAD140 that have been reported from medical case studies. Young men have presented clinically after taking RAD140 with acute myocarditis (Padappayil et al., 2022) and drug-induced liver injury (Barbara et al., 2020). Abuse of other SARMs has also been related to liver injury, such as Ligandrol (Koller et al., 2021) and Ostarine/Enobosarm (Bedi et al., 2021; Koller et al., 2021). These toxicities may be due to the consumption of higher-than-recommended doses, but also due to the impurity of SARMs purchased online (Leaney et al., 2021). These case studies highlight the risks of SARMs not related to traditional therapeutic use.[10]

5.7.7 Limitations of this work

The relatively short study duration of 6-weeks is a limitation to the primary study

[10] SARMs pose a unique risk for young people, particularly men searching for unrealistic body images of hyper-muscular physiques, given the health risks during abuse and their availability online (Heinze-Milne & Joy, 2020). They are often sold as "safer" than steroids and have a strong online presence in fitness communities. Their unregulated nature makes them a dangerous compound that is easily marketed to young adults. While the United States Congress has been presented the SARMs Control Act of 2019 (S.2895) aimed to regulate SARMs like anabolic steroids, the bill has not been passed as of July 18th, 2022.

objective to investigate the impact of RAD140 on frailty. As reviewed earlier, longer periods of time (*e.g.*, multiple months) may be necessary to observe a difference in frailty score. The beneficial changes, particularly for males in chronic inflammation, cardiac function, and lean mass after RAD140 treatment suggests that longer treatment may have led to reductions in frailty scores. Investigations into the effect of RAD140 on cardiac function could have been improved by measuring baseline parameters too, strengthening the power of these intriguing observations. Another consideration is the weekly assessment of grip strength, which declined significantly over the 6-week period by approximately 30 g. Grip strength in a different study was only 20 g lower in 24-month-old C57BL/6 mice versus 18-month-old mice (Takeshita et al., 2017). Thus, although possible, the decline of ~20g/month in the present study is much steeper than the ~3.33 g/month decline reported elsewhere. Motivation to pull is a key determinant of grip strength test validity (Takeshita et al., 2017) and weekly measurements may have led to familiarity and reduced motivation during testing. This could lead to steeper declines in grip strength than what is true, although this is not proven.

5.7.8 Conclusions

Six-weeks of RAD140 treatment in older male and female C57BL/6 mice did not lead to improvements in frailty scores compared to vehicle treated controls as measured by a non-invasive FI, an FI-Lab, or combined values. However, there were key beneficial changes in bodily systems relating to frailty that support future investigations. Notably, lean mass and BMD was preserved, particularly in males. Similarly, the pro-inflammatory cytokine IL-6 was present in lower concentrations in RAD140 treated males versus controls, but not in females. Several markers of systolic function as well as IVRT were better in RAD140 treated males too, but not in females. Measurement of gene expression was inconclusive in elucidating the mechanisms of these changes in cardiac and skeletal muscle, however. Further, whether these changes, especially those in lean mass, will lead to meaningful differences in frailty is not known. Future studies investigating the effects of longer-term administration of RAD140 in males and females on frailty and mechanisms of frailty would be enlightening.

5.8 Tables

Table 5.1 Summary of clinical studies investigating selective androgen receptor modulators regarding musculoskeletal health measures related to frailty and safety profiles.

Year	Reference	Methods	Results	Safety profile versus control
2021	Pencina et al.	OPK-88004 (1, 5, or 15 mg) for 12-weeks vs placebo in prostate cancer survivors	↔ 6MWT ↑ App. LBM ↑ LBM ↔ Fat mass ↓ Percent fat mass ↔ Stair climb power (loaded and unloaded) ↔ Strength, leg press	↑ ALT ↑ AST ↑ Hematocrit (5 mg)
2018	Neil et al.	GSK2881078 (men=0.75, 1.5, and 4 mg; women=0.5, 0.75, and 1.5 mg) for 28-56 days in people aged ≥50 years	↑ App. LBM (men and women) ↑ LBM (men and women) ↑ Thigh muscle volume (men and women)	↑ ALT
2013	Papanicolau et al.	MK-0773 (50 mg/day) in older sarcopenic women vs. placebo for 6-months	↔ AMPAC – Physical movement ↑ App. LBM ↔ Gait speed ↑ LBM ↔ SPPB ↔ Strength, leg press	↑ ALT ↑ AST ↑ Hematocrit
2013	Basaria et al.	LGD-4033 (0.1, 0.3, or 1 mg) in adult men vs placebo for 21-days	↔ 6MWT ↔ App. LBM ↔ Fat mass ↑ LBM ↔ Stair climb speed and power ↔ Strength, leg press	↔ Hematocrit ↔ Liver enzymes ↔ PSA
2013	Dobs et al.	GTx-024 (1 or 3 mg) in adult cancer patients (aged ≥45 years) for 113 days	↔ 6MWT ↑ Anorexia cachexia subscale (1 mg) ↔ Fat mass ↔ Grip strength ↔ IL-6 levels	↔ ALT ↔ Malignant neoplasm progression ↓ PSA (1 mg)

Year	Reference	Methods	Results	Safety profile versus control
			↑ LBM ↑ Stair climb power ↓ Stair climb time ↓TNF-α levels (1 mg)	
2011	Dalton et al.	GTx-024 (0.1, 0.3, 1, or 3 mg) for 12-weeks for older men and women	↓ Fat mass (3mg) ↑ LBM (men and women; 3mg for both) ↑ Stair climb power (3mg) ↔ Stair climb speed	↑ ALT (3mg) ↓ HDL

Notes: 6MWT = 6-minute walk test; AMPAC = Activity Measure for Post Acute Care; App. LBM = Appendicular lean body mass;

Table 5.2 Items included to create a frailty index based on laboratory measures.

	Males			Females			
Analyte	**-1.5SD**	**Mean***	**+1.5SD**	**-1.5SD**	**Mean***	**+1.5SD**	**Unit**
Anion Gap[a]	8.30	12.80	17.30	11.85	16.80	21.75	mmol/L
Blood urea nitrogen[b]	0	24.00	67.50	27.25	31.00	34.75	mg/dL
Carbon dioxide (total)[c]	23.70	26.40	29.10	20.05	22.30	24.55	mmol/L
Chloride[b]	105.17	109.00	112.83	105.79	110.00	114.21	mmol/L
Creatinine[b]	0.65	0.80	0.95	0.35	0.80	1.25	mg/mL
Eotaxin[d]	249.94	670.65	1091.36	187.86	518.28	848.71	pg/mL
Glucose[a]	199.00	236.80	274.60	133.55	180.50	227.45	mg/dL
Granulocyte colony stimulating factor[d]	20.85	56.02	91.19	21.39	49.82	78.26	pg/mL
Granulocyte-macrophage colony stimulating factor[d]	9.63	20.65	31.67	11.09	22.54	33.99	pg/mL
Hematocrit[b]	42.79	46.00	49.21	43.52	45.90	48.29	% Packed cell volume
Hemoglobin[a]	12.71	13.60	14.49	13.49	13.90	14.31	g/dL
Interferon-γ[d]	1.75	15.07	28.39	3.09	12.22	21.34	pg/mL
Interleukin-10[d]	4.52	19.05	33.59	2.28	20.01	37.74	pg/mL
Interleukin-12(p40)[d]	31.96	55.37	78.78	32.98	53.43	73.88	pg/mL
Interleukin-12(p70)[d]	0	70.54	152.48	14.85	62.21	109.56	pg/mL
Interleukin-13[d]	5.06	99.79	194.51	19.98	99.08	178.19	pg/mL
Interleukin-17A[d]	0	46.58	99.28	8.26	37.58	66.89	pg/mL
Interleukin-1α[d]	3.40	5.38	7.36	2.02	4.82	7.61	pg/mL
Interleukin-1β[d]	0	163.29	328.08	163.11	230.48	297.85	pg/mL
Interleukin-2[d]	1.77	12.10	22.42	1.69	7.33	12.98	pg/mL
Interleukin-3[d]	0	5.20	10.47	0.92	4.24	7.56	pg/mL
Interleukin-4[d]	1.41	4.20	6.99	1.18	4.31	7.43	pg/mL
Interleukin-5[d]	1.79	4.96	8.13	1.56	6.47	11.37	pg/mL
Interleukin-6[d]	0.91	4.07	7.24	1.46	3.73	5.99	pg/mL
Interleukin-9[d]	36.92	66.66	96.41	21.03	69.41	117.79	pg/mL
Ionized calcium[c]	0.95	1.10	1.25	0.85	1.00	1.15	mmol/L
Keratinocyte-derived chemokine[d]	19.32	26.23	33.14	16.47	26.96	37.44	pg/mL
Macrophage inflammatory protein-1α[d]	3.69	6.56	9.43	2.97	5.69	8.41	pg/mL
Macrophage inflammatory protein-1β[d]	0.14	13.73	27.31	1.73	9.77	17.81	pg/mL
Monocyte chemoattractant protein-1[d]	22.67	92.10	161.52	4.23	70.50	136.78	pg/mL
Potassium[c]	3.60	4.10	4.60	3.23	3.80	4.37	mmol/L
RANTES[d]	8.00	14.52	21.04	7.52	15.38	23.24	pg/mL
Sodium[a]	141.88	148.00	154.12	139.49	146.00	152.51	mmol/L
Tumor necrosis factor-α[d]	0	204.25	444.45	24.53	138.33	252.13	pg/mL

Notes: [a] Denotes Kane et al., 2019. [b] Denotes Schnell et al., 2002. [c] Denotes Otto et al., 2016. [d] Denotes values obtained from our lab group. * Values are all mean except for those from Otto et al., 2016. RANTES = Regulated on Activation, Normal T Expressed and Secreted; SD = Standard deviation.

Table 5.3 Results from t-tests or Mann-Whitney U-tests comparing circulating cytokine values between vehicle and RAD140-treated male and female mice when including all values and datum substitutions. See section 5.3.4 for arbitration between parametric and non-parametric tests. Significant results are in bold, and their test statistics can be found in section 5.3.4.

	Males		**Females**	
Cytokine	***t*-test p-value**	**MW-*U* test p-value**	***t*-test p-value**	**MW-*U* test p-value**
IL-1α	0.420		0.867	
IL-1β	0.825		0.083	
IL-2	0.993		0.280	
IL-3	0.146		0.866	
IL-4	0.986		0.822	
IL-5	0.935		0.484	
IL-6		**0.043**		1.000
IL-9	0.635		0.657	
IL-10	0.600		0.880	
IL-12(p40)	0.618		0.345	
IL-12(p70)		0.468		1.000
IL-13		0.918		1.000
IL-17A	0.093		0.883	
Eotaxin	0.470		0.082	
G-CSF	0.178		0.262	
GM-CSF		0.512		0.383
IFN-γ	0.409		0.298	
KC	0.073		0.150	
MCP-1	0.355		0.367	
MIP-1α	0.116		**0.016**	
MIP-1β	0.585		**0.040**	
RANTES	0.848		**0.039**	
TNF-α	0.105		0.618	

Table 5.4 Relationship between a 31-item non-invasive frailty index and log-transformed circulating cytokines

	Males				Females			
Cytokine	**Pearson's *r***	**p-value**	**Kendall's τ-b**	**p-value**	**Pearson's *r***	**p-value**	**Kendall's τ-b**	**p-value**
IL-1α	0.109	0.638			**-0.674**	**0.011**		
IL-1β	-0.153	0.507			0.090	0.770		
IL-2	-0.057	0.811			0.196	0.502		
IL-3	-0.180	0.447			0.314	0.275		
IL-4	-0.190	0.409			0.134	0.647		
IL-5	-0.020	0.935			**0.593**	**0.025**		
IL-6			0.108	0.531			**0.584**	**0.007**
IL-9	-0.232	0.311			0.293	0.309		
IL-10	0.059	0.804			-0.437	0.156		
IL-12(p40)	-0.312	0.181			-0.165	0.590		
IL-12(p70)			-0.189	0.259			0.186	0.392
IL-13			-0.110	0.519			0.212	0.328
IL-17A	0.060	0.796			0.197	0.519		
Eotaxin	0.081	0.726			0.081	0.793		
G-CSF	0.249	0.277			0.207	0.497		
GM-CSF			-0.067	0.698			**0.455**	**0.039**
IFN-γ	-0.258	0.260			0.094	0.749		
KC	-0.051	0.836			-0.388	0.171		
MCP-1	**0.560**	**0.008**			-0.160	0.601		
MIP-1α	0.291	0.226			0.169	0.581		
MIP-1β	-0.083	0.720			0.255	0.400		
RANTES	0.054	0.822			**0.593**	**0.033**		
TNF-α	-0.100	0.675			0.269	0.352		

Table 5.5 Relationships between bone mineral density and log-transformed circulating cytokines

	Males				Females			
Cytokine	**Pearson's *r***	**p-value**	**Kendall's τ-b**	**p-value**	**Pearson's *r***	**p-value**	**Kendall's τ-b**	**p-value**
IL-1α	-0.105	0.679			0.139	0.701		
IL-1β	**-0.469**	**0.050**			-0.518	0.125		
IL-2	-0.292	0.255			-0.292	0.413		
IL-3	0.287	0.264			-0.271	0.449		
IL-4	-0.202	0.423			**-0.632**	**0.050**		
IL-5	0.160	0.541			-0.342	0.333		
IL-6			-0.033	0.862			-0.304	0.266
IL-9	-0.031	0.902			-0.248	0.490		
IL-10	-0.069	0.794			-0.238	0.508		
IL-12(p40)	0.434	0.082			-0.143	0.694		
IL-12(p70)			-0.191	0.290			-0.328	0.212
IL-13			-0.097	0.597			-0.277	0.291
IL-17A	**-0.708**	**0.001**			-0.434	0.244		
Eotaxin	-0.120	0.636			-0.472	0.168		
G-CSF	-0.092	0.716			-0.456	0.186		
GM-CSF			-0.176	0.338			-0.435	0.104
IFN-γ	-0.082	0.746			-0.386	0.270		
KC	-0.064	0.814			-0.563	0.090		
MCP-1	-0.332	0.178			**-0.697**	**0.025**		
MIP-1α	-0.356	0.161			-0.415	0.233		
MIP-1β	-0.039	0.877			-0.352	0.319		
RANTES	-0.384	0.128			-0.403	0.248		
TNF-α	-0.158	0.544			-0.535	0.111		

Table 5.6 Relationships between global circumferential strain and circulating cytokines

	Males				Females			
Cytokine	**Pearson's *r***	**p-value**	**Kendall's τ-b**	**p-value**	**Pearson's *r***	**p-value**	**Kendall's τ-b**	**p-value**
IL-1α	**0.509**	**0.031**			-0.041	0.916		
IL-1β	-0.020	0.938			-0.116	0.766		
IL-2	-0.010	0.970			-0.165	0.671		
IL-3	0.387	0.125			-0.079	0.840		
IL-4	-0.014	0.955			-0.051	0.897		
IL-5	-0.234	0.367			-0.084	0.830		
IL-6			**0.425**	**0.024**			-0.182	0.531
IL-9	-0.150	0.551			0.333	0.381		
IL-10	0.221	0.395			0.252	0.514		
IL-12(p40)	0.112	0.670			0.349	0.358		
IL-12(p70)			0.177	0.327			-0.183	0.511
IL-13			0.052	0.776			0.183	0.511
IL-17A	0.287	0.248			-0.264	0.527		
Eotaxin	-0.329	0.182			0.132	0.735		
G-CSF	0.424	0.079			-0.194	0.617		
GM-CSF			0.234	0.202			-0.065	0.818
IFN-γ	-0.014	0.957			0.069	0.861		
KC	0.358	0.174			-0.113	0.772		
MCP-1	**0.604**	**0.008**			-0.238	0.537		
MIP-1α	0.199	0.444			0.130	0.739		
MIP-1β	0.057	0.822			-0.058	0.882		
RANTES	0.199	0.444			0.018	0.963		
TNF-α	0.373	0.140			-0.061	0.876		

5.9 Figures

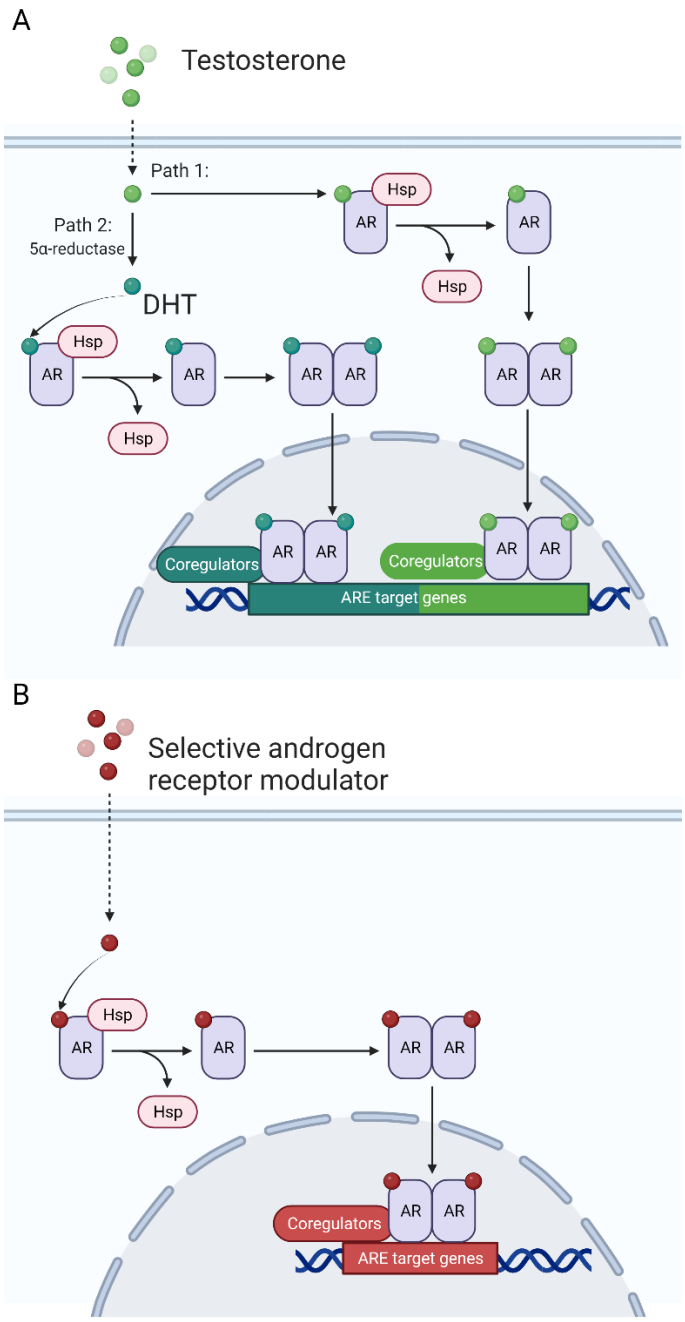


Figure 5.1 Testosterone signalling versus selective androgen receptor modulator signalling. A) After entering the cell, testosterone can either bind directly to the androgen receptor (AR) or be converted into dihydrotestosterone (DHT) by the enzyme 5α-reductase before binding. Once either ligand is bound, heat shock proteins (Hsp) dissociate from the AR, which enables homodimerization. Then, this dimer translocates to the nucleus and binds to androgen response elements (ARE) that lead to selective gene transcription. Coregulators

are proteins that impact which genes are upregulated and their presence can differ by tissue type. Further, different coregulators are recruited based on the binding of different ligands to the AR (denoted by different colours of coregulators and ARE target genes). B) On the other hand, SARMs are thought to attract a unique blend of coregulators (depicted in red) that differ in overall profile than those attracted by the endogenous ligands testosterone or DHT and help tailor their selective effects on gene expression. Image created with BioRender.com.

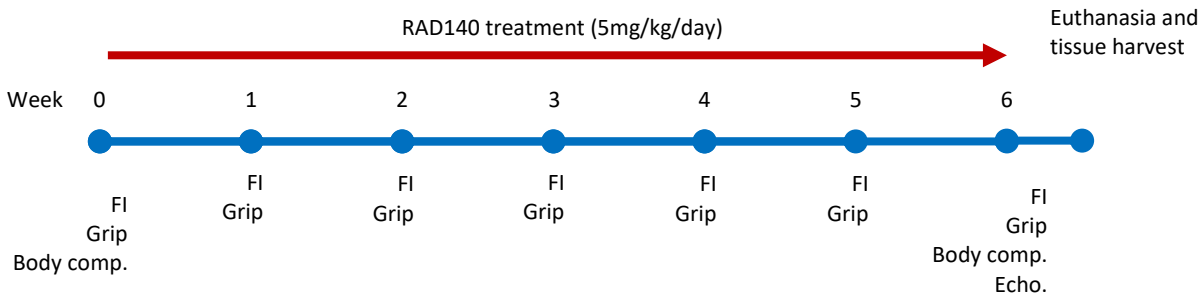


Figure 5.2 Study timeline for the experiments in Chapter 5 investigating the impact of a selective androgen receptor modulator (RAD140) on frailty and other bodily systems. FI=Frailty index; Body comp.=Body composition; Echo.= Echocardiography.

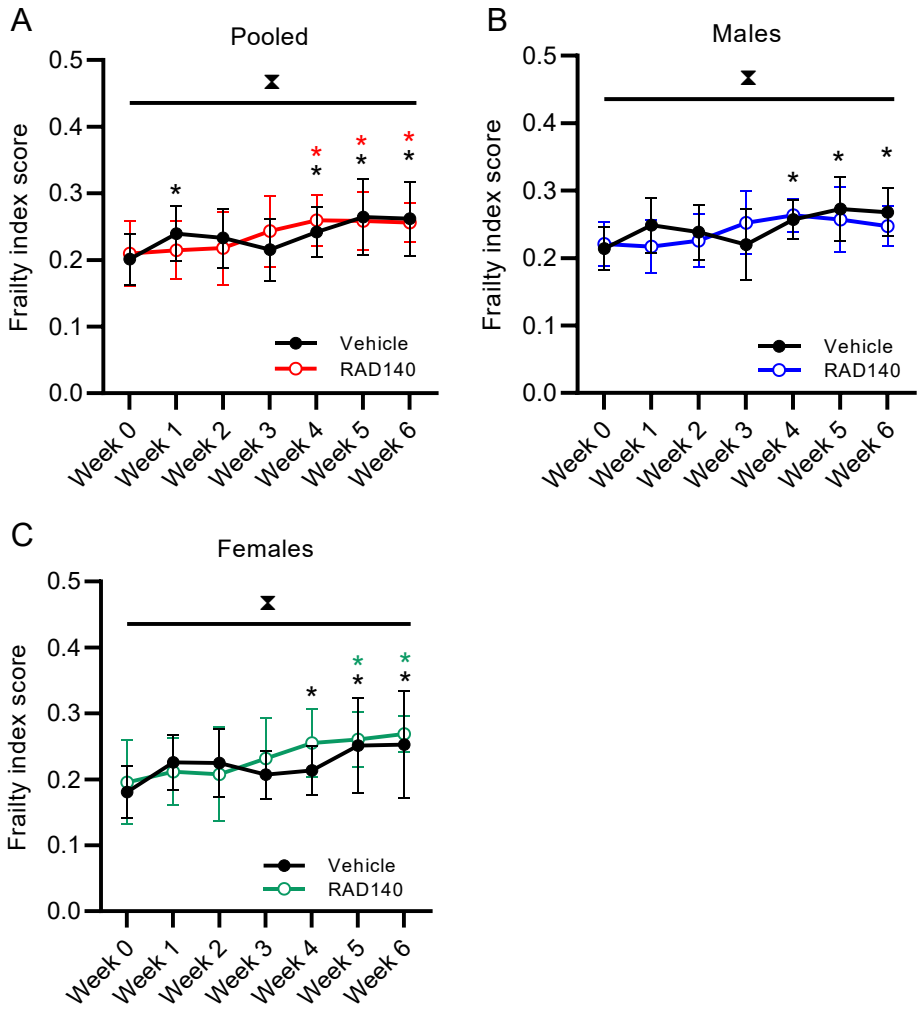


Figure 5.3 Non-invasive frailty index scores calculated using a 31-item index (Whitehead et al., 2014) increased over time in a pooled sample (A) and stratified by sex (B + C). ⧗ Denotes a significant effect of time overall. * Denotes a significant difference according to a Šídák's multiple comparisons test relative to baseline (Week 0) within the group.

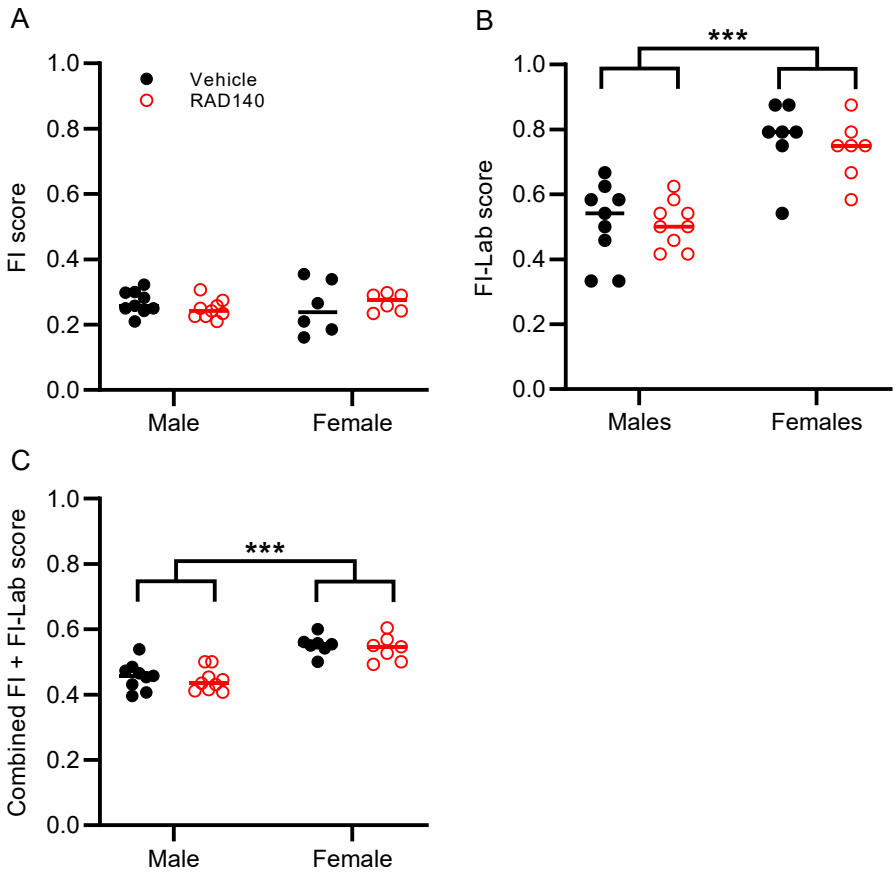


Figure 5.4 Frailty scores from a non-invasive index (FI score) and a laboratory-based index (FI-Lab score). A) Frailty index (FI) scores (31-item, Whitehead et al., 2014) or FI-Lab scores (34-item, see Table 5.2 for items) in male and female mice after 6-weeks of treatment with RAD140 did not differ between treatment groups. Females had significantly higher FI-Lab scores compared to males (B). The same result was found when combining both the FI and FI-Lab to create an FI-Combined (C). *** Denotes a main effect of sex following a 2-way analysis of variance (sex × treatment) at $p<0.001$.

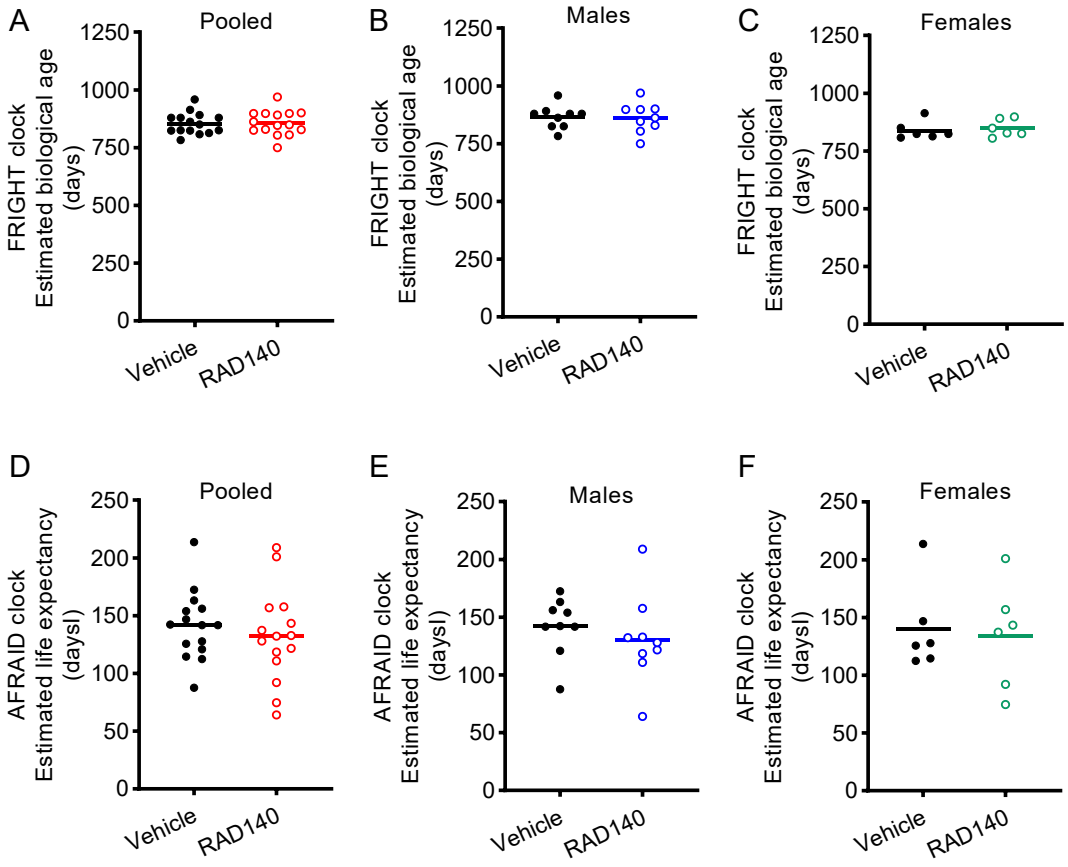


Figure 5.5 Estimated biologic age and time to death. Frailty Inferred Geriatric Health Timeline (FRIGHT; A-C) and Analysis of Frailty and Death (AFRAID; D-F) clocks predicting biologic age and estimated time to death between vehicle and RAD140 treated mice after 6-weeks of treatment were not different in pooled or sex-specific analysis.

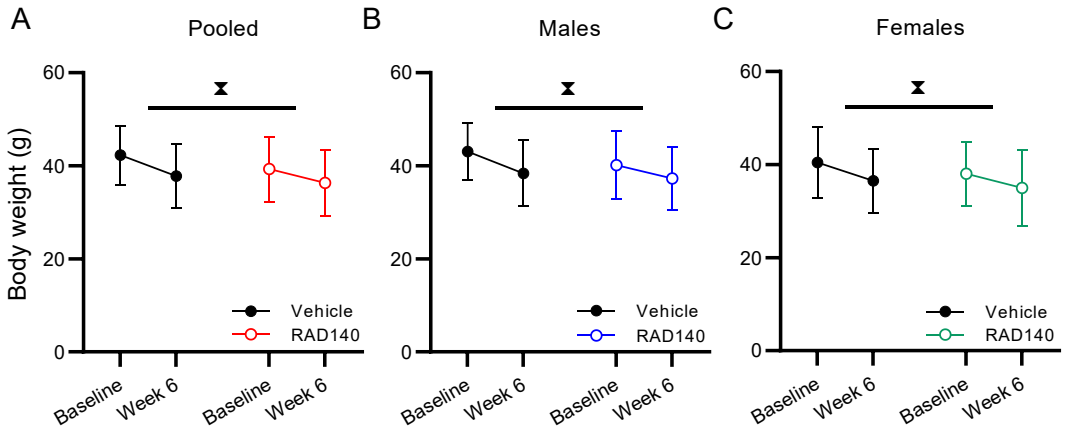


Figure 5.6 Body weight in all mice and stratified by sex. ⧗ Denotes a significant effect of time overall.

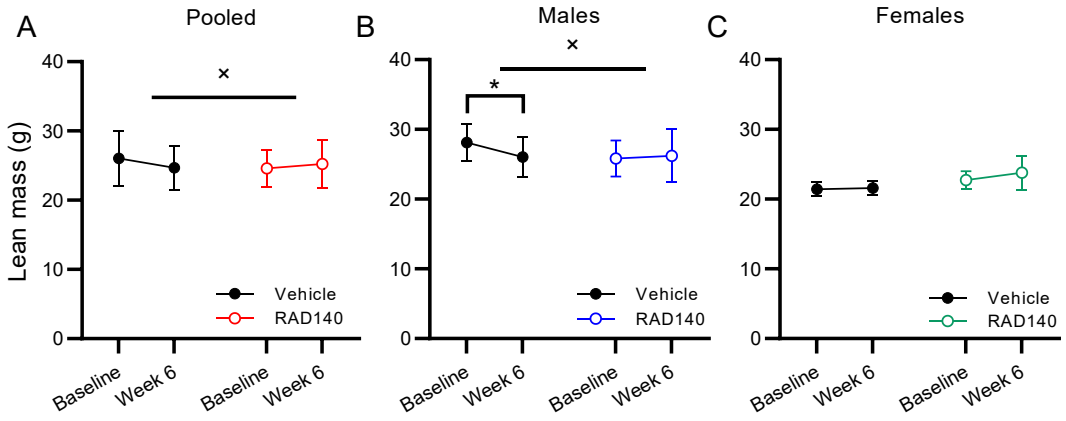


Figure 5.7 Lean mass in all mice and stratified by sex. × Denotes a significant 2-way (treatment × time) interaction. * Denotes a significant difference according to a Šídák's multiple comparisons test relative to baseline within the group.

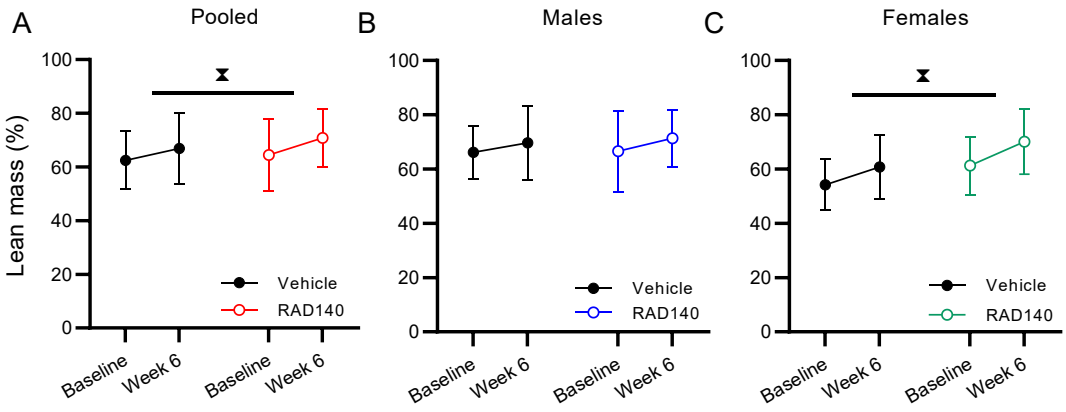


Figure 5.8 Percent lean mass of total body weight in all mice and stratified by sex. ⧗ Denotes a significant effect of time overall.

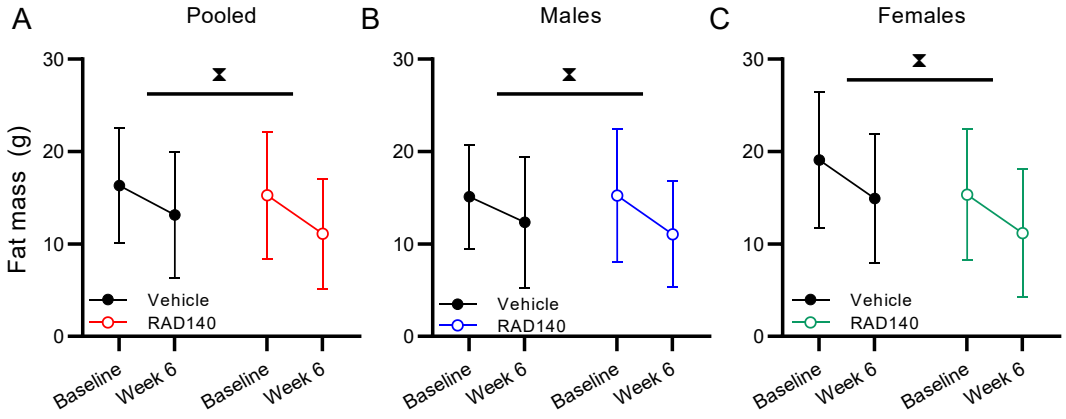


Figure 5.9 Fat mass in all mice and stratified by sex. ⧗ Denotes a significant effect of time overall.

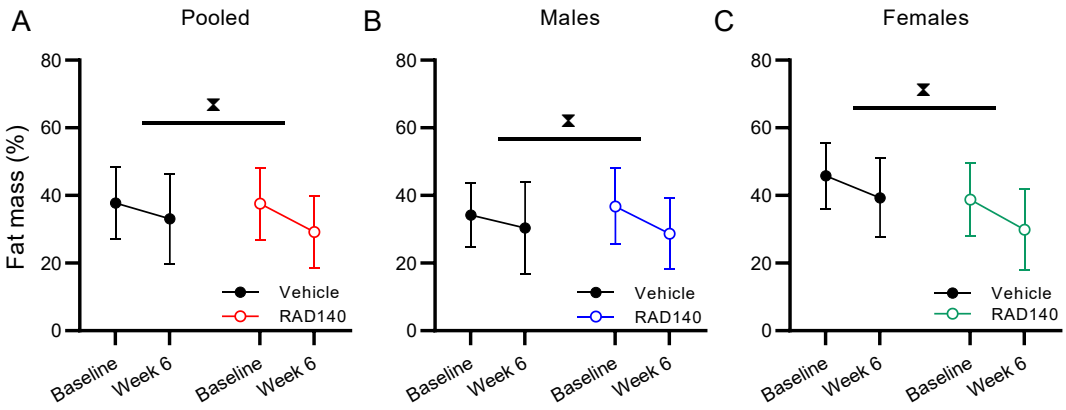


Figure 5.10 Percent fat mass of total body weight in all mice and stratified by sex. ⧗ Denotes a significant effect of time overall.

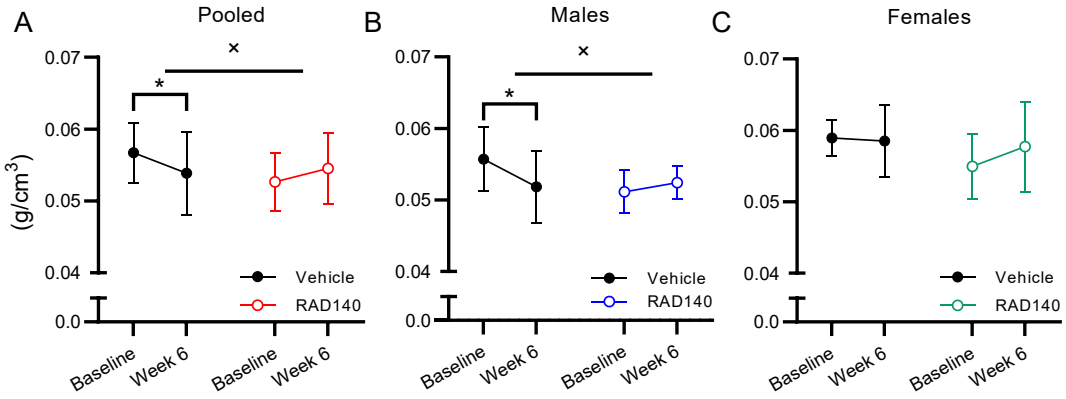


Figure 5.11 Bone mineral density in all mice and stratified by sex. × Denotes a significant 2-way (treatment × time) interaction. * Denotes a significant difference according to a Šídák's multiple comparisons test relative to baseline within the group.

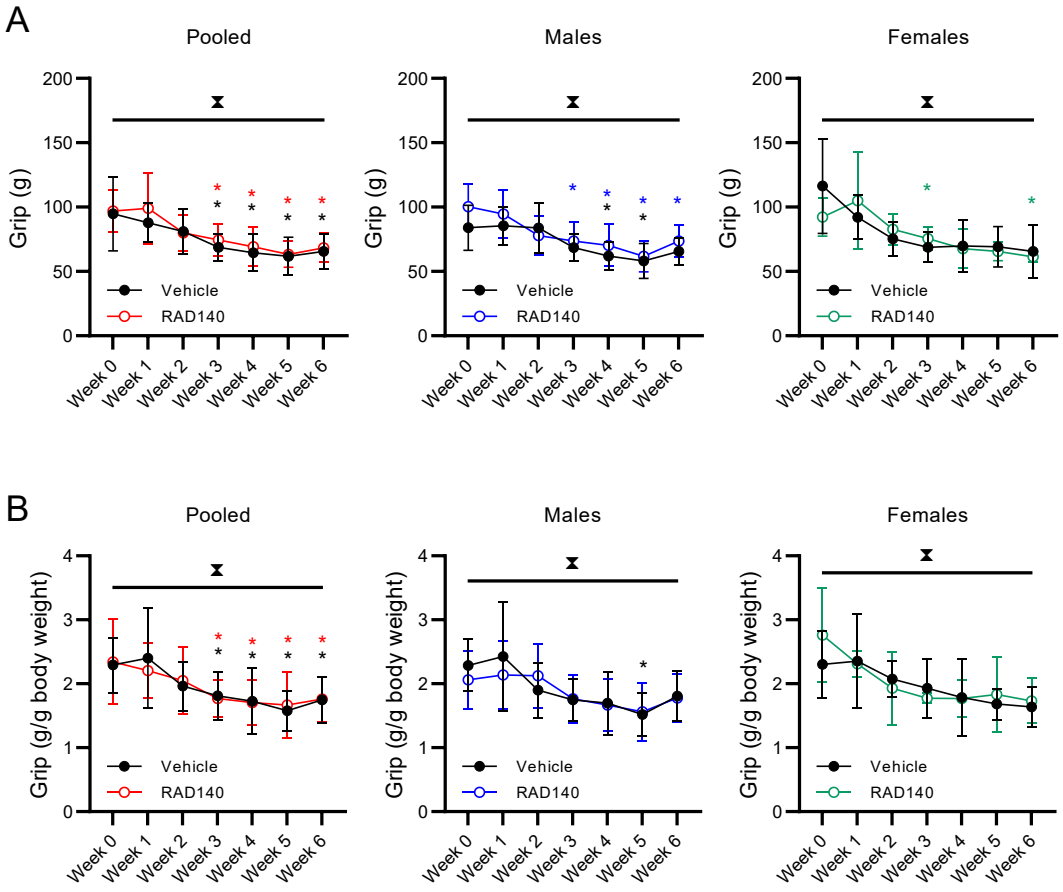


Figure 5.12 Grip strength measured in grams (A) and normalized grip strength (B) declined over time but did not change between treatment groups in a pooled analysis using all mice or when stratified by sex. ⧗ Denotes a significant effect of time overall. * Denotes a significant difference according to a Šídák's multiple comparisons test relative to baseline within the group.

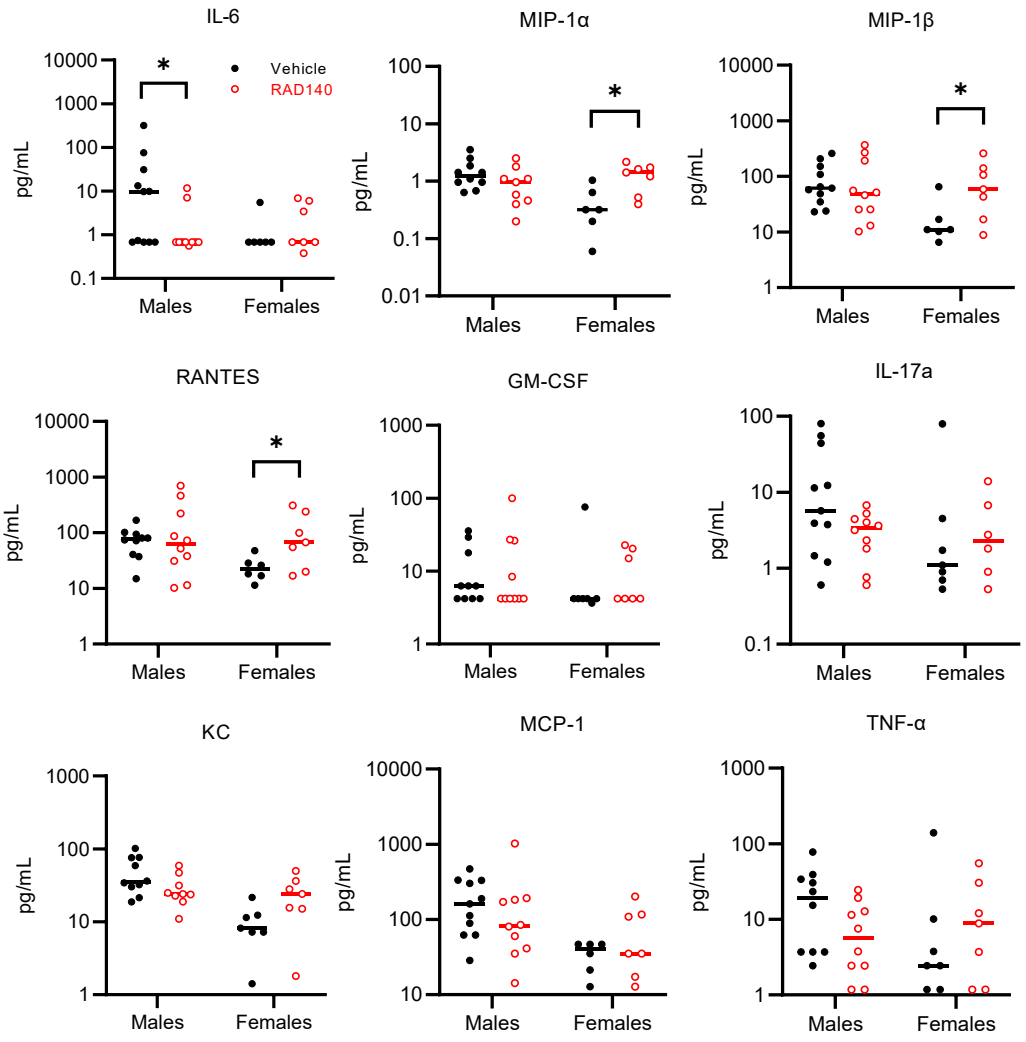


Figure 5.13 Selected circulating cytokines after 6-weeks of treatment with or without RAD140. * Denotes statistical significance following a t-test or Mann-Whitney U-test (see 5.3.4 for test arbitration; p-values in table 5.3).

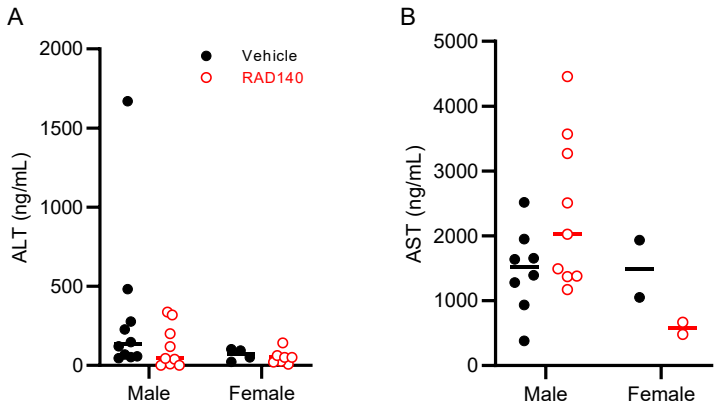


Figure 5.14 Serum concentrations of alanine aminotransferase and aspartate aminotransferase (ALT & AST) in male and female mice with or without 6-weeks of RAD140 treatment.

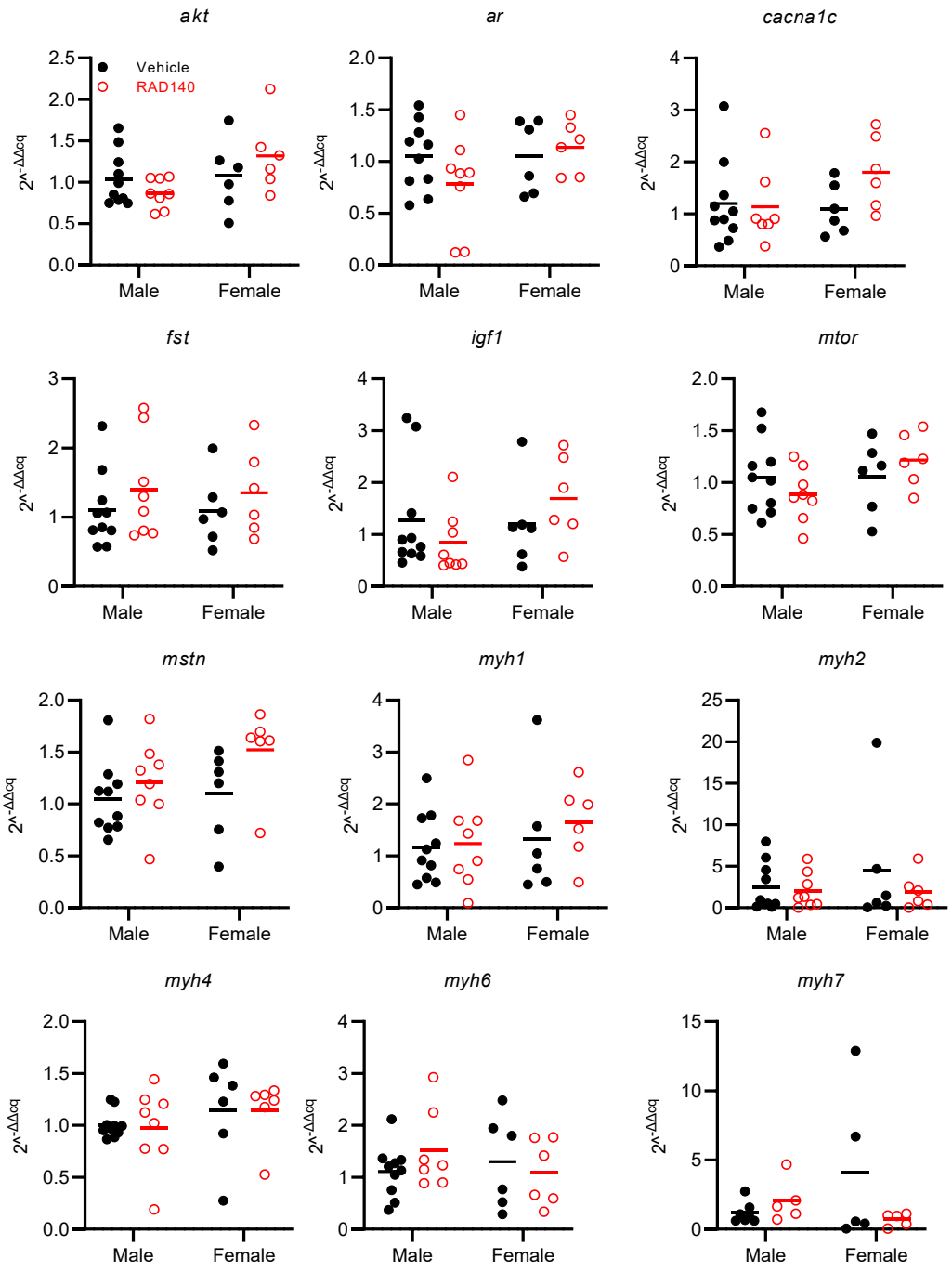


Figure 5.15 mRNA expression levels from quadriceps femoris skeletal muscle samples relative to reference gene averages within each mouse (each circle).

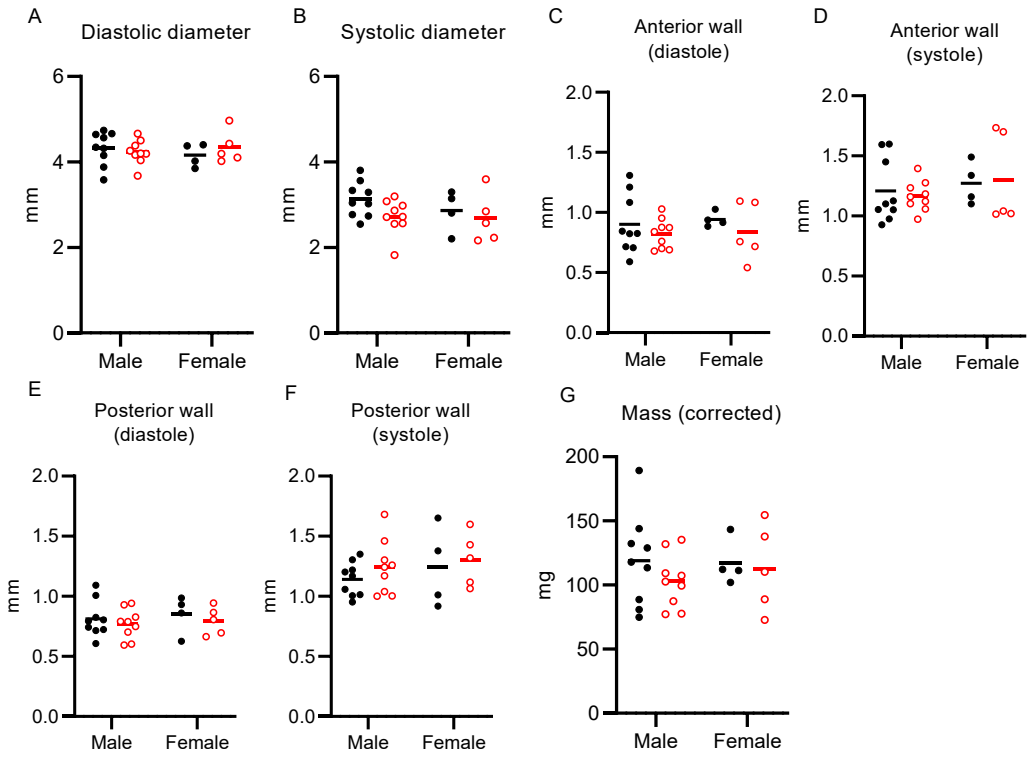


Figure 5.16 Left ventricular structure as measured using echocardiography in older male and female with or without 6-weeks of treatment with RAD140.

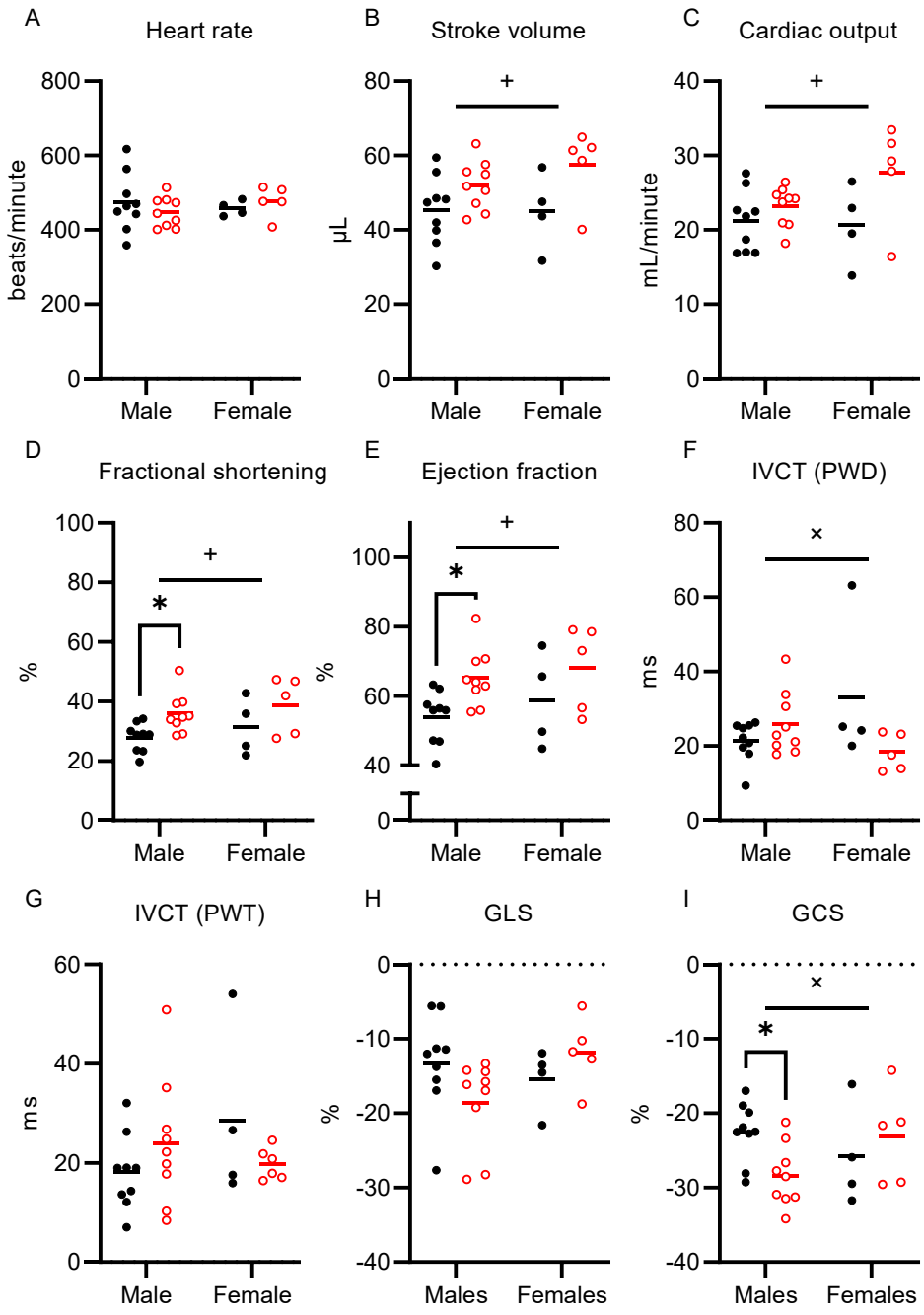


Figure 5.17 Left ventricular systolic function as measured using echocardiography in older male and female with or without 6-weeks of treatment with RAD140. + Denotes a significant effect of RAD140 treatment. × Denotes a significant (sex × treatment) interaction. * Denotes a significant difference according to a Šídák's multiple comparisons test relative to baseline within the group.

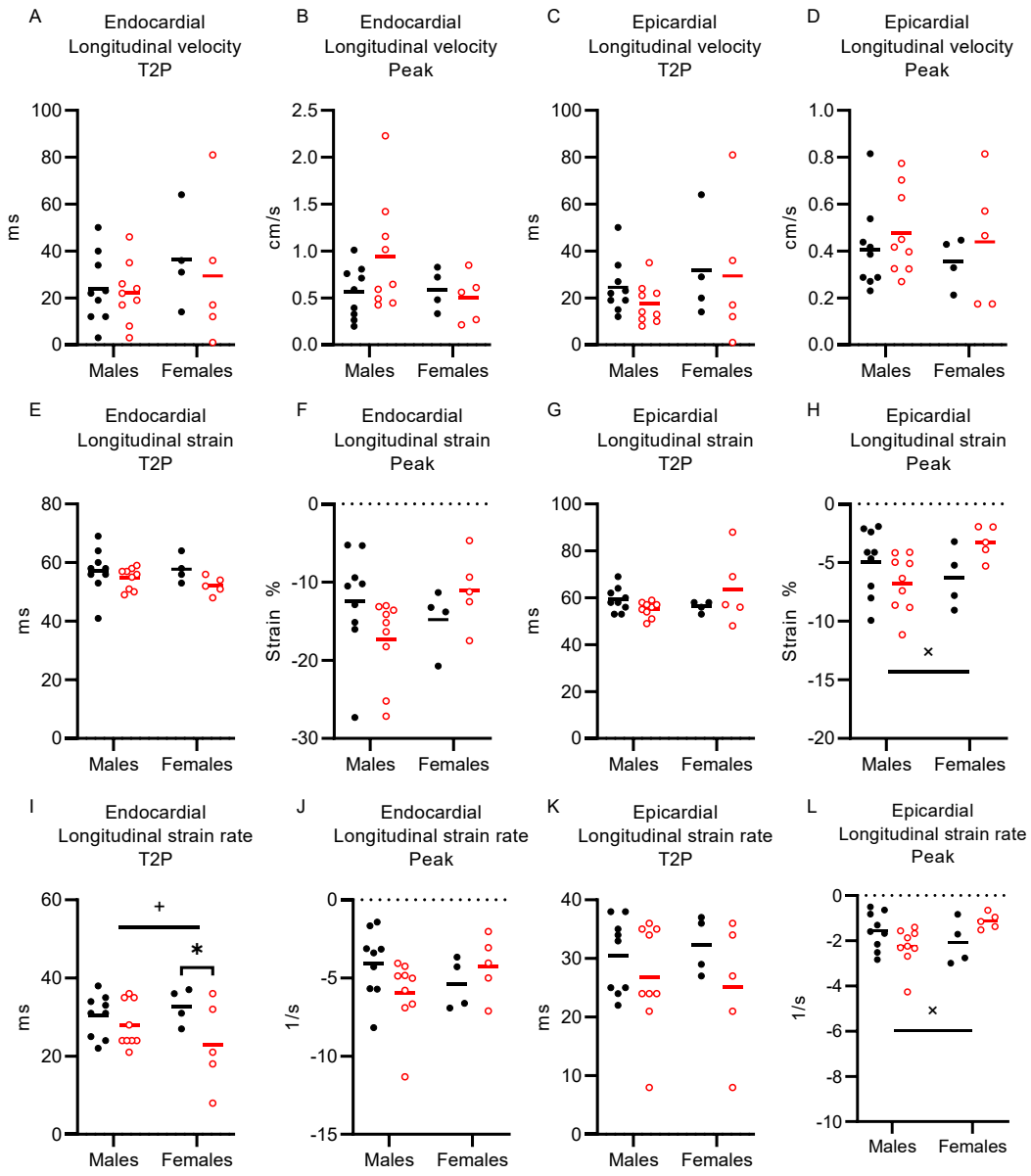


Figure 5.18 Longitudinal strain analysis as measured from the longitudinal axis during echocardiography in older male and female with or without 6-weeks of treatment with RAD140. + Denotes a significant effect of RAD140 treatment. * Denotes a significant difference according to a Šídák's multiple comparisons test relative to baseline within the group.

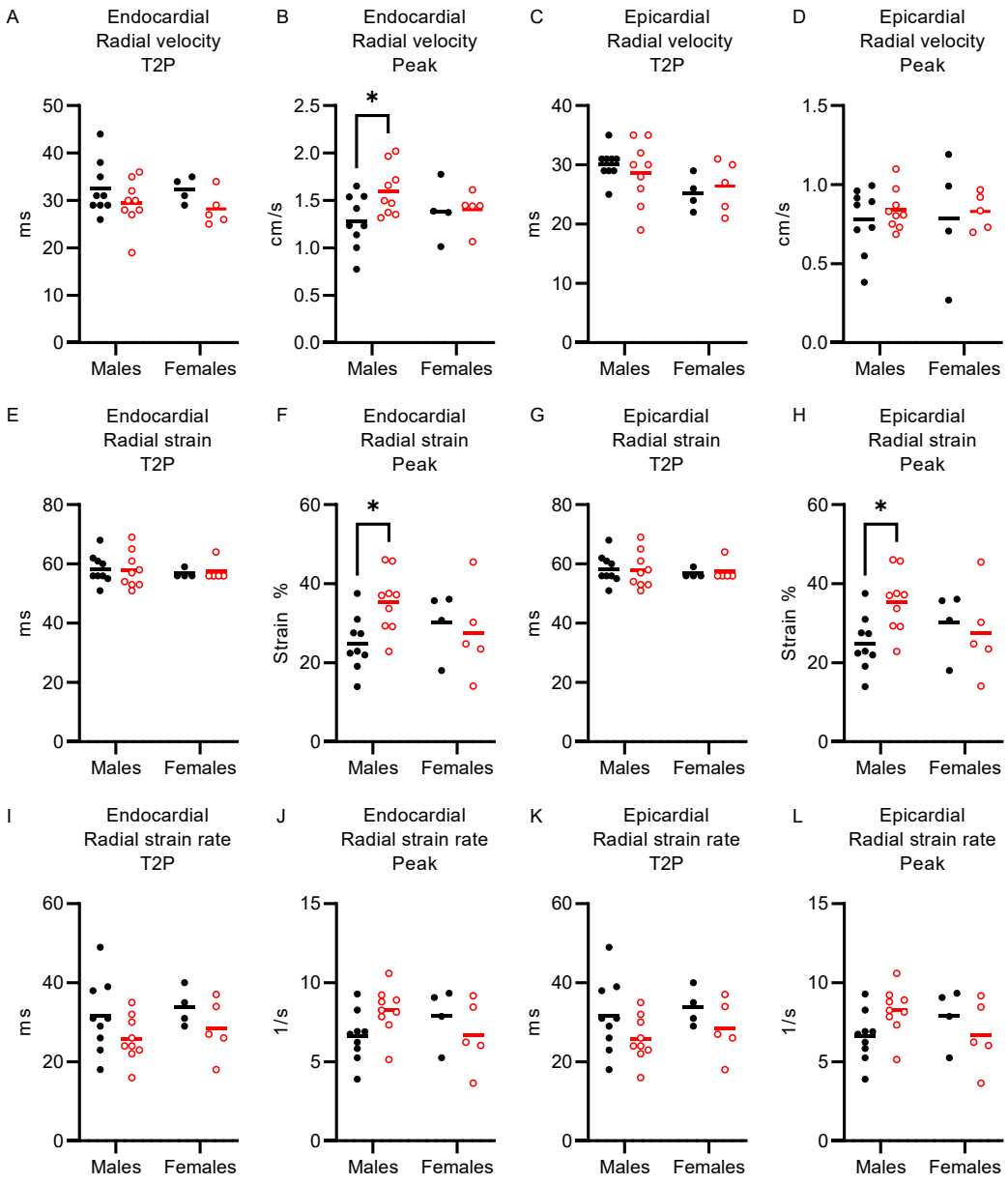


Figure 5.19 Radial strain analysis as measured from the short axis during echocardiography in older male and female with or without 6-weeks of treatment with RAD140. * Denotes a significant difference according to a Šídák's multiple comparisons test relative to baseline within the group.

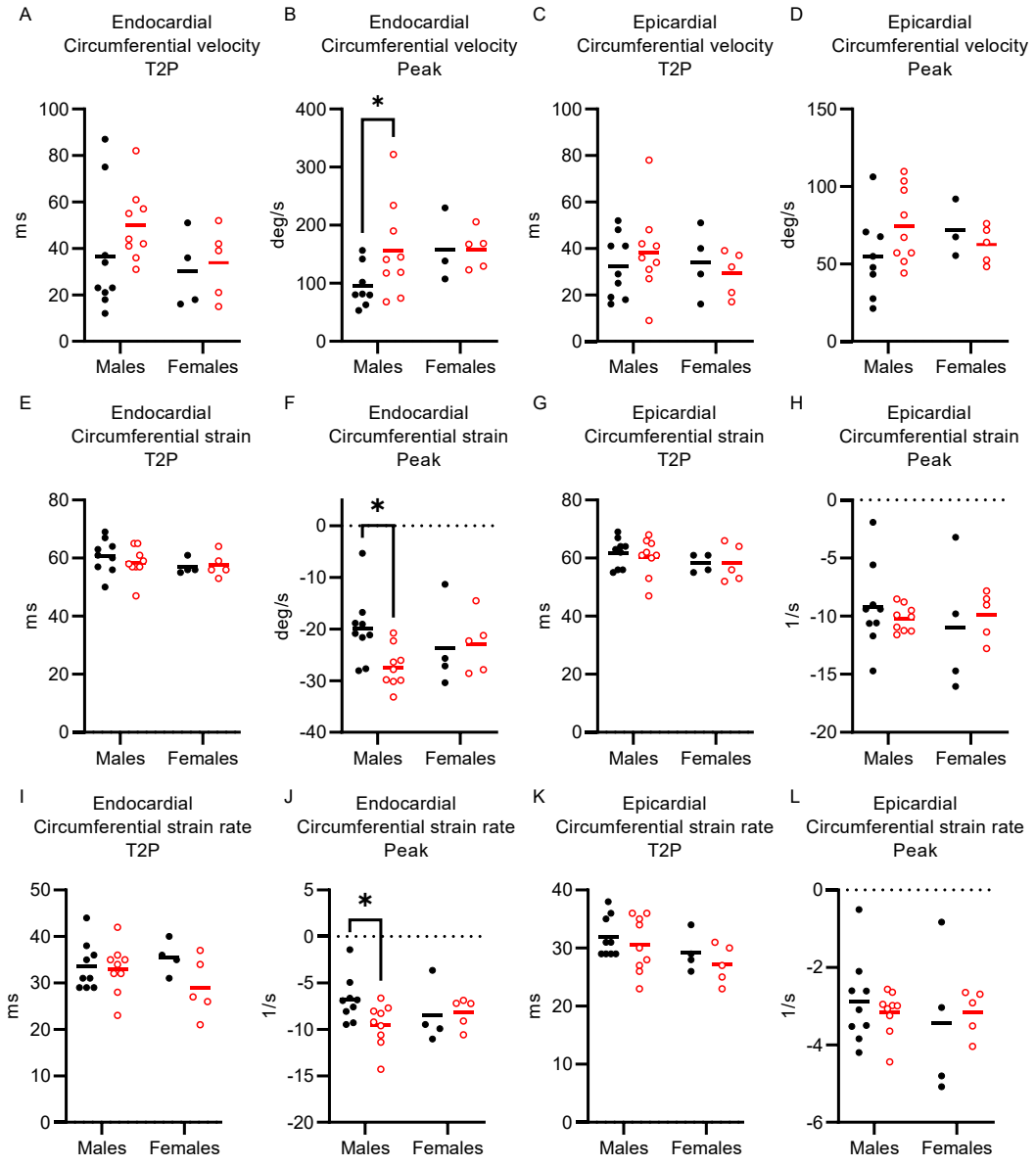


Figure 5.20 Circumferential strain analysis as measured from the short axis during echocardiography in older male and female with or without 6-weeks of treatment with RAD140. * Denotes a significant difference according to a Šídák's multiple comparisons test relative to baseline within the group.

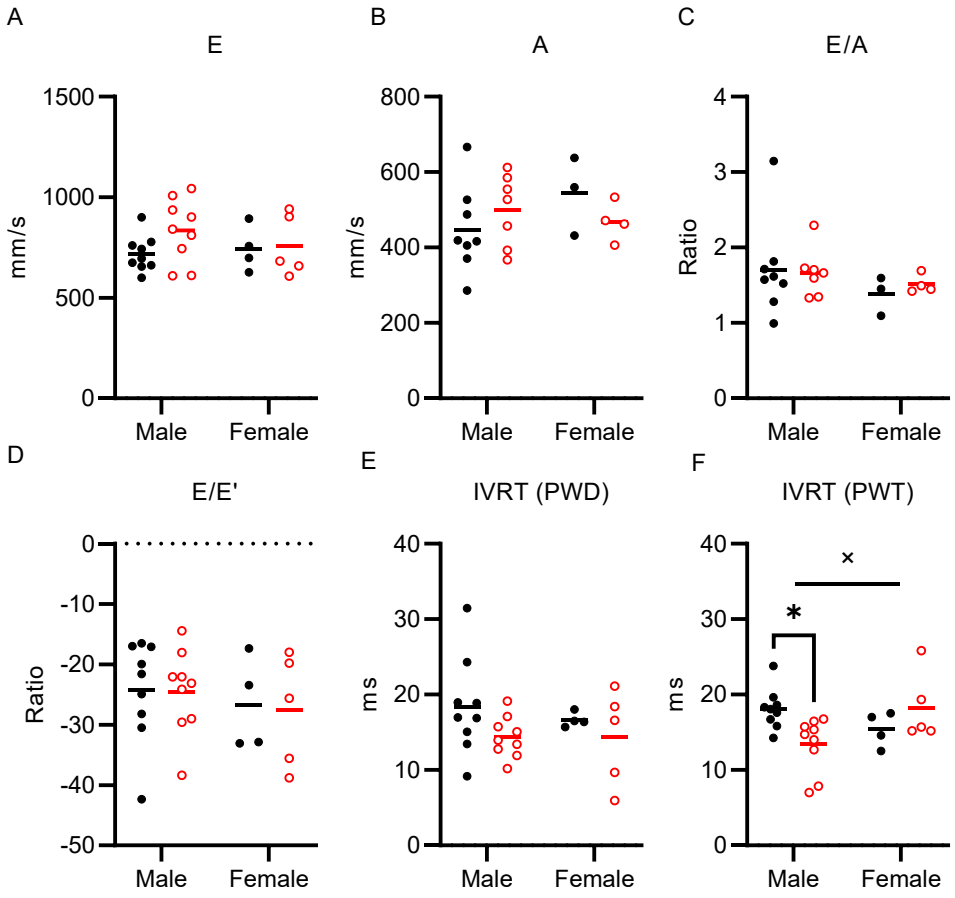


Figure 5.21 Left ventricular diastolic function measured using echocardiography in older male and female with or without 6-weeks of treatment with RAD140. × Denotes a significant (sex × treatment) interaction. * Denotes a significant difference according to a Šídák's multiple comparisons test relative to baseline within the group.

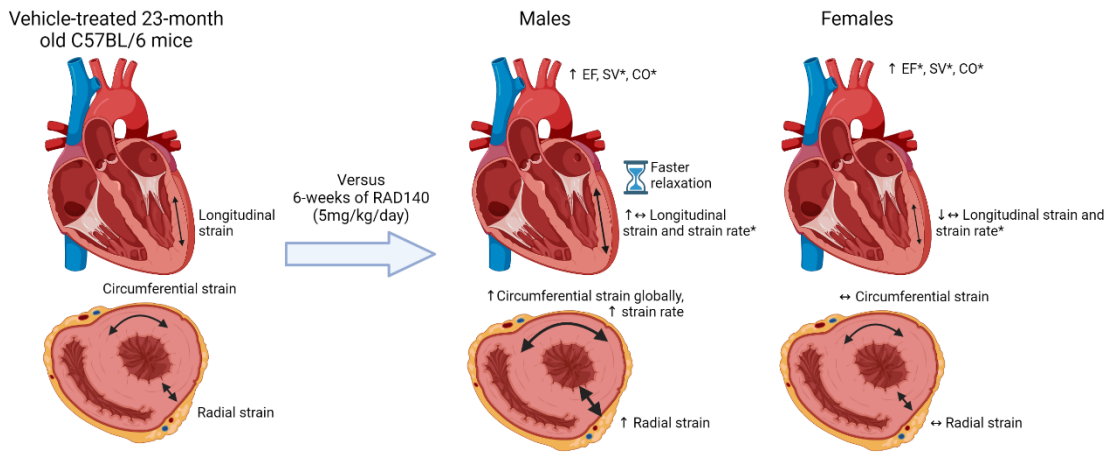


Figure 5.22 Summary figure for echocardiographic results. Male mice treated for six-weeks RAD140 had better systolic function in the form of myocardial strain, ejection fraction, stroke volume, and cardiac output, and better isovolumic relaxation times compared to controls. Differences in myocardial strain were not observed in females, although ejection fraction, stroke volume, and cardiac output trended upward. * Denotes a sex × treatment effect through an analysis of variance, but effects otherwise stand alone for the specified sex (*e.g.*, ejection fraction in males). ↓↔ and ↑↔ denote differences in some ventricle strain analysis sections but not others. CO = cardiac output; EF = ejection fraction; SV = stroke volume. Image created in BioRender.com.

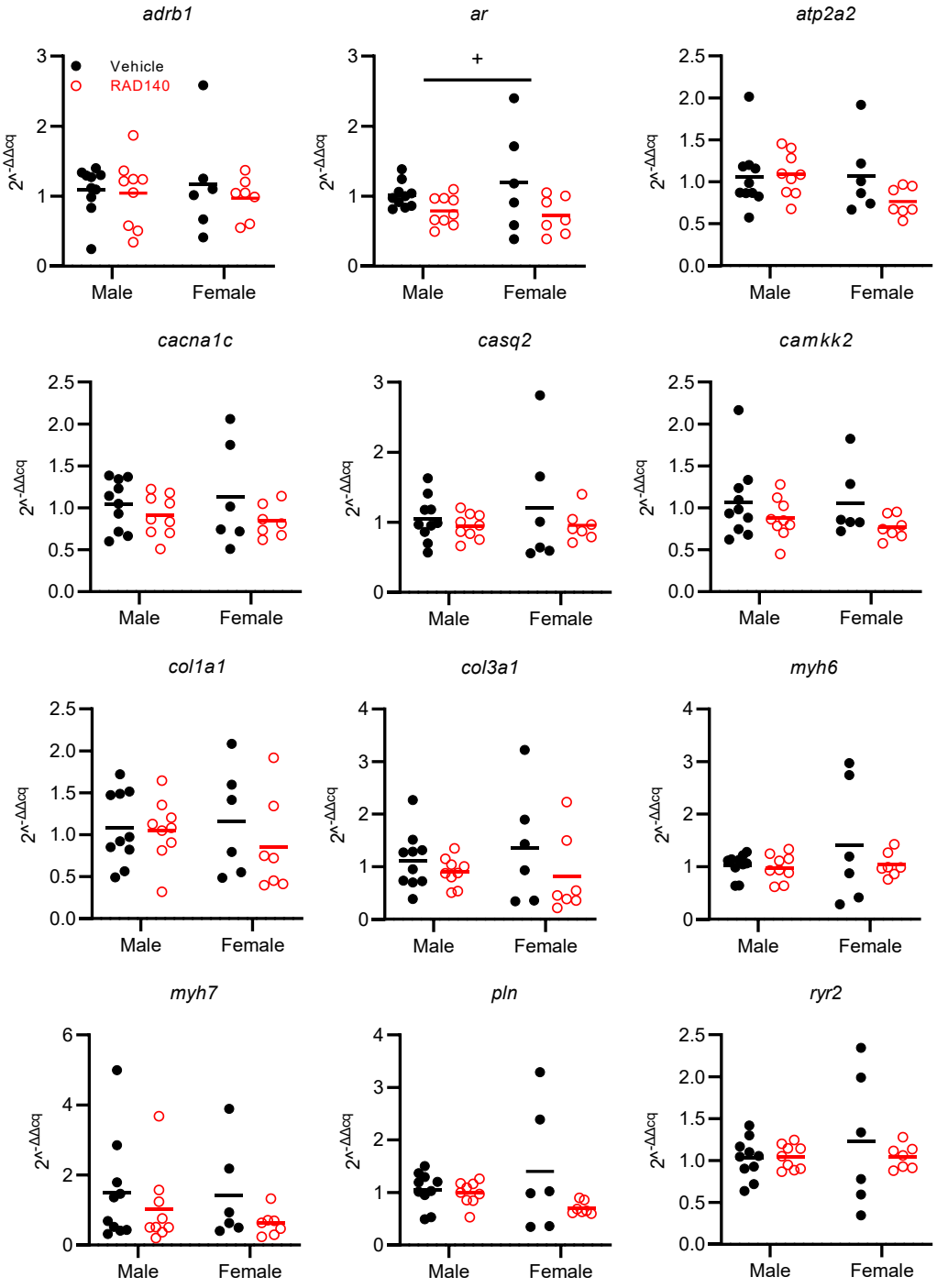


Figure 5.23 mRNA expression levels from ventricular heart tissue relative to reference gene averages within each mouse (each circle). Proteins encoded by these genes are named in Chapter 5.3.8. + Denotes a significant effect of RAD140 treatment.

Chapter 6 Conclusion

6.1 General summary

Frailty describes a state of increased risk to adverse health outcomes and is reviewed in detail in Chapter 2. It can be measured in humans and model organisms, such as mice, using a multi-dimensional FI or a more physicality-focused FP. An increasingly popular version of the FI is one based primarily upon laboratory values, termed the FI-Lab, which can predict mortality and adverse health events in a variety of populations and can be constructed using a diverse array of analytes. A systematic review and meta-analysis of the FI-Lab is presented in Chapter 2. The analytes in the FI-Lab related to various underlying mechanisms of frailty, from molecular agents like elevated circulating cytokines (*i.e.*, IL-6) to macroscopic parameters like abnormal cardiovascular parameters (*i.e.*, high blood pressure). High chronic levels of inflammatory markers play an important role in frailty progression and parallel declines in both musculoskeletal health and cardiac function. These mechanisms are reviewed with regards to frailty in Chapter 3.

The first objective in this dissertation was to examine how chronic testosterone deficiency contributes to frailty in a preclinical mouse model of aging, which was presented in Chapter 4. A 6-month longitudinal study evaluating frailty levels of older male mice with or without their testicles since 4-weeks of age suggested that low testosterone is not a potent driver of frailty. This finding was unexpected, given that low testosterone regularly (but not always) relates to higher frailty levels in humans. Frailty scores of the GDX mice were in fact lower than the intact, sham-operated mice, but the progression of frailty was not different over time. In human studies, older men with low testosterone often have chronic conditions that confound the relationship between testosterone and frailty. Here, the mice with low testosterone were otherwise healthy. Thus, this study provided mechanistic evidence that low testosterone may not substantially drive frailty progression. This result should be interpreted with caution, given the smaller sample size, but nonetheless helps answer important questions regarding how low testosterone alone impacts frailty, suggesting that naturally low levels in the absence of disease do not strongly promote frailty.

Although this finding suggested that low testosterone levels do not accelerate

frailty progression, there are benefits of androgen therapy to combat mechanisms of frailty. The SARM drug class is a novel set of drugs that act via the same receptor as testosterone, but are more anabolic than androgenic, making them an appealing drug in the context of frailty. This led to the dissertation's second objective, which was to explore how treatment with a SARM affects frailty and underlying mechanisms in a mouse model of aging. Both male and female mice were investigated in older age (~23-months-old), while two different FIs were used (non-invasive and an FI-Lab) to evaluate frailty. SARM treatment did not lower frailty scores between groups using either measurement. However, mechanisms of frailty including chronic inflammation and age-related lean mass wasting were also investigated. A promising finding was that IL-6 levels in SARM treated male mice were lower than those in the vehicle group. This is important, because IL-6 is the best translational cytokine between humans and mice regarding shared mechanisms of frailty, where higher IL-6 levels are detrimental (Chapter 3 reviews this). Further, SARM treatment was associated with improved lean mass and BMD, particularly in males. Females did not seem to derive as much benefit as the males, and RAD140 treated females had elevated levels of multiple inflammatory cytokines, one of which was associated with higher frailty scores in females. These results importantly suggest that RAD140 is a potential therapeutic to help treat these mechanisms of frailty in males, although they may be less effective in females. More work is needed to elucidate effects in both sexes.

The third objective was to investigate how treatment with a SARM affected cardiac left ventricular structure and function in an aging mouse model. This was in part due to the strong relationship between higher frailty and left ventricular dysfunction, but also because of other research from our lab using the GDX and sham mice, which suggests low testosterone is detrimental to left ventricular function (Banga et al., 2021). Interestingly, SARM treatment was associated with improved systolic function (EF, SV, CO) in both males and females. SARM treated male mice had better myocardial strains and faster ventricular relaxation than vehicle treated mice, although this was not observed in females (Figure 5.22). These results raise important questions about how SARMs may benefit cardiac function, especially in males. It is uncertain if they could help during pathologic conditions, such as heart failure, but the results presented in Chapter 5 suggest

a beneficial effect.

6.2 Future directions

The possibility that SARMs, specifically RAD140 can act to improve multiple aspects of frailty like muscle and bone wasting, chronic inflammation, and even left ventricular dysfunction is intriguing. Future research building on this work would benefit by closely examining the impact of RAD140 on frailty progression and frailty mechanisms in longer-term studies with even greater mechanistic rigor. A minimum of three months is recommended based on other studies requiring at least between two to seven months of treatment to see differences in frailty scores between treatment groups (Asadi Shahmirzadi et al., 2020; Keller et al., 2019). Mixing RAD140 into chow is one labor-saving technique that may enable larger sample sizes, as has been done before (Keller et al., 2019), although this makes accurate dosing more difficult. Further, adding an exercise intervention concomitantly with RAD140 treatment could help to confirm the theory that functional training is needed alongside an anabolic agent to impart physical benefit beyond lean mass gains.

Investigating how RAD140 may suppress IL-6 production in males, possibly using transgenic mice with elevated IL-6 levels would help define whether RAD140 is anti-inflammatory for this important cytokine. Further investigation and confirmation regarding the anti-inflammatory effects on other pro-inflammatory cytokines would also be of interest. For example, it may be the case that lower levels of IL-17a in the RAD140 treated males positively affected BMD (Weitzmann, 2017). Further, examining whether RAD140 actually promotes chronic inflammation in females is important in understanding the effects of RAD140 in women. Evidence suggests that testosterone acts differently between women and men, with it being anti-inflammatory in men but pro-inflammatory in women (Di Stasi et al., 2022). This suggests that the sex differences in chronic inflammation are genuine but investigating any impact on frailty would be an important next step. Confirming and elucidating how RAD140 imparts a male-specific beneficial impact on myocardial strain, and determining effects in females, would also be enlightening.

Lastly, investigating the effect of SARM treatment in pathologic conditions, such

as treatment-induced cachexia (*i.e.*, from cancer chemotherapy), or heart failure would help to advance research efforts targeting frailty progression during chronic disease. A model of heart failure may enable investigations relating to both cardiac dysfunction and muscle wasting, given that the two often occur together (Pandey et al., 2019). In another sense, using a model of prostate cancer would be an excellent choice, because of the possible anti-cancer effects SARMs possess and their putative muscle-sparing abilities. It would be interesting to investigate whether SARMs could help treat cancer while also maintaining muscle mass and/or physical function, perhaps in combination with an exercise intervention.

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Appendix A: List of peer-reviewed publications during degree and author contributions for manuscripts used in dissertation.

1. Sapp, D. G., Cormier, B. M., Rockwood, K., Howlett, S. E., **Heinze-Milne, S. D**. (Unpublished). The Frailty Index based on laboratory test data as a tool to investigate the impact of frailty on health outcomes in humans and in preclinical models: A systematic review and meta-analysis. *Submitted to Age and Ageing.*

2. **Heinze-Milne, S. D.**, Banga, S., & Howlett, S. E. (2022). Frailty and Cytokines in Preclinical Models: Comparisons With Humans. *Mechanisms of ageing and development*, 111706. Advanced online publication. https://doi.org/10.1016/j.mad.2022.111706

3. **Heinze-Milne, S. D.**, Banga, S., Godin, J., & Howlett, S. E. (2022). Serum testosterone concentrations are not associated with frailty in naturally ageing and testosterone-deficient older C57Bl/6 mice. *Mechanisms of ageing and development*, *203*, 111638. https://doi.org/10.1016/j.mad.2022.111638

4. **Heinze-Milne, S.**, Banga, S., Howlett, S. E. (2022). Low testosterone concentrations and risk of ischaemic cardiovascular disease in ageing: not just a problem for older men. *The Lancet: Healthy Longevity*. *3*(2):E83-E84. doi: https://doi.org/10.1016/S2666-7568(22)00008.
Invited commentary.

5. Bisset, E. S., **Heinze-Milne, S.**, Grandy, S. A., & Howlett, S. E. (2022). Aerobic Exercise Attenuates Frailty in Aging Male and Female C57Bl/6 Mice and Effects Systemic Cytokines Differentially by Sex. *The journals of gerontology. Series A, Biological sciences and medical sciences*, *77*(1), 41–46. https://doi.org/10.1093/gerona/glab297

6. Banga, S., **Heinze-Milne, S. D.**, Godin, J., & Howlett, S. E. (2021). Signs of diastolic dysfunction are graded by serum testosterone levels in aging C57BL/6 male mice. *Mechanisms of ageing and development*, *198*, 111523. https://doi.org/10.1016/j.mad.2021.111523

7. **Heinze-Milne, S. D.**, Keats, M. R., Blanchard, C., Giacomantonio, N., MacDonald, D., Rajda, M., ... & Grandy, S. A. (2021). Exercise to Prevent Anthracycline-Based Cardiotoxicity (EXACT): A Feasibility Study. *Translational Journal of the American College of Sports Medicine*, *6*(3), 1-11.
Work published from during Master's degree

8. Kane, A. E., Bisset, E. S., **Heinze-Milne, S.**, Keller, K. M., Grandy, S. A., & Howlett, S. E. (2021). Maladaptive Changes Associated With Cardiac Aging Are Sex-Specific and Graded by Frailty and Inflammation in C57BL/6 Mice. *The journals of gerontology. Series A, Biological sciences and medical sciences*, *76*(2), 233–243. https://doi.org/10.1093/gerona/glaa212

9. **Heinze-Milne, S. D.**, Banga, S., & Howlett, S. E. (2019). Frailty Assessment in Animal Models. *Gerontology*, *65*(6), 610–619. https://doi.org/10.1159/000501333

10. Robinson, S. A., O'Brien, M. W., Grandy, S. A., **Heinze-Milne, S**., & Kimmerly, D. S. (2019). Short-term supplement of virgin coconut oil improves endothelial-dependent dilation but not exercise-mediated hyperemia in young adults. *Nutrition research (New York, N.Y.)*, *67*, 17–26. https://doi.org/10.1016/j.nutres.2019.03.016
Work published from during Master's degree

11. Banga, S., **Heinze-Milne, S. D.**, & Howlett, S. E. (2019). Rodent models of frailty and their application in preclinical research. *Mechanisms of ageing and development*, *179*, 1–10. https://doi.org/10.1016/j.mad.2019.01.008

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Work published from during Master's degree

14. Kane, A. E., Keller, K. M., **Heinze-Milne, S.**, Grandy, S. A., & Howlett, S. E. (2019). A Murine Frailty Index Based on Clinical and Laboratory Measurements: Links Between Frailty and Pro-inflammatory Cytokines Differ in a Sex-Specific Manner. *The journals of gerontology. Series A, Biological sciences and medical sciences*, *74*(3), 275–282. https://doi.org/10.1093/gerona/gly117
Work published from during Master's degree

DALHOUSIE UNIVERSITY

Faculty of Graduate Studies

STUDENT CONTRIBUTION TO MANUSCRIPTS IN THESIS

MUST BE WORD-PROCESSED OR TYPEWRITTEN.

NAME: Stefan Heinze-Milne	STUDENT ID #: B00584481
DEPARTMENT: Pharmacology	PROGRAMME: PhD
PHONE: 902 292 3310	E-MAIL: stefan.heinze.milne@dal.ca

MANUSCRIPT AUTHORS: Heinze-Milne, S. D., Banga, S., & Howlett, S. E.
MANUSCRIPT TITLE: Frailty Assessment in Animal Models.
JOURNAL: Gerontology
STUDENT CONTRIBUTION: Mr. Heinze-Milne researched and drafted all sections of this narrative review and critically revised the document based on feedback from Dr. Howlett (supervisor).
SUPERVISOR SIGNATURE:

MANUSCRIPT AUTHORS: Heinze-Milne, S. D., Banga, S., & Howlett, S. E.
MANUSCRIPT TITLE: Frailty and Cytokines in Preclinical Models: Comparisons with Humans
JOURNAL: Mechanisms of Aging and Development
STUDENT CONTRIBUTION: Mr. Heinze-Milne researched and drafted the section involving human research and critically reviewed and edited the remaining sections involving mouse models alongside Dr. Howlett (supervisor).
SUPERVISOR SIGNATURE:

MANUSCRIPT AUTHORS: Heinze-Milne, S. D., Banga, S., Godin, J., & Howlett, S. E.
MANUSCRIPT TITLE: Serum testosterone concentrations are not associated with frailty in naturally ageing and testosterone-deficient older C57Bl/6 mice.
JOURNAL: Mechanisms of Aging and Development
STUDENT CONTRIBUTION: Mr. Heinze-Milne analyzed the data included and drafted the manuscript, which was critically reviewed and revised by co-authors. Data collection was done collectively with Mr. Banga.
SUPERVISOR SIGNATURE:

Appendix B: List of non-peer-reviewed publication during degree

1. **Heinze-Milne, S.** & Joy, P. **Men are buying potentially risky steroid substitutes online to get the 'ideal body'.** *The Conversation.* https://theconversation.com/men-are-buying-potentially-risky-steroid-substitutes-online-to-get-the-ideal-body-144249

Appendix C: Copyright permissions

AW: Requested Karger Material

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Fri 6/3/2022 11:13 AM
To: Stefan Heinze-Milne <Stefan.Heinze.Milne@Dal.Ca>

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Heinze-Milne S, D, Banga S, Howlett S, E: Frailty Assessment in Animal Models. Gerontology 2019;65:610-619. doi: 10.1159/000501333

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Hopefully, I have been of assistance to you with the above.

Kind regards
Samuel Lei
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ePartner Manager
+41 61 306 12 76
s.lei@karger.com

S. Karger AG · Allschwilerstrasse 10 · 4009 Basel · Switzerland
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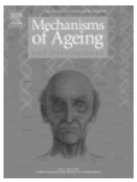


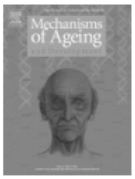
Frailty and cytokines in preclinical models: Comparisons with humans

Author: Stefan D. Heinze-Milne,Shubham Banga,Susan E. Howlett

Publication: Mechanisms of Ageing and Development

Publisher: Elsevier

Date: September 2022



Serum testosterone concentrations are not associated with frailty in naturally ageing and testosterone-deficient older C57Bl/6 mice

Author: Stefan D. Heinze-Milne,Shubham Banga,Judith Godin,Susan E. Howlett

Publication: Mechanisms of Ageing and Development

Publisher: Elsevier

Date: April 2022

Appendix D: Supplemental documentation for Sapp & Cormier et al., unpublished

Supplementary information for: The Frailty Index based on laboratory test data as a tool to investigate the impact of frailty on health outcomes in humans and in preclinical models: A systematic review and meta-analysis

Detailed methods for performing the systematic review and meta-analysis

Duplicate removal and full-text assessment were completed by reviewer DGS. In secondary screening, abstracts were reviewed independently by reviewers DGS and BMC. If these reviewers disagreed, reviewer SEH made the final decision.

Risk of Bias Assessment

The Newcastle-Ottawa Quality Assessment Scale for Cohort Studies was modified and used to assess the risk of bias in the mortality and adverse events subgroups by authors BMC and DGS (Appendix B). The Newcastle-Ottawa Quality Assessment Scale assesses bias in three domains: Selection, Comparability, and Outcome. They are evaluated using a 'star system' by which stars were awarded for each domain based on specified criteria. We replaced the second item in the Selection domain (Selection of the non-exposed cohort) with "appropriately constructed FI-Lab." Also, we eliminated the third item in the Selection domain (Ascertainment of exposure). These changes were made because studies in our review assessed a tool rather than comparing exposed and non-exposed cohorts. Additionally, we modified the options for the fourth item in the Selection domain (Demonstration that the outcome of interest was not present at the start of the study). This change allowed the risk of bias assessment to differentiate between samples starting with disease/adverse events or older populations that did not share a common disease/adverse event. The risk of bias assessment was summarized in a table format indicating the 'star rating' for each paper. Any paper that received four or fewer

stars was considered to have a high risk of bias.

Data Synthesis and Analysis

A meta-analysis of studies was completed on the human mortality subgroup. The analysis was conducted using the statistical program R (Version 3.6.1, Vienna, Austria) with its package 'meta'. The inverse variance method was used as a fixed-effects model on log-transformed hazard ratios based on a 0.01 change in frailty index scores. Studies that reported changes in FI-Lab scores at the 0.1 level were included by re-calculating parameter estimates and confidence intervals. To do this, hazard ratios and confidence intervals were ln-transformed, converted to values corresponding to a 0.01 change in FI-Lab score by dividing the transformed value by 10, followed by raising *e* to the power of the newly calculated value. Heterogeneity of studies was assessed using Cochrane's Q-test (α=0.05) and the I^2 statistic. If studies were found to be heterogeneous, a random-effects model was used.

Within the mortality meta-analysis, sub-subgroups were formed to examine the effects of study characteristics on the HR. The included studies were divided into binary groups for the following characteristics: mean age, follow-up time, community dwelling, number of FI-Lab items measured, sample size, percent female, age adjusted model, and sex adjusted model. For continuous characteristics, cut-offs were made as though the trait was distributed roughly bimodally (Supplemental Figure 2).

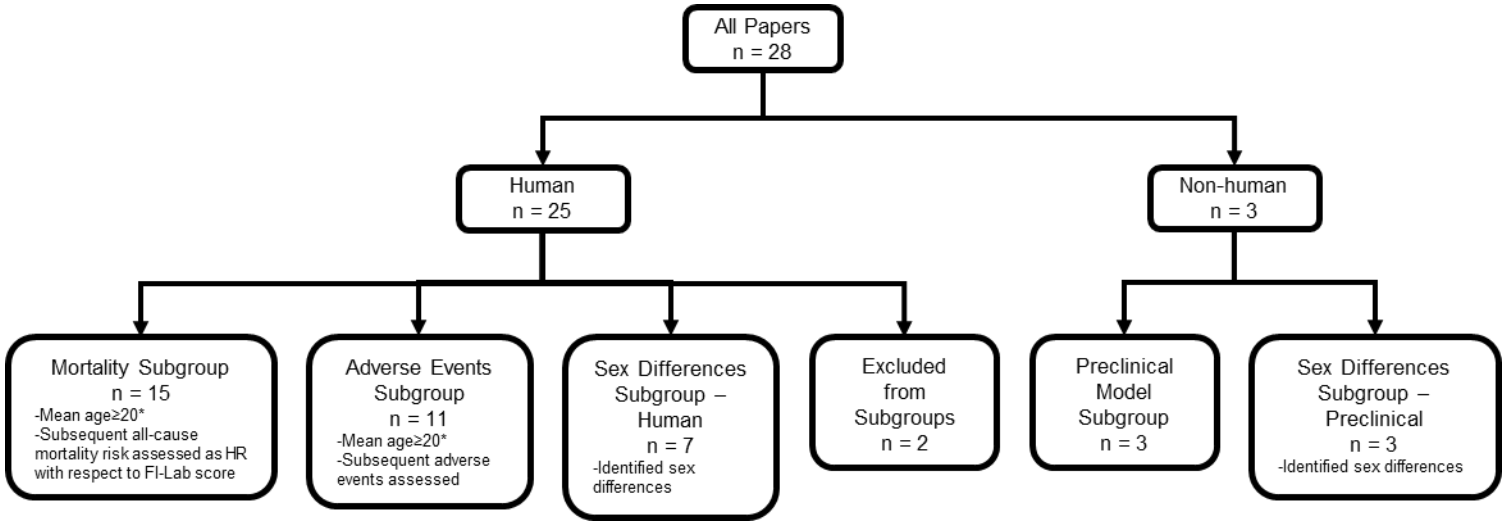
Narrative synthesis described the distribution of publication year, location, sample size, sex, mean age, number of deficits used, and follow-up period in all papers. A narrative synthesis was also completed in each subgroup according to their conclusions. FI-Lab items were summarized in a frequency bar chart.

Meta-bias Assessment

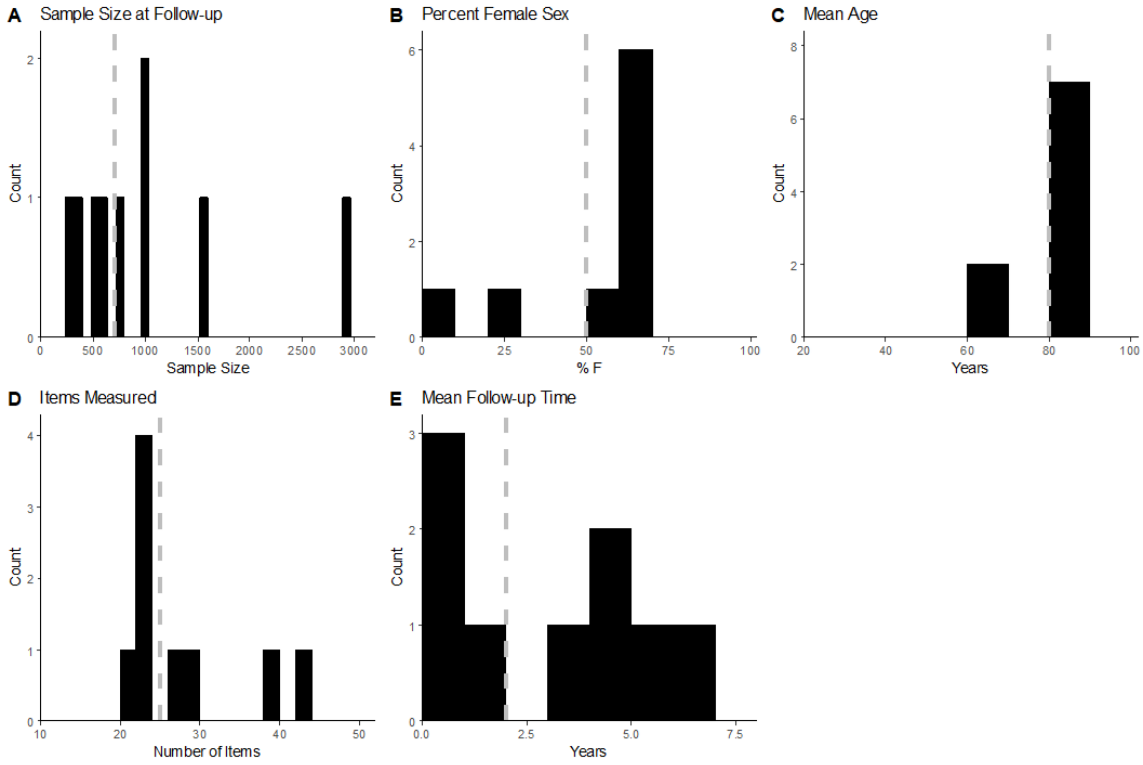
Meta-bias was assessed statistically using a Begg-Mazumdar's test and an Egger's test, alongside visual inspection of a funnel plot. Two-tailed confidence intervals have been reported alongside back-transformed hazard ratios and statistical significance for tests was determined at α=0.05.

Certainty of Cumulative Evidence

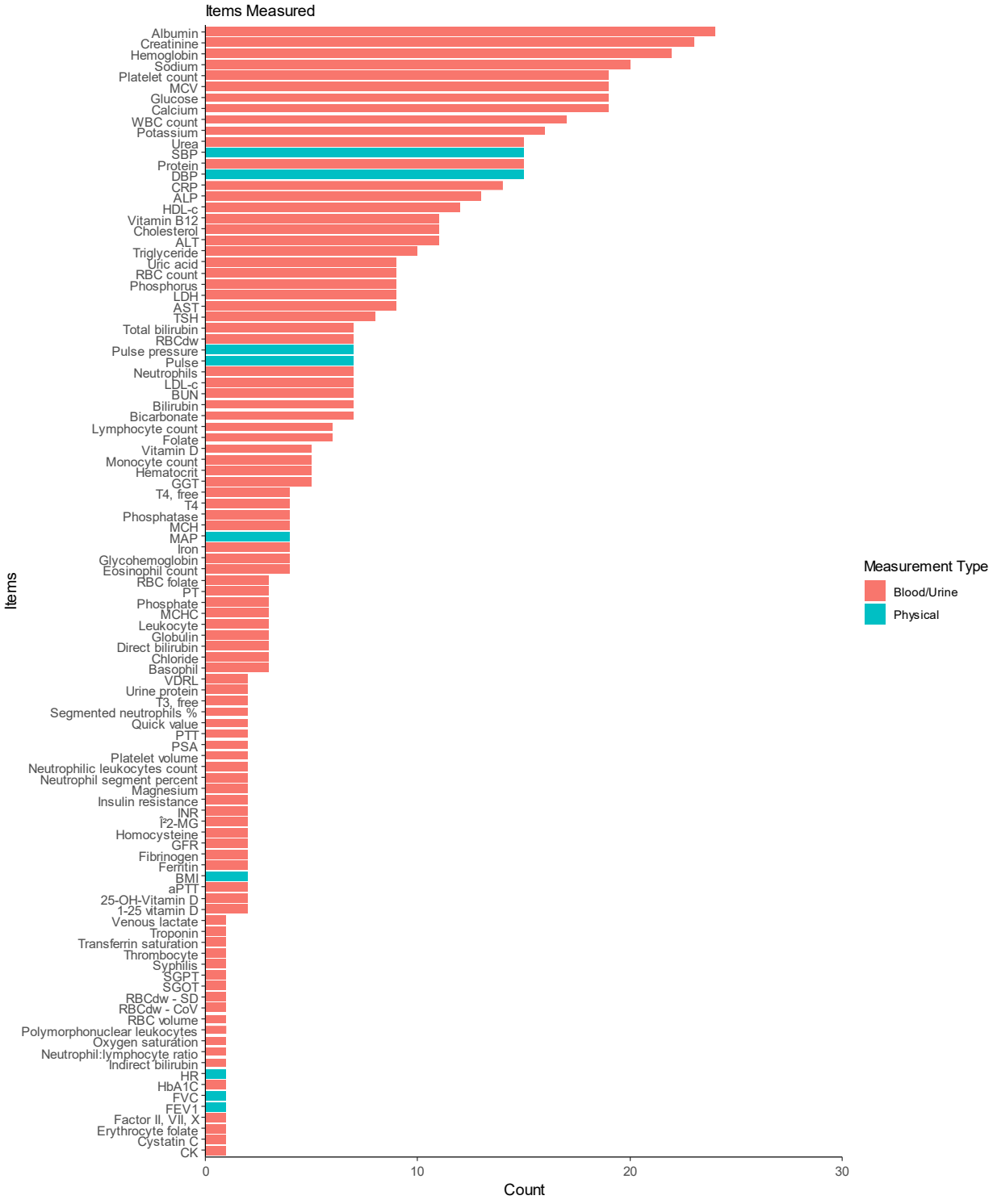
Authors BMC, DGS, and SHM assessed the certainty of the quality of evidence using the Grading of Recommendations, Assessment, Development, and Evaluation (GRADE) methodology. We evaluated the evidence as high, moderate, low, or very low according to the Grade handbook. Evidence was graded on the mortality, adverse events, sex differences, and preclinical models' subgroups from a narrative synthesis perspective. Evidence was further graded on the studies included in the meta-analysis and studies included in the age subgroup of the meta-analysis; these are subsets of the mortality subgroup. After the evidence was graded, a summary of findings table was constructed. The assumed risk, corresponding risk, and relative effects sections were removed from the table as most of our outcomes are being assessed from a narrative synthesis perspective. All decisions to downgrade or upgrade evidence are explained in the footnotes of the table.



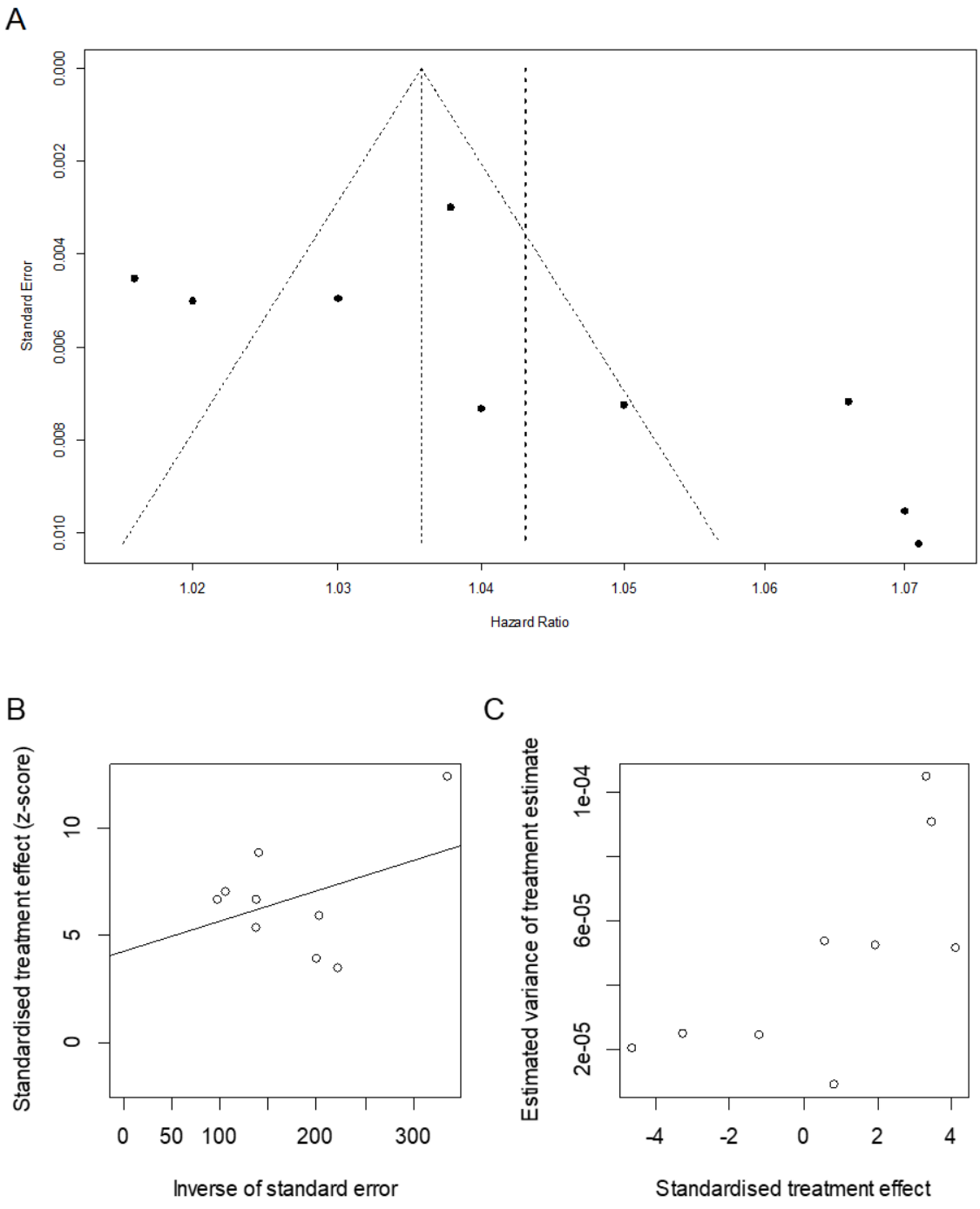
Supplemental Figure 1. Subgroup sorting of studies flow chart for systematic review. *NB:* One study can appear in multiple subgroups (*e.g.,* the mortality subgroup and the sex differences subgroup).



Supplemental Figure 2. Histograms of study distribution for summary data used to create separate analyses for the mortality subgroup meta-analysis. The grey dashed lines represent the cut-offs used to create the subgroups for analysis.

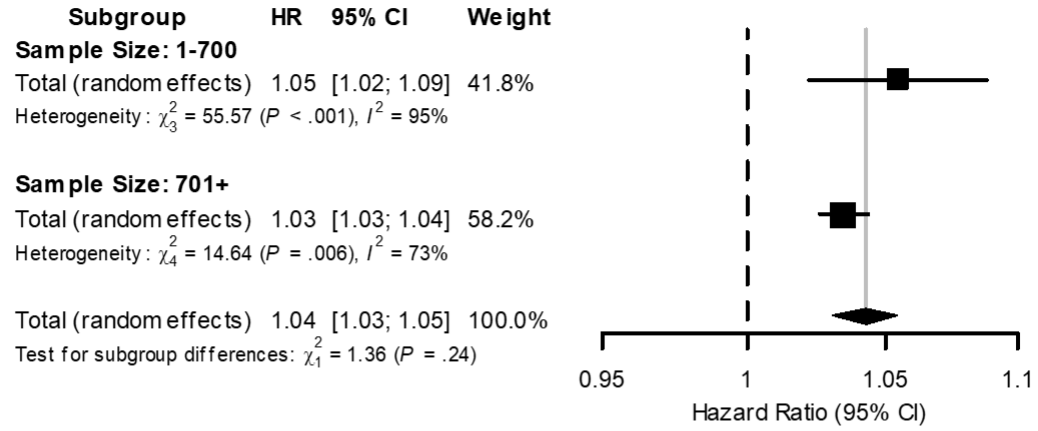


Supplemental Figure 3. Number of FI-Labs items that were included from human studies in the systematic review. For studies that included multiple FI-Labs, each FI-Lab was included in the above count. Blood/urine measures were any measures from blood/urine samples. Physical measures were assessed externally, primarily including cardiovascular measures.

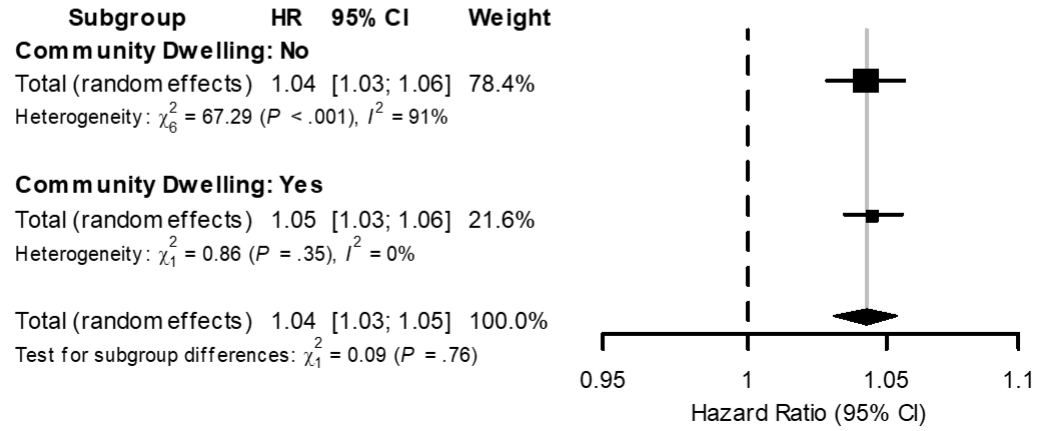


Supplemental Figure 4. Meta-bias for hazard ratios in mortality meta-analysis subgroup. A) Funnel plot. B) Egger's test plot. C) Begg-Mazumdar's test plot.

A



B



Supplemental Figure 5. Forest plots of mortality risk by hazard ratio (HR) according to a 0.01 increase in frailty measured by the FI-Lab. All forest plots used HRs from all studies included in the mortality subgroup meta-analysis. Data presented on a $\log_{10}$, dotted-black line represents no effect, solid-grey line indicates the overall effect of all studies. CI: confidence interval. A) Analysis separated by sample size. B) Analysis separated by the presence or absence of a community dwelling cohort.

Supplemental Table 1. Risk of bias assessment for studies included in the mortality and adverse events subgroups.

Paper	Representativeness of exposed cohort	Appropriately constructed FI-Lab	Demonstration that outcome of interest was not present at start of study	Comparability of cohorts on the base of the design or analysis	Assessment of outcome	Was follow-up long enough for outcomes to occur?	Adequacy of follow-up of cohorts	Selection (/***)	Comparability (/**)	Outcome (/***)
Mortality										
Blodgett et al. 2016	B	A	A	A*	B	A	B	***		***
Blodgett et al. 2017	A	A	A	A, B	B	A	B	***	**	***
Ellis et al. 2020	C	A	C	A, B	B	A	D	*	**	**
Gu et al. 2021										
Hao et al. 2019	B	A	B	A, B	B	A	C	***	**	**
Heikkila et al. 2021	B	A	A	A, B	B	A	A	***	**	***
Howlett et al. 2014	C	A	B	A, B	B	A	B	**	**	***
Jager et al. 2017	C	A	B	A, B	A	A	A	**	**	***
Klausen et al. 2017	C	A	C	A	B	A	A	*	*	***
Mitnitski et al. 2015	B	A	A	B	B	A	D	***	*	**
Ritt et al. 2017	C	A	B	A, B	D	A	A	**	**	**
Rockwood et al. 2015	C	A	B	A, B	B	A	D	**	**	**
Sohn et al. 2019	C	A	C	C	B	A	D	*		**
Wang et al. 2019	C	A	C	A, B	B	A	B	*	**	***

Paper	Representativeness of exposed cohort	Appropriately constructed FI-Lab	Demonstration that outcome of interest was not present at start of study	Comparability of cohorts on the base of the design or analysis	Assessment of outcome	Was follow-up long enough for outcomes to occur?	Adequacy of follow-up of cohorts	Selection (/***)	Comparability (/**)	Outcome (/***)
Yang et al. 2019	C	A	B	A, B	B	A	B	**	**	***
Adverse Events										
Bello et al. 2018	C	A	C	A, B	A	N/A	N/A	*	**	*
Blodgett et al. 2016	B	A	A	A	C	A	B	***	*	**
Blodgett et al. 2019	A	A	A	A, B	B	N/A	N/A	***	**	*
Cheung et al. 2017	C	A	C	A	B	A	B	*	*	***
Ellis et al. 2020	C	A	C	A, B	D	A	D	*	**	*
Heikkila et al. 2021	B	A	B	A, B	B	A	B	***	**	***
Justice et al. 2019	C	A	C	C	A	A	A	*		***
Ma et al. 2018	B	A	A	A, B	A	N/A	N/A	***	**	*
Nixon et al. 2019	C	A	C	C	A	A	D	*		**
Sohn et al. 2019	C	A	C	A, B	B	A	B	*	**	***
Wang et al. 2019										

Appendix D, Sub-appendix A PRISMA 2020 checklist

Section and Topic	Item #	Checklist item	Location where item is reported
TITLE			
Title	1	Identify the report as a systematic review.	Title
ABSTRACT			
Abstract	2	See the PRISMA 2020 for Abstracts checklist.	
INTRODUCTION			
Rationale	3	Describe the rationale for the review in the context of existing knowledge.	Introduction
Objectives	4	Provide an explicit statement of the objective(s) or question(s) the review addresses.	Introduction
METHODS			
Eligibility criteria	5	Specify the inclusion and exclusion criteria for the review and how studies were grouped for the syntheses.	Methods: Data Source and Search Strategy, Subgroups
Information sources	6	Specify all databases, registers, websites, organisations, reference lists and other sources searched or consulted to identify studies. Specify the date when each source was last searched or consulted.	Methods: Data Source and Search Strategy
Search strategy	7	Present the full search strategies for all databases, registers and websites, including any filters and limits used.	Methods: Data Source and Search Strategy
Selection process	8	Specify the methods used to decide whether a study met the inclusion criteria of the review, including how many reviewers screened each record and each report retrieved, whether they worked independently, and if applicable, details of automation tools used in the process.	Methods: Data Source and Search Strategy
Data collection process	9	Specify the methods used to collect data from reports, including how many reviewers collected data from each report, whether they worked independently, any processes for obtaining or confirming data from study investigators, and if applicable, details of automation tools used in the process.	Methods: Data Collection and Management
Data items	10a	List and define all outcomes for which data were sought. Specify whether all results that were compatible with each outcome domain in each study were sought (*e.g.* for all measures, time points, analyses), and if not, the methods used to decide which results to collect.	Methods: Data Collection and Management

Section and Topic	Item #	Checklist item	Location where item is reported
	10b	List and define all other variables for which data were sought (*e.g.* participant and intervention characteristics, funding sources). Describe any assumptions made about any missing or unclear information.	Methods: Data Collection and Management
Study risk of bias assessment	11	Specify the methods used to assess risk of bias in the included studies, including details of the tool(s) used, how many reviewers assessed each study and whether they worked independently, and if applicable, details of automation tools used in the process.	Methods: Risk of Bias Assessment
Effect measures	12	Specify for each outcome the effect measure(s) (*e.g.* risk ratio, mean difference) used in the synthesis or presentation of results.	Methods: Data Collection and Management
Synthesis methods	13a	Describe the processes used to decide which studies were eligible for each synthesis (*e.g.* tabulating the study intervention characteristics and comparing against the planned groups for each synthesis (item #5)).	Methods: Data Synthesis
	13b	Describe any methods required to prepare the data for presentation or synthesis, such as handling of missing summary statistics, or data conversions.	Methods: Data Synthesis
	13c	Describe any methods used to tabulate or visually display results of individual studies and syntheses.	Methods: Data Synthesis
	13d	Describe any methods used to synthesize results and provide a rationale for the choice(s). If meta-analysis was performed, describe the model(s), method(s) to identify the presence and extent of statistical heterogeneity, and software package(s) used.	Methods: Data Synthesis
	13e	Describe any methods used to explore possible causes of heterogeneity among study results (*e.g.* subgroup analysis, meta-regression).	Methods: Data Synthesis
	13f	Describe any sensitivity analyses conducted to assess robustness of the synthesized results.	N/A
Reporting bias assessment	14	Describe any methods used to assess risk of bias due to missing results in a synthesis (arising from reporting biases).	Methods: Meta-bias Assessment
Certainty assessment	15	Describe any methods used to assess certainty (or confidence) in the body of evidence for an outcome.	Methods: Certainty of Cumulative Evidence
RESULTS			
Study selection	16a	Describe the results of the search and selection process, from the number of records identified in the search to the number of studies included in the review, ideally using a flow diagram.	Results: Search and Selection
	16b	Cite studies that might appear to meet the inclusion criteria, but which were excluded,	N/A

Section and Topic	Item #	Checklist item	Location where item is reported
		and explain why they were excluded.	
Study characteristics	17	Cite each included study and present its characteristics.	Results: Characteristics of Included Studies
Risk of bias in studies	18	Present assessments of risk of bias for each included study.	Results: Certainty of Evidence
Results of individual studies	19	For all outcomes, present, for each study: (a) summary statistics for each group (where appropriate) and (b) an effect estimate and its precision (*e.g.* confidence/credible interval), ideally using structured tables or plots.	Results
Results of syntheses	20a	For each synthesis, briefly summarise the characteristics and risk of bias among contributing studies.	Results
	20b	Present results of all statistical syntheses conducted. If meta-analysis was done, present for each the summary estimate and its precision (*e.g.* confidence/credible interval) and measures of statistical heterogeneity. If comparing groups, describe the direction of the effect.	Results: FI-Lab as a Predictor of Mortality
	20c	Present results of all investigations of possible causes of heterogeneity among study results.	Results
	20d	Present results of all sensitivity analyses conducted to assess the robustness of the synthesized results.	N/A
Reporting biases	21	Present assessments of risk of bias due to missing results (arising from reporting biases) for each synthesis assessed.	Results: FI-Lab as a Predictor of Mortality
Certainty of evidence	22	Present assessments of certainty (or confidence) in the body of evidence for each outcome assessed.	Results: Certainty of Evidence
DISCUSSION			
Discussion	23a	Provide a general interpretation of the results in the context of other evidence.	Discussion
	23b	Discuss any limitations of the evidence included in the review.	Discussion: Quality of evidence
	23c	Discuss any limitations of the review processes used.	Discussion: Quality of evidence

Section and Topic	Item #	Checklist item	Location where item is reported
	23d	Discuss implications of the results for practice, policy, and future research.	Discussion
OTHER INFORMATION			
Registration and protocol	24a	Provide registration information for the review, including register name and registration number, or state that the review was not registered.	Methods: PRISMA guidelines
	24b	Indicate where the review protocol can be accessed, or state that a protocol was not prepared.	Methods: PRISMA guidelines
	24c	Describe and explain any amendments to information provided at registration or in the protocol.	Methods: PRISMA guidelines
Support	25	Describe sources of financial or non-financial support for the review, and the role of the funders or sponsors in the review.	Support and Conflicts of Interest
Competing interests	26	Declare any competing interests of review authors.	Support and Conflicts of Interest
Availability of data, code and other materials	27	Report which of the following are publicly available and where they can be found: template data collection forms; data extracted from included studies; data used for all analyses; analytic code; any other materials used in the review.	N/A

From: Page MJ, McKenzie JE, Bossuyt PM, Boutron I, Hoffmann TC, Mulrow CD, et al. The PRISMA 2020 statement: an updated guideline for reporting systematic reviews. BMJ 2021;372:n71. doi: 10.1136/bmj.n71

Appendix D, Sub-appendix B Modified Newcastle-Ottawa Risk of Bias Assessment

Risk of Bias Assessment

Note: A study can be awarded a maximum of one star for each numbered item within the Selection and Outcome categories. A maximum of two stars can be given for Comparability.

Selection

1) Representativeness of the exposed cohort
   a) truly representative of the average 20+ year old community-dwelling population *
   b) somewhat representative of the average 20+ year old community-dwelling population *
   c) selected group of users
   d) no description of the derivation of the cohort

2) Appropriately constructed FI-Lab
   a) FI-Lab items meet all 5 criteria specified by Searle et al. 2008, contains 10 or more items, and at least 70% of items are non-arbitrary laboratory measures *
   b) FI-Lab meets fewer than 5 criteria specified by Searle et al. 2008, contains fewer than 10 items, or fewer than 70% of items are non-arbitrary laboratory measures
   c) no description

3) Demonstration that outcome of interest was not present at start of study
   a) Sample is community-dwelling and does not have a common disease or acute adverse event *
   b) Sample is non-community-dwelling and does not have a common disease or acute adverse event *
   c) Sample has a common disease or acute adverse event

Comparability

1) Comparability of cohorts on the basis of the design or analysis
   a) study controls for age in analysis/cohorts *
   b) study controls for sex in analysis/cohorts *
   c) study doesn't control for any factors

Outcome

1) Assessment of outcome
   a) independent blind assessment *
   b) record linkage *
   c) self-report
   d) no description

2) Was follow-up long enough for outcomes to occur

a) yes (6 months for the Mortality Subgroup) *
b) no

3) Adequacy of follow up of cohorts
a) complete follow up - all subjects accounted for *
b) subjects lost to follow up unlikely to introduce bias - small number lost - > 95% follow up, or description provided of those lost *
c) follow up rate < 95% and no description of those lost
d) no statement

Appendix E: Supplemental data for Chapter 5

Table 1 Primers used for RT-qPCR in Chapter 5. F=Forward; R=Reverse.

Adrb1	F	5’	GTTTACTCAAGACCGAAAGCAG	3’
Adrb1	R	5’	CACTCTCCCAACTCCTCCTAA	3’
Akt1	F	5’	GACGTAGCCATTGTGAAGGAG	3’
Akt1	R	5’	GCCGTTCCTTGTAGCCAAT	3’
Ar	F	5’	CTGCCTTGTTATCTAGCCTCA	3’
Ar	R	5’	AAATACCATCAGTCCCATCCAG	3’
Atp2a2	F	5’	TGGTTGTACTTCTGTTATCTGCTC	3’
Atp2a2	R	5’	TGTCACCTTCTACTTTGTCCAG	3’
Camkk2	F	5’	CCCACAGTAGAGTCACACCA	3’
Camkk2	R	5’	GCCCTTTCCAATTTCATCCTTC	3’
Casq2	F	5’	CTGTCTCTATTACCACGAACCTG	3’
Casq2	R	5’	GAATCCACCATCACAAAGCCTA	3’
Col1a1	F	5’	CGCAAAGAGTCTACATGTCTAGG	3’
Col1a1	R	5’	CATTGTGTATGGCAGCTGACTTC	3’
Col3a1	F	5’	TTCTTCTCACCCTTCTTCATCC	3’
Col3a1	R	5’	TCTCTAGACTCATAGGACTGACC	3’
Fst	F	5’	GAAGTGAAGCATTCTGGATCTTG	3’
Fst	R	5’	GGAAGAGATAGGAAAGCTGTAGTC	3’
Gapdh	F	5’	AATGGTGAAGGTCGGTGTG	3’
Gapdh	R	5’	GTGGAGTCATACTGGAACATGTAG	3’
Hprt	F	5’	CCCCAAAATGGTTAAGGTTGC	3’
Hprt	R	5’	AACAAAGTCTGGCCTGTATCC	3’
Igf1	F	5’	CACTGACATGCCCAAGACTC	3’
Igf1	R	5’	GCTCACCTTTCCTTCTCCTTT	3’
Mstn	F	5’	GCCATCATCTTGCTGTAACCT	3’
Mstn	R	5’	CAGTCAAGCCCAAAGTCTCT	3’
Mtor	F	5’	AAGTCATCACATCCAAGCAGA	3’
Mtor	R	5’	TGCATCACTCGTTCATCCTG	3’
Myh1	F	5’	GGACAAACTGCAATCAAAGGTC	3’
Myh1	R	5’	CTGGATCTTGCGGAATTTGG	3’
Myh2	F	5’	TCAGGCTTCAGGATTTCCTG	3’
Myh2	R	5’	GGATCTTGCGGAACTTGGATA	3’
Myh4	F	5’	GGACTTGGTGGACAAACTACA	3’
Myh4	R	5’	TGCTGGATCTTACGGAACTTG	3’
Myh6	F	5’	GCGCATTGAGTTCAAGAAGATAG	3’
Myh6	R	5’	AAGTAGAGCTTCATCCATGGC	3’
Myh7	F	5’	CAACATGGAGCAGATCATCAAG	3’
Myh7	R	5’	CTGGTCAGGTCATTGACAGAA	3’
Pln	F	5’	ATGACGACGATTCAAATCTCTTGG	3’
Pln	R	5’	TGGGTTTGCAAAGTTAGGCATAA	3’
Ryr2	F	5’	GGTGGATGTGGAAAAGTGGA	3’
Ryr2	R	5’	CTGTAGGAATGGCGTAGCAA	3’