The Effects of Obesity and Exercise on Healthspan, Cancer Incidence, and Lifespan in a Mouse Model of Radiation-Induced Cancer

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General Abstract

Background: The number of cancer survivors across North America is increasing, with estimates indicating that this population will grow to nearly 19 million individuals by the end of 2020. This increase can be attributed, in part, due to improvements in cancer treatments, of which radiation therapy is most commonly used. Unfortunately, exposure to radiation also increases the risk of secondary cancer development long-term. Moreover, obesity and physical inactivity are prevalent, modifiable, risk factors among cancer survivors, with both factors being linked to decrements in quality of life, increased cancer risk, and greater mortality risk. To date, there has been promising epidemiological and clinical data highlighting the role of exercise as a way to mitigate cancer risk and improve survival; however, longitudinal studies are lacking and the effects of radiation in these studies have been largely ignored. Therefore, there is a major clinical need to directly evaluate the combinatory long-term effects of radiation, exercise, and/or obesity to reveal their implications on healthspan, cancer incidence, and survival. Recent pre-clinical work from our group has shown that after being exposed to radiation, endurance exercise prevented several negative alterations to hematopoietic stem cells and their niche caused by high-fat diet (HFD)-induced obesity. We also showed that leukemic blast viability *in vitro* was greater when cultured in bone marrow supernatant from mice with HFD-induced obesity compared to bone marrow supernatant from mice without HFD-induced obesity. It is unknown, however, whether these findings extend to alterations in cancer risk across the lifespan. As such, the purpose of this study was to evaluate the effects of lifelong exercise and diet-induced obesity on healthspan, cancer incidence, and survival in an established mouse model of radiation-induced cancer.

Methods: Male CBA mice (n=80) were randomly divided into either a control diet (CTRL; n=40) of 45% high-fat diet (HFD; n=40) and then further divided into either a sedentary group (SED; n=20) or exercise-trained group (EX; n=20). At age 13 weeks, all mice were exposed to a cancer inducing dose of whole-body ionizing radiation (3 Gy). A healthspan index score and endpoint monitoring were conducted throughout the study by blinded investigators.

Results: When normalized to CTRL/SED, the highest healthspan score was in the CTRL/EX (score = +2.5), followed by HFD/EX (score = +1) and lastly HFD/SED (score = -0.5). Cancer incidence was significantly higher in the HFD/SED group when compared to the CTRL/EX group ($p<0.05$) and a trend for higher cancer incidence for HFD/SED was observed when compared to the CTRL/SED group ($p=0.079$). There was no significant difference between the HFD/SED and HFD/EX group in cancer incidence ($p>0.05$). Overall survival was significantly higher in the HFD/SED group compared to CTRL/SED group ($p<0.05$); however, risk of cancer-related mortality was 1.6-times higher in the HFD/SED group compared to the CTRL/SED group (RR=1.60; 95% CI, 1.00-2.56; $p=0.0495$) and 1.68-times that of the CTRL/EX group (RR=1.68; 95% CI, 1.02-2.78; $p=0.0415$).

Conclusion: Our findings show that lifelong exercise training resulted in higher healthspan index, lower cancer incidence, and lower risk of cancer-related mortality following radiation exposure, with these effects being largely reversed by HFD-induced obesity. This study provides the rationale for future studies to uncover cellular and molecular mechanisms that could be underlying these results. Moreover, this study presents a proof of concept for the consideration of clinical studies in cancer survivors examining exercise as an intervention to reduce the long-term effects of radiation.

Chapter I: Literature Review

Introduction

According to the Canadian Cancer Society (2019), the 5-year survival rate following cancer diagnosis is currently 63% and cancer mortality rates have decreased by 32% and 17% in males and females, respectively. One reason for this increase in survivorship can be attributed to advances in cancer screening, detection, and therapy. One such treatment is radiation therapy which is used by two-thirds of cancer patients (American Cancer Society, 2013). Radiation is an effective anti-tumour therapy, however, it also can lead to adverse long-term health effects in healthy tissues including toxicity and increased cancer risk (Dracham et al., 2018; Kleinerman et al., 1995). Indeed, evidence from studies following atomic bomb survivors have shown that radiation is strongly associated with increased cancer incidence later in life (D. L. Preston et al., 2007; Dale L Preston et al., 1994). The most common radiation-induced malignancy is acute myeloid leukemia (AML) (Preston et al., 1994), which is derived from mutations in the extremely radiation-sensitive hematopoietic stem cells (HSCs) (Williams & McBride, 2011). While AML is the most common radiation-induced cancer, several other types of cancer have also been strongly linked with radiation exposure (Braunstein & Nakamura, 2013; D. L. Preston et al., 2007). Despite these strong links between radiation and cancer, there is a large amount of interindividual variability in radiation-induced cancer latency with some persons exposed to radiation never developing cancer at all. As such, recent research efforts have focused on identifying genetic and modifiable risk factors that contribute to radiation-induced cancer risk.

Two modifiable risk factors that have been epidemiologically linked to increased risk of a variety of cancers, and which are prevalent among cancer survivors, are obesity and physical inactivity. Further, obesity and physical inactivity contribute to reduced quality of life (Wolin et al., 2010), and longevity (Schmid & Leitzmann, 2015) among cancer survivors. According to the National Cancer Institute (2017), the number of cancer survivors with obesity is increasing

steadily. There is a limited understanding, however, with regards to the extent in which obesity and physical inactivity impact healthspan, the incidence of radiation-induced malignancies, and longevity following radiation exposure. As such, a *critical need* exists to develop a better understanding of how modifiable risk factors interact with radiation exposure, such that we can provide cancer survivors with personalized care to enhance overall quality of life and decrease secondary cancer risk induced by radiation.

The Hematopoietic Stem Cell (HSC) Niche

HSCs are the most primitive cells in the hematopoietic system and exist in our bone marrow within a specialized microenvironment or niche. A niche can be defined as a local tissue microenvironment that contains all the components required for stem cell function and regulation (Morrison & Spradling, 2008). The idea that an HSC niche existed was first introduced by Schofield in 1978. Prior to the postulate of an HSC niche, it was believed that HSCs were derived from the spleen according to several colony forming units – spleen (CFU-S) assays. Schofield demonstrated that the HSC population in the bone marrow had better hematopoietic reconstitution than in the spleen after radiation exposure (Schofield, 1978). As such, Schofield's work was the first to provide support for an HSC niche in the bone marrow that contains cells with enhanced reconstitution potential. The HSC niche is composed of a variety of different cell types of which the most prominent are HSCs and their progeny, mesenchymal stem cells (MSCs), and endothelial cells (Asada et al., 2017). These cell types work together to maintain and regulate hematopoiesis within the bone marrow during steady state and in response to stress.

HSCs can exist in a quiescent or active state and exhibit their stemness via their ability to self-renew and differentiate into all mature hematopoietic cells. Briefly, HSCs can differentiate directly into platelets, down the myeloid lineage to form red blood cells and innate immune cells, or down the lymphoid lineage to form adaptive immune cells (Y. Zhang et al., 2018). The distribution of HSCs throughout the bone marrow, however, is not homogeneous. Earlier work indicated that roughly 20% of HSCs reside in an endosteal niche, with the other 80% residing in a perivascular

niche, highly enriched in sinusoidal vessels (Kiel et al., 2005). It was observed that the more quiescent HSCs resided near arterioles in the endosteal niche, whereas the more active and proliferative HSCs resided near the leaky sinusoids and perivascular cells in the perivascular niche (Itkin et al., 2016; Kunisaki et al., 2013). Both niches played important yet functionally distinct roles. Briefly, the endosteal niche had been shown to be important in supporting HSC regulation and expansion, as well as enhancing HSC engraftment post-transplantation (L. M. Calvi et al., 2003; J. Zhang et al., 2003), whereas the perivascular niche was an excellent source of stem cell factor (SCF) which is secreted by perivascular cells and is important in the promotion and maintenance of HSCs (Ding et al., 2012). This notion of two distinct HSC niches has been recently challenged, with more recent evidence suggesting that these sub-niches are more dynamic than originally thought and can adapt to various environmental stimuli such as stress or cancer (Laura M. Calvi & Link, 2015). In addition, it has been shown that nearly all HSCs reside near sinusoidal vessels and away from bone surfaces (Acar et al., 2015), but this does not rule out the importance of the endosteal niche as it too is supplied by sinusoidal vessels and stromal cells that are vital for HSC function (Baccin et al., 2020). Taken together, the HSC niche is represented by a highly complex, dynamic and heterogeneous network of cells and regulating factors that help to achieve homeostasis in response to different environmental stimuli (Figure A).

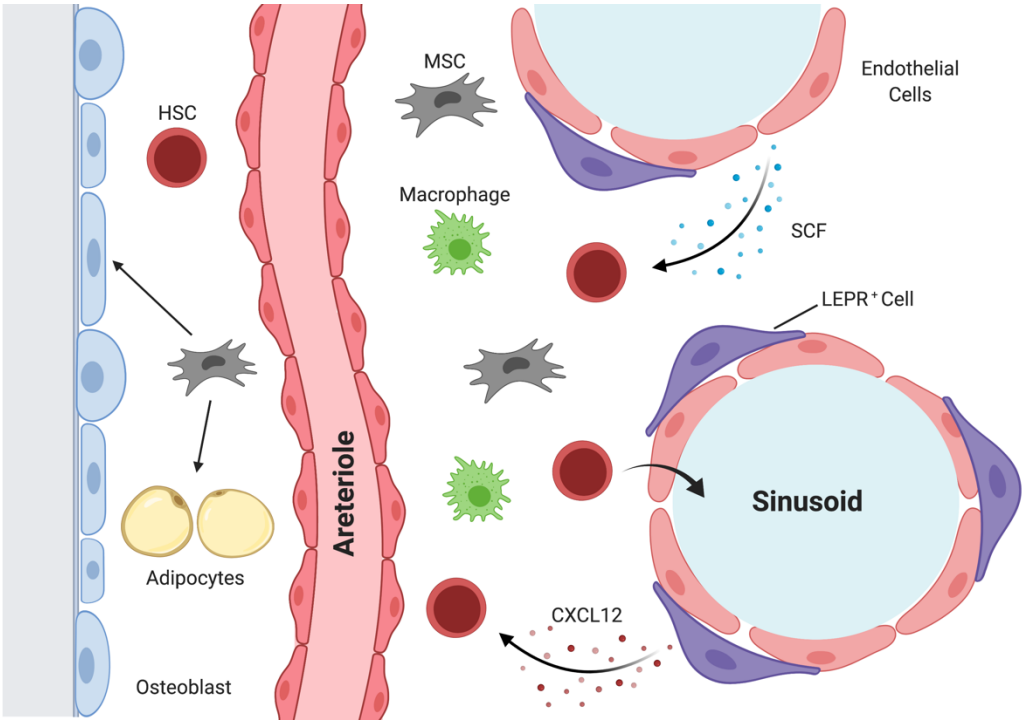

Figure A. Simplified overview of the HSC niche. The HSC niche is composed of a highly complex, dynamic and heterogeneous network of cells and regulating factors that help in the maintenance, maturation and/or mobilization of HSCs. Common regulating factors released predominantly from endothelial cells and LEPR$^+$-MSCs include CXCL12 and SCF. MSCs have also been shown to differentiate into either osteoblasts or adipocytes. The coordination of cellular communication among the various cell types in the HSC niche helps to achieve homeostasis in response to different environmental stimuli. Created with BioRender.com.

At the same time as Schofield was developing his stem cell niche hypothesis, work by Dexter, Allen and Lajtha (1977) provided new evidence that another population of stem cells found in the HSC nice – MSCs - play a large role in HSC regulation. Specifically, they showed that in a co-culture of HSCs and MSCs, HSCs had enhanced proliferation and expansion compared to HSCs cultured without MSCs (Dexter et al., 1977). MSCs within the bone marrow are also heterogeneous (Severe et al., 2019), with some of these subsets (i.e. leptin receptor; LEPR$^+$-

MSCs) acting as sources of SCF and CXCL12, which is also important in the promotion and maintenance of HSCs (Crane et al., 2017; Zou et al., 1998). In addition to their paracrine role in HSC regulation, MSCs are able to self-renew and possess the ability to differentiate into osteoblasts, adipocytes, or chondrocytes. Osteoblasts and adipocytes in particular, have also been proposed to be important in HSC regulation. While it was originally thought that osteoblasts were directly involved in HSC maintenance, osteoblast ablation showed subsequent depletion of early lymphoid progenitors but no effect on HSC frequency (Visnjic et al., 2004; J. Zhu et al., 2007). In a gain of function study using strontium treatment to increase osteoblasts, there was no acute change to HSC content (Lymperi et al., 2008). In addition, osteoblasts express minimal amounts of CXCL12 and SCF (Ding et al., 2012; Ding & Morrison, 2013; Greenbaum et al., 2013), providing support that osteoblasts either do not regulate HSCs at all or rather, indirectly regulate them via other niche cells. On the other hand, there is much stronger support for the direct role of adipocytes (which are derived primarily from $LEPR^{+}$-MSCs) on HSC regulation. Marrow adipose tissue (MAT) is distributed throughout the long bone cavities in a distal to proximal manner during postnatal development (Emery & Follett, 1964). By age 25 in humans, MAT makes up approximately 70% of the bone marrow and its content continues to increase throughout the lifetime (Scheller et al., 2014). A seminal study by Naveiras et al. (2009), evaluated the effect of MAT on hematopoiesis. Using A-ZIP/F1 “fatless” mice which are incapable of forming adipocytes, they showed that decreased MAT contributed to greater HSC frequency, cycling capacity, and CFUs, as well as enhanced engraftment of donor cells post bone marrow transplant, compared to wild-type mice (Naveiras et al., 2009). Furthermore, when mice were treated with a known inhibitor of adipogenesis (bisphenol A diglycidyl ether - BADGE), there was an increase in hematopoietic recovery and function compared to mice not treated with the inhibitor (Naveiras et al., 2009; R. J. Zhu et al., 2013). While these studies suggest that adipocytes in the bone marrow are negative regulators of hematopoiesis, relatively newer evidence from Boyd and colleagues (2017) showed that MAT may have some benefits on hematopoiesis as well, especially in the

context of AML. In their study, patients with AML had lower MAT relative to healthy controls and concurrently, a decrease in HSC maturation down the myeloid lineage. Further, MSCs collected from patients with AML lacked adipogenic differentiation. On the other hand, in healthy controls, MAT supported myelo-erythroid maturation while simultaneously inhibiting AML progenitors. Importantly, they also demonstrated that the administration of PPARγ (an agonist of adipogenesis) to AML mice, induced MAT which in turn improved myelo-erythroid maturation (Boyd et al., 2017). Taken together, while MAT appears to negatively regulate hematopoiesis under homeostatic conditions, these new findings suggest that it may play a key role in HSC regulation in the context of AML.

Ionizing Radiation and its Effects on the HSC Niche

Ionizing radiation (IR) is the energy released by atoms as they disintegrate. This energy can then travel via electromagnetic waves – either gamma or x-rays. Humans are exposed to low levels of background IR daily; however, in the past several decades human radiation exposure has increased dramatically, mostly due to its increased use for medical applications. Indeed, radiation therapy is used in roughly 67% of cancer patients in the United States (American Cancer Society, 2013). The units for IR are Gray (Gy - the amount of energy (Joules) absorbed per kilogram (kg) of matter, and the cancer type, tumour size, tumour location, and cancer stage dictate the amount of IR administered to a patient during radiation therapy (American Cancer Society, 2014). Depending on these factors, patients can receive IR doses ranging from 20-80 Gy, which is delivered in fractionated doses of typically 1.5-2 Gy over several treatments to reduce toxicity to healthy cells (Benderitter et al., 2014). In many cancer cases, radiation therapy is focally administered, however, in the case of hematological cancers, a bone marrow transplant may be required which involves myeloablative total body irradiation. Typically, total body irradiation is delivered in 8-12 fractionated doses, three times a day, over the course of four days for a total administration of between 12-15 Gy (Seung et al., 2013; Storb et al., 1994).

IR is geno- and cytotoxic which makes it an effective cancer therapy. Unfortunately, these characteristics also increase the likelihood of malignant transformations in healthy cells which could develop into secondary cancers. According to the Life Span Study, which longitudinally tracked close to 95 thousand Hiroshima and Nagasaki atomic bomb survivors, there were 231 confirmed cases of leukemia - of which acute myeloid leukemia (AML) made up 103 of those cases (45%) - amongst those exposed to between 0 and 4 Gy of IR (Dale L Preston et al., 1994). Furthermore, Richardson and colleagues (2009) showed that among these atomic bomb survivors, it can be expected that 310 of them will develop and die of some form of leukemia secondary to the late effects of radiation exposure, of which 124 of these deaths (40%) will be caused by radiation-induced AML (Richardson et al., 2009). Together, these results suggest that AML is the most common radiation-induced malignancy, although other malignancies are also prevalent (Dale L Preston et al., 1994). Indeed, other studies have shown that persons exposed to radiation at age 30 have increased radiation-associated cancer risk (35% per Gy in men and 58% per Gy in women) at multiple sites throughout the body by age 70 (D. L. Preston et al., 2007). Specifically, other cancer types that have been associated with radiation exposure include (but are not limited to): breast, colorectal, thyroid, endometrial, cervical, and central nervous system cancers (Braunstein & Nakamura, 2013), as well as liver cancer secondary to radiation-induced liver disease (Kim & Jung, 2017; Koay et al., 2018) Combined with the fact that radiation-induced cancer risk has not improved despite advances in radiotherapy technology (Schaapveld et al., 2015), it is evident that the late effects of radiation therapy, including radiation-induced cancer, are of major clinical concern.

Radiation-induced malignancy is a multi-stage process that involves a combination of the immediate, direct and long-term, indirect effects of IR. The direct effects of IR include an increase in oxidative stress leading to DNA damage and mutations (Azzam et al., 2012). HSCs are particularly susceptible to IR. Mice exposed to 6.5 Gy of total body IR had a decrease in HSC quantity, impaired HSC expansion, and an increased expression of senescence markers (Wang,

Schulte, LaRue, Ogawa & Zhou, 2006). HSCs exposed to 4 Gy of IR *in vitro* had an increase in reactive oxygen species (ROS; indicator of oxidative stress), DNA damage (gamma-H2AX foci per cell), and cell death compared to controls (0 Gy) (Yamaguchi & Kashiwakura, 2013). Moreover, exposure to total body IR has been shown to impair HSC self-renewal, repopulating capacity, and induce myeloid skewing (Shao et al., 2014). This in turn, may increase the risk of developing AML. Interestingly, even low doses of IR (0.02 Gy) produced persistent oxidative stress, decreased HSC self-renewal, an increase in myeloid-primed quiescent HSCs, and an increase in myeloid cells (Rodrigues-Moreira et al., 2017). The indirect effects of IR can exacerbate cancer risk introduced by the direct effects. Indirect effects are the result of a prolonged inflammatory response produced by surviving irradiated cells and/or the progeny of previously irradiated cells (Lorimore et al., 2003). These cells release various inflammatory cytokines (i.e. IL-1β, IL-6, TNF-α, CXCL-12, TGF-β, SDF-1, CXCR4), which lead to an inflammatory environment, oxidative stress, and tissue damage/cell death (Kim et al., 2014). Taken together, both the direct and indirect effects of IR, even at low doses, help to produce a pro-inflammatory and oncogenic milieu in the bone marrow which can contribute to an increase in the incidence of radiation-induced cancers.

It is clear that IR, through both its direct and indirect effects, is detrimental to the bone marrow and plays a large role in cancer promotion. Given the increasing number of cancer survivors and frequency in which radiation therapy is used in cancer treatments, it is no surprise that the long-term effects of such treatments are a major clinical concern. Perhaps, even more concerning is that despite improvements in IR delivery, radiation-induced cancer incidence in cancer survivors treated with radiation therapy has not improved (Schaapveld et al., 2015). While these late effects of radiation on their own present new and worrisome challenges for the cancer survivor population, it should be noted that not all persons exposed to radiation therapy will develop secondary cancers, and there is large variability in the timespan between radiation exposure and secondary cancer incidence in those who do. As such, developing a better understanding of the

genetic and modifiable risk factors responsible for altered cancer risk following radiation exposure would provide the opportunity for increased surveillance and the development of targeted interventions in individuals who are at increased risk.

The Effects of Obesity on the HSC Niche

Obesity in both Canada and the United States is an epidemic. Specifically, in Canada, we have seen a marked increase in individuals with obesity in the past three decades. According to Obesity Canada (2019), over 30% of Canadians have obesity which is predicted to result in nearly $9 billion dollars in healthcare costs by 2021 (Obesity Canada, 2019). The obesity epidemic extends to cancer survivors as the National Cancer Institute (2017) indicates that the number of cancer survivors with obesity is increasing. This is problematic as obesity has been epidemiologically linked to about 20% of cancer cases including breast, liver, colorectal, and leukemic cancers, to name a few (Wolin et al., 2010). Moreover, evidence from pre-clinical models have shown that aged mice with obesity have increased mortality, overall reduced healthspan, and increased spontaneous cancer incidence (Schafer et al., 2019). Despite the increasing incidence of cancer survivors with obesity, relatively little attention has been paid to the effects of obesity on cancer recurrence in long-term cancer survivors; particularly those exposed to radiation.

Obesity is known as a chronic low-grade inflammatory condition. It is characterized by an abnormal or excessive increase in adipose tissue, which in turn leads to the release of pro-inflammatory cytokines (IL-6, TNF-α), and is associated with insulin resistance (Ellulu et al., 2017; Meijer et al., 2011). Obesity is associated with an increase in circulating leukocytes, in humans (Dixon & O'Brien, 2006; Niemiro et al., 2016) and mice (Singer et al., 2014). Since these mature circulating blood cell populations are short-lived and derived from HSCs, recent work has focused on the effects of obesity on hematopoiesis. Mice fed a 45% high-fat diet (HFD) for 18 weeks experienced a significant decrease in self-renewing HSCs, an increase in multipotent progenitor

cells (MPPs), and impaired HSC reconstitution upon transplantation into control diet fed mice (Van Den Berg et al., 2016). Obesity also induces myeloid lineage skewing as mice fed a HFD for 16 weeks had significant increases in pro-inflammatory monocytes and cytokines, as well as an increase in myeloid cells after serial bone marrow transplantation compared to mice fed a control diet (Singer et al., 2014). This aberrant myelopoiesis was found to begin as early as six weeks following commencement of a HFD and was dependent on cell-autonomous Toll-like receptor 4 (TLR4) (Liu et al., 2018). Importantly, the authors also showed that a HFD contributed to delayed leukocyte recovery following bone marrow injury using fluorouracil (5-FU) (Liu et al., 2018). Together these findings suggest that obesity induces activation and aberrant myelopoiesis in HSCs resulting in premature exhaustion of the most primitive HSC pool. Since AML is a result of uncontrolled expansion of myeloid lineage cells, these findings suggest that obesity may accelerate AML by enhancing myelopoiesis. This hypothesis has been supported by epidemiological data indicating that obesity is strongly associated with several types of leukemia, particularly AML (Castillo et al., 2012).

Recent work from our group has begun to address the effects of obesity on hematopoietic recovery following acute radiation-induced hematopoietic stress. We recently showed that mice who were fed a 45% HFD for 12 weeks, remained sedentary, and were exposed to a 3 Gy dose of IR at age 13 weeks, had higher MAT and a pro-inflammatory bone marrow environment, as well as lower bone marrow cellularity and pro-hematopoietic paracrine factors (Emmons et al., 2019). Moreover, there was greater viability of KG-1α leukemic blasts when cultured in bone marrow supernatant from those HFD-fed mice (Emmons et al., 2019). These results indicate that obesity delays hematopoietic recovery, potentially by creating pro-inflammatory alterations in the HSC niche, and a paracrine bone marrow environment that promotes the expansion of leukemic blasts *in vitro*. It remains unknown, however, whether these short-term alterations in hematopoiesis and the bone marrow result in increased incidence of AML or other cancers long-term after being exposed to radiation.

The Effects of Exercise on the HSC Niche

Exercise is generally accepted as anti-inflammatory, with studies suggesting that it may be beneficial at reducing the degree of inflammation associated with chronic pro-inflammatory conditions such as obesity (You et al., 2013), and in cancer patients and survivors (LaVoy et al., 2016). Although the effects of exercise on mature hematopoietic cells have received considerable attention, limited work has focused on the role of exercise in regulating hematopoiesis. Our lab recently examined the effects of an acute exercise bout on HSCs. We found that acute treadmill exercise increased HSC content in the spleen within 48 hours post-exercise (Emmons et al., 2016). In addition, 15 minutes post-exercise, there was an increase in HSC content in circulation, which closely mirrors the results from human studies (Bonsignore et al., 2002). Furthermore, we showed an increase in gene expression for pro-hematopoietic factors such as CXCL12 in muscle, and proliferating HSCs, MPPs, and MSCs in the bone marrow (Emmons et al., 2016). Conversely, a more recent study showed that mice who participated in voluntary wheel-running for 6 weeks had a decrease in circulating leukocytes at baseline compared to sedentary mice, but after undergoing cecal ligation and puncture (sepsis model), had improved emergency hematopoiesis (increase in circulating leukocytes) and survival compared to sedentary mice (Frodermann et al., 2019). We also previously showed that mice exposed to 10 weeks of treadmill exercise training had significantly more HSCs and CFUs, and an associated decrease in MAT (Baker et al., 2011). Similarly, we showed that mice exposed to eight weeks of treadmill exercise training had higher HSC quantity and whole bone marrow cell proliferation, as well as a decrease in pro-inflammatory cytokines in the bone marrow compared to sedentary mice (De Lisio & Parise, 2012). This therefore suggests that exercise is involved is altering the HSC niche and acts as a regulator of hematopoietic mobilization.

Exercise has also been shown to attenuate or reverse the effects of obesity on the bone marrow. Using a mouse model of colorectal cancer (CRC), our lab showed that mice who were fed a HFD for 8 weeks to induce obesity, then permanently switched to a control diet both during

and post CRC induction, and then started a 22 week long treadmill exercise training intervention, had reduced CRC incidence, MAT, bone marrow inflammatory markers, and myeloid skewing, as well as more quiescent and active HSCs compared to mice undergoing the same dietary intervention except remained sedentary (Emmons, Xu, et al., 2019). A main way in which exercise exerts its effects is through the MSC populations. For instance, exercise-trained mice have more osteoblasts and less adipocytes compared to sedentary mice (Baker et al., 2011; Marędziak et al., 2015). Additionally, work by Styner and colleagues showed in mice that 6-weeks of voluntary wheel running was able to attenuate increases in MAT and decrements to bone structure induced by a MAT agonist (Styner et al., 2015) or HFD (Styner et al., 2014, 2017). This suggests that exercise induces positive changes to the bone marrow, in part, by stimulating MSC differentiation down the osteoblastic region and reducing MAT.

It is therefore suggested that in both disease-free and disease contexts, exercise can play a role in positively stimulating hematopoiesis. In the context of radiation, however, there have only been a few studies to analyze the effects of exercise on hematopoiesis. We demonstrated that exercise-trained mice exposed to an acute challenge of radiation (1 and 2 Gy), had less expression of apoptotic markers and cell death in the bone marrow compared to sedentary controls, suggesting that exercise may protect bone marrow cells from radiation-induced death (De Lisio et al., 2011). We also demonstrated that pre-conditioning mice with treadmill exercise training before being exposed to radiation for bone marrow transplant, resulted in a greater hematopoietic reconstitution and overall survival compared to sedentary mice (De Lisio et al., 2013). To more precisely elucidate the cellular response to radiation in the bone marrow, our lab recently exposed exercise-trained mice and those with obesity to a single high dose of radiation and quantified various hematopoietic and stromal cell populations (Emmons et al., 2019). In this study, we demonstrated that exercise-trained mice, regardless of diet, had lower MAT and inflammatory cytokines in the marrow, as well as higher bone marrow cellularity and HSC content. Importantly, with respect to cancer incidence, there was an increase in KG-1a leukemic blast cell

viability *in vitro*, when cultured in bone marrow paracrine factors from mice with obesity after 24 hours (Emmons, Ngu, et al., 2019). Taken together, there is some evidence that shows support for exercise as a possible strategy to mitigate the effects of radiation of the HSC niche, especially at acute timepoints. Yet, it still remains unknown whether these effects can translate long-term to decrease cancer incidence and mortality.

Statement of Problem and Rationale

To summarize the above literature: The number of physically inactive cancer survivors with obesity who are exposed to radiation is rising. This is problematic as these factors can increase the lifetime risk of developing various secondary cancers, but specifically those that arise from HSCs, such as AML. HSCs are extremely radio-sensitive and they reside in a highly complex and diverse niche that dynamically adapts to environmental stimuli. Radiation is geno- and cytotoxic, exerting its deleterious effects both directly by causing DNA damage and indirectly through exacerbating the inflammatory profile of the HSC niche and inducing myeloid skewing. Similarly, obesity has been shown to increase HSC niche inflammation and induce myeloid skewing. This suggests that obesity can exacerbate an already oncogenic environment induced by radiation. Exercise on the other hand, has been shown to be beneficial for the HSC niche and mitigates both the negative cellular effects of radiation and obesity, suggesting that it could be a good strategy for improving healthspan as well as decreasing radiation-induced cancer incidence and mortality.

Within the exercise oncology field, most of the work has focused on physical and mental health-related outcomes (Campbell et al., 2019), while studies that have looked at exercise, cancer incidence and survival, have been largely epidemiologically based and focused on populations of patients with breast, prostate, and colorectal cancer, irrespective of treatment (Cormie et al., 2017). No previous studies have examined the direct effects of exercise, or obesity in the context of long-term effects of radiation exposure on healthspan, cancer incidence, and

survival. As such, the purpose of this study will be to address this gap using a pre-clinical established model of radiation-induced cancer (Major & Mole, 1978; Rivina et al., 2016).

Objective & Hypotheses

Our *overall objective* is to directly quantify the effects of lifelong-exercise training and/or HFD-induced obesity, in combination with a cancer inducing dose of radiation, on healthspan, cancer incidence, and survival in a mouse model of radiation-induced cancer. We *hypothesize* that sedentary mice with HFD-induced obesity, will exhibit greater cancer incidence as well as decreased healthspan and survival, compared to lean, exercise-trained mice after radiation exposure. Furthermore, we predict that exercise-trained mice with HFD-induced obesity will have greater healthspan and survival, as well as decreased cancer incidence compared to sedentary mice with HFD-induced obesity, but similar to that in lean, sedentary mice after radiation exposure.

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Chapter II: Research Article

The Effects of Obesity and Exercise on Healthspan, Cancer Incidence, and Lifespan in a Mouse Model of Radiation-Induced Cancer

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Abstract

The increasing number of long-term cancer survivors exposed to radiation therapy as part of their treatment is increasing the risk of secondary cancer development. Furthermore, obesity and physical inactivity are prevalent, modifiable, risk factors in this population that have also been shown to increase cancer risk. However, whether obesity and strategies such as lifelong exercise alter radiation-induced cancer incidence, overall healthspan, and survival, remains to be explored. As such, we evaluated the effects of lifelong exercise and diet-induced obesity on these outcomes in a mouse model of radiation-induced cancer. Male CBA mice were randomly divided into either a control diet/sedentary group (CTRL/SED), 45% high fat diet/sedentary group (HFD/SED), control diet/exercise group (CTRL/EX), or 45% high fat diet/exercise group (HFD/EX), and exposed to a cancer-inducing dose of whole body ionizing radiation (3 Gy). Healthspan index scores and endpoint monitoring was conducted throughout the study. When normalized to CTRL/SED, the highest healthspan score was in CTRL/EX (score = +2.5), followed by HFD/EX (score = +1), and HFD/SED (score = -0.5). Cancer incidence was higher in HFD/SED compared to CTRL/EX ($p<0.05$) and there was a trend for lower incidence when compared to CTRL/SED ($p=0.079$). There was no difference between the HFD/SED and HFD/EX groups ($p>0.05$). Overall survival was higher in HFD/SED compared to CTRL/SED ($p<0.05$); however, risk of cancer-related mortality was increased in the HFD/SED group by 1.6-times that of CTRL/SED (RR=1.60; 95% CI, 1.00-2.56; $p=0.0495$) and 1.68-times that of CTRL/EX (RR=1.68; 95% CI, 1.02-2.78; $p=0.0415$). Here we show that lifelong treadmill exercise training improved healthspan, lowered cancer incidence, and reduced the risk of cancer-related mortality with these effects being reversed in HFD-induced obesity in a mouse model of radiation-induced cancer. This study presents a proof of concept for the consideration of clinical studies in cancer survivors examining exercise as an intervention to reduce the long-term effects of radiation.

Introduction

Cancer survival rates have been steadily increasing in North America, with the 5-year survival rate reported to be 63% and 69% in Canada and the United States, respectively (Canadian Cancer Society, 2019; American Cancer Society 2019). Radiation therapy, which is undergone by two-thirds of patients with cancer (American Cancer Society, 2013), is a standard anti-tumour therapy. Conversely, its geno- and cytotoxicity and lack of specificity result in adverse long-term health effects in non-cancer tissues, thereby increasing the likelihood of developing secondary cancers. Despite improvements in radiation delivery, the incidence of radiation-induced cancers and morbidity have not improved (Dracham et al., 2018; Rubinsak et al., 2018; Schaapveld et al., 2015). The most common radiation-induced malignancy is acute myeloid leukemia (AML) (Preston et al., 1994). AML derives from mutations in hematopoietic stems cells (HSCs) - found in the HSC niche within the bone marrow - which are particularly susceptible to radiation damage (Williams & McBride, 2011). As such, the late effects of radiation therapy, including increased secondary cancer risk, organ toxicity, and morbidity, are an emerging major clinical concern.

A large body of evidence has focused on explaining the cellular changes induced by radiation, as well as the effects of radiation dose that could lead to secondary cancers (Braunstein & Nakamura, 2013; Little, 2000; Shah et al., 2012). However, interindividual variability in both the incidence and latency of cancer development in persons exposed to radiation suggests that individual modifiable risk factors should also be considered when investigating the potential long-term impact and response to radiation exposure. To date, the interactions between modifiable risk factors and radiation-induced cancer incidence have been largely ignored. Two modifiable risk factors that have been linked to increased cancer risk, which are prevalent among cancer survivors, are obesity and physical inactivity (Greenlee et al., 2016). In the context of radiation, previous work from our group has demonstrated that high fat diet (HFD)-induced obesity increased marrow adipose tissue, pro-inflammatory cytokines, and leukemic blast viability *in vitro* after radiation exposure (Emmons et al., 2019). In addition, it has been shown that a HFD alone

can increase myeloid skewing and decrease hematopoietic recovery after injury (Liu et al., 2018), mimicking the effects of radiation (Shao et al., 2014). Thus, obesity may exacerbate the deleterious long-term effects of radiation and as such, strategies such as exercise training, may mitigate these effects. Clinical evidence indicates that exercise training in cancer survivors is safe, and has numerous physical and psychological cancer health-related benefits (Campbell et al., 2019), while epidemiological (Cormie et al., 2017), and preclinical studies using various cancer models have shown that exercise training can reduce cancer incidence and cancer growth (Ashcraft et al., 2016). Of note, there have been a few exploratory follow-up studies that have provided more direct evidence that exercise may improve survival in cancer patients (Courneya et al., 2014, 2015). Importantly, the bulk of both the preclinical and clinical evidence has looked primarily at these outcomes in breast, colorectal, and prostate cancer and have largely ignored the effects of radiation in their analysis.

As such, there is a need to directly evaluate the combinatory long-term effects of radiation, exercise, and/or obesity to reveal their implications in cancer incidence and thus concomitant effects on healthspan and survival. Therefore, using an established mouse model of radiation-induced cancer (Major & Mole, 1978; Rivina et al., 2014, 2016), we aimed to evaluate the effects of lifelong treadmill exercise training and HFD-induced obesity on healthspan, cancer incidence, and survival following radiation exposure. We hypothesized that sedentary mice with HFD-induced obesity, will exhibit greater cancer incidence as well as decreased healthspan and survival, compared to lean, exercise-trained mice after radiation exposure. Furthermore, we predicted that exercise-trained mice with HFD-induced obesity will have greater healthspan and survival, as well as decreased cancer incidence compared to sedentary mice with HFD-induced obesity, but similar to that in lean, sedentary mice after radiation exposure.

Materials and Methods

Ethical Approval

Ethical approval for this project was obtained from the University of Ottawa Animal Care and Veterinary Service Committee.

Study Design

Male CBA mice (n=80) were purchased from The Jackson Laboratory (Bar Harbor, ME) at four weeks of age. Mice were allowed to acclimatize to the animal facility for one week, and provided food and water *ad libitum,* maintained on a 12-hour light: dark cycle, and housed under specific pathogen free conditions for the duration of the study. Male CBA mice are the favoured model of radiation-induced AML (Rivina et al., 2016) because it is the primary type of radiation-induced cancer in this strain, the morphology of the disease in this strain has been reported to closely resemble that in humans (Rithidech et al., 1999), and this strain exhibits a low spontaneous AML frequency (less than 1%) (Rivina et al., 2016).

At five weeks of age, the mice were randomly divided into either a control diet group (CTRL, n=40) or high fat diet group (HFD, n=40) and housed at no more than four mice per cage. At nine weeks of age, each dietary group was divided in half and randomly assigned to either an exercise training intervention (EX) or sedentary (SED) group, making the following 4 groups: CTRL/EX, CTRL/SED, HFD/EX, HFD/SED (n=20/group) (Figure 1A).

Dietary Intervention

The HFD group was fed a diet consisting of 45% kcal from fat (D12451, Research Diet Inc.) and the CTRL group was fed an AIN-93M diet (D10012M, Research Diet Inc.) consisting of 9.4% kcal from fat with matched micronutrient content to the HFD. Body weight and food intake (in grams and kcal) were measured weekly while body fat percentage and lean mass were measured monthly using an EchoMRI-900 (EchoMRI LLC, Texas, United States). This dietary intervention continued until endpoint.

Exercise Intervention

At nine weeks of age, mice began a treadmill exercise intervention (Exer6; Columbus Instruments, Columbus, OH) or remained sedentary as previously described by our group (Baker et al., 2011; De Lisio et al., 2011, 2013; De Lisio & Parise, 2012; Emmons et al., 2019). Mice were exercise-trained at approximately the same time each day for 3 days/week (Monday, Wednesday, Friday) for 1 hour per day up until 14 months of age, at which point their exercise frequency was reduced to 2x/week. Each session began with a 10-minute warm-up period at 10 m/min, followed by a 45-minute training period starting at 10 m/min and increasing by 1 m/min every 3 training sessions up to a maximum of 24 m/min. Mice were encouraged to run by gentle tapping with the bristles of a paintbrush. Electric shock was not used. Sedentary mice were placed in a sham treadmill with similar dimensions to that of the motorized treadmill. The sham treadmill was placed on top of the actual treadmill for the duration of the exercise training session to control for any stress associated with treadmill exercise. This exercise intervention continued until endpoint.

Radiation Challenge

At age 13 weeks, all mice were exposed to a single 3 Gy dose of total body gamma radiation over 4.32 minutes using an XRAD 320 radiation unit (Precision X-Ray Inc., East Haven, CT). This radiation dose administered at this age is sublethal and has been shown to induce AML in ~25% of male CBA mice by 18 months after radiation exposure (Major & Mole, 1978). Mice were irradiated in a small container to prevent them from moving (non-anesthetized) during radiation. The container was then placed in the radiation unit and a uniform dose of radiation was administered with the field of the beam being larger than the container to ensure that all mice received the same dose. Following radiation exposure, mice were transferred to sterile cages, given Baytril water (50 mg/mL) *ad libitum* for three weeks to prevent infection secondary to immunosuppression, and then missed the subsequent exercise-training session.

Cancer Incidence

Primary (bone marrow) and secondary (spleen and liver) sites of hematopoiesis were evaluated by a veterinary pathologist blinded to experimental conditions, for AML and other microscopic tumours. A positive diagnosis of AML was made based on the established criteria outlined in the "*Bethesda Proposal for Classification of Nonlymphoid Hematopoietic Neoplasms in Mice*" (Kogan et al., 2002). Specifically, a positive diagnosis of AML was made if (1) the complete blood count (CBC) revealed a low platelet/neutrophil count with or without high white blood cell quantity, (2) there was detection of ≥ 20% myeloid blast cells in Wright-Giemsa stained hematopoietic tissue, and/or (3) tissue pathology revealed AML infiltration in either the liver or spleen. Bi-monthly facial vein blood collection began at 6 months after radiation for CBC analysis to begin screening for cancer development and this continued until endpoint. Blood was collected in an EDTA-coated microvette capillary tube (Thermo Fisher Scientific, Waltham, MA, NC9141704), mixed, and then immediately transferred to a 2 mL Eppendorf tube and stored on ice. From each sample, 4 μL of blood was used to prepare each peripheral blood smear which were stained in Wright-Giemsa for quantification of blast cells. The remaining blood was used for CBC analysis (Centre for Phenogenomics, Toronto, ON). At endpoint, the livers and spleens were excised, weighed, visually inspected for macroscopic tumours, and stored in 10% neutral buffered formalin. In addition, both femurs were collected and thoroughly cleaned of muscle and other connective tissue. Both femurs were fixed in 10% neutral buffered formalin for 48 hours under gentle agitation. After fixation, the bones were washed for 1 hour under cool running water and then placed in a 14% EDTA decalcification solution for 14 days (Scheller et al., 2014). Once fully decalcified, the bones were washed again for 1 hour under cool running water and then stored in 70% ethanol at 4°C. The samples were then sectioned at a thickness of 4 μm and stained with hematoxylin and eosin (H & E) staining for evaluation of blast cell quantity.

Endpoint Monitoring

Health checks were performed on average three to four times per week. Endpoint monitoring criteria was created based on previously established guidelines (Burkholder et al., 2012; De Lisio et al., 2013; Ullman-Culleré & Foltz, 1999), with minor modifications. Specifically, endpoint criteria included: body condition less than three, hunched, ruffled coat, skin lesions, dehydration, lethargy, pale mucous membranes, masses, moribund, or a seizure (either lasting longer than 30 seconds or more than three seizures in less than one minute). Any abnormalities discovered during a health check that were potential grounds for veterinary intervention or euthanasia, were first referred to veterinary technicians blinded to experimental groups.

Behaviour Testing

Exercise Endurance Test

Mice underwent endurance testing at baseline, 5 days prior to radiation exposure, 5 days after radiation exposure, and 5 months after radiation exposure as previously described (Chorghade et al., 2017; De Lisio & Parise, 2012). Prior to starting the test, mice were habituated to the testing room for 30 minutes then placed in the treadmill, one mouse per lane. The test began at a speed of 11 m/min and increased by 1 m/min every 2 minutes. The bristles of a paint brush were used to motivate the mice to continue to run, and electric shock was not used. A mouse was deemed to have completed the test if it: (1) was resistant to stimulation, (2) remained on the platform for longer than two minutes or, (3) stayed approximately one body length away from the platform for longer than five continuous seconds. The speed and time at which each mouse finished the test was documented. Analyses were conducted by investigators blinded to experimental group.

Metabolic Activity

Oxygen consumption (VO2 and VCO2), heat production, and respiratory exchange ratio (RER) were measured using the OXYMAX Comprehensive Lab Animal Monitoring System (CLAMS, Columbus Instruments, Columbus, OH). Mice were tested at age 17 months at thermal

neutral conditions. Mice were individually housed in CLAMS-specific housing chambers and tested for 72 hours. The first 48 hours were used to allow the mice to habituate to the new environment and only data collected in the final 24 hours were analyzed**.** For the entire duration of the test, each mouse was provided with water and their specialized diet in powdered form *ad libitum* and maintained on their regular 12-hour light: dark cycle.

Beam Break (BBK)

The BBK test was used to assess anxiety-like behaviour (Crawley & Bailey, 2008). Mice were tested at age 17 months. Mice were allowed to habituate to the testing room for 30 minutes. They were then removed from their original cages and individually housed in new cages placed between photocell emitters which sent horizontal beams of infrared light through the cage. Mice were monitored for 24 hours to track anxiety-like behaviour throughout their entire circadian cycle. BBK data was analyzed using the Fusion 5.3 software for Micromax (Omnitech Electronics, Inc., Columbus, OH) by measuring the ambulatory movement, per hour, per each mouse (i.e. number of infrared beams broken during ambulation).

Forelimb Grip Strength

The forelimb grip strength test was used to measure the maximal grip strength (Pincu et al., 2016; Zou et al., 2015). Mice were tested at 17 months of age using the Chantillion DFE II (Columbus Instruments, Columbus, OH). Prior to testing, mice were allowed to habituate to the testing room for 30 minutes. Mice were then placed, one at a time, on the force gauge grid until they grabbed it with their front paws. Each mouse was then gently pulled horizontally off the grid with a constant and consistent speed. The test was performed by the same experimenter blinded to the experimental groups for each mouse to reduce variability. This protocol was repeated for a total of 8 trials per mouse and then the grip strength was averaged after removing the highest and lowest measurement and normalized to body weight.

Tail Suspension

The tail suspension test is a commonly used test to assess depression-like behaviour in rodents (Cryan et al., 2005; Steru et al., 1985; Vagena et al., 2019). Mice were tested at age 17 months. Prior to testing, mice were allowed to habituate to the testing room for 30 minutes. Mice were taped upside down by their tail to a bar connected to a strain gauge. The total test duration was 6 minutes with immobility time measured as the total amount of time (in seconds) that a mouse spent below the pre-set lower threshold.

Lipoprotein Levels

At 17 months of age, plasma was collected from the mice to analyze the high-density to low-density lipoprotein ratio (HDL:LDL), which is often used to assess cardiovascular disease risk (Millán et al., 2009). The mice were not fasted. Blood was collected in an EDTA-coated microvette capillary tube (Thermo Fisher Scientific, Waltham, MA, NC9141704), mixed, and then transferred into a 2 mL Eppendorf tube which was stored on ice. The blood samples were then centrifuged at 5000 rcf for 10 minutes at 8^{o}C. Afterwards, the plasma was removed with a pipette and transferred to a new 2 mL Eppendorf tube. The samples were then stored at -80^{o}C until they were ready to be shipped and analyzed by investigators blinded to the experimental conditions (Centre for Phenogenomics, Toronto, ON). Samples were shipped on dry ice.

Data Analysis

Body weight, body fat percentage, lean mass, food consumption, and endurance exercise capacity were assessed using a 3-Factor (exercise x diet x time) Repeated Measures ANOVA. Tukey's post hoc test was used to determine significant differences between means for all significant main effects and interactions. Metabolic activity, beam break, grip strength, and tail suspension were assessed using a 2-Factor (exercise x diet) ANOVA. Sidak's post hoc test was used to determine significant differences between means for all significant main effects and interactions.

The healthspan index table was adapted from Schafer et al. (2019). It is composed of 10 different parameters including 4 metabolic, 2 performance, 2 behavioural, and 2 morbidity outcomes. A score ranging from -1 to +1 was assigned to each group based on the p-value as determined by 2-Factor ANOVA for the anthropometric/metabolic, performance, and behavioural outcomes, or Fisher's Exact test for the morbidity outcome. For outcomes analyzed by 2-Way ANOVA, Dunnett's post hoc test was used to determine significant differences between means relative to the CTRL/SED group. Positive outcomes were assigned positive values and negative outcomes were assigned negative values. Specifically, when compared to the CTRL/SED group, a p-value greater than 0.10 was given a score of 0, a p-value between 0.05 and 0.10 was given a score of ±0.5, and a p-value less than 0.05 was given a score of ±1. The cumulative healthspan index was then determined by adding the scores for each outcome together for each group.

Cancer incidence was determined as the number of new cancer cases over the number of mice that were at risk of developing radiation-induced cancer. Mice that died and whose tissue was unable to undergo histological analysis (i.e. tissue autolysis as a result of being found dead after a prolonged period of time), were removed from this calculation. As such, one mouse from the CTRL/SED group, two mice from the CTRL/EX group, one mouse from the HFD/SED group, and three mice from the HFD/EX group were excluded as their tissue was unable to be analyzed histologically to confirm whether or not they were tumour positive. An additional mouse from the HFD/EX group was excluded as it died prior to being exposed to radiation. For seizure burden analysis, nine mice from the CTRL/SED group and four mice each from the CTRL/EX group, HFD/SED group, and HFD/EX group were excluded as their causes of death were not related to either seizures or cancer in the different tissues that were analyzed. Data are presented as mean ± SEM with a p-value less than 0.05 considered to be statistically significant. All statistical analyses above were conducted with GraphPad Prism 8.0 software (Graphpad Software, San Diego, CA).

Kaplan-Meier survival curves were created for each group to assess overall survival. The log-rank test was conducted to determine significant differences between curves. Cumulative incidence and incidence rate of cancer-related mortality curves were created for each group to determine the incidence and rates of cancer development at different points throughout the study. Cumulative incidence of cancer-related mortality was calculated as the number of mice that died from cancer over the number of mice at risk of dying from cancer at a given timepoint. The incidence rate of cancer-related mortality was calculated as the number of cancer cases divided by follow time broken up into three-month intervals, measured in mouse-months (m-m). The average incidence rate was also calculated for each group across the entire study and then used to determine the incidence rate ratios for each group relative to the CTRL/SED group. One mouse was removed from the HFD/EX group from all of these analyses except overall survival as it died prior to being exposed to radiation. Relative risk was calculated by dividing the cancer incidence of each group by that of the CTRL/SED group. Analysis of survival and cumulative incidence of cancer-related mortality was conducted using Graphpad Prism 8.0 software (Graphpad Software, San Diego, CA). Incidence rates at three-month intervals were calculated using Microsoft Excel (Microsoft Corporation, Redmond, WA). Lastly, incidence rate ratios, relative risks, and their respective 95% confidence intervals (95% CI) were determined using the epi.2by2 package in RStudio (RStudio Computer Program, Boston, MA).

Results

Lifelong exercise and HFD-induced obesity differentially influenced body composition throughout the lifespan after radiation exposure.

Body weight was significantly increased in both HFD groups compared to the CTRL/EX group from 2-13 months of age and compared to the CTRL/SED group from 2-7 months, as well as at 9 and 11 months of age. There was also a significant increase in body weight in the HFD/SED group compared to the CTRL/SED group at 8, 10, and 12 months of age and compared to the CTRL/EX group at 13 months of age ($p<0.05$, Figure 1B). Body fat percentage was significantly increased in both HFD groups compared to the CTRL/EX group from 2-10 months of age and compared to the CTRL/SED group at 2, 3, and 5 months of age. There was also a significant increase in body fat percentage in the HFD/SED group compared to the CTRL/SED group at 4 months of age, as well as between the HFD/EX and CTRL/SED at 6 and 8 months of age ($p<0.05$, Figure 1C). Absolute lean mass was significantly increased in both HFD groups compared the CTRL/EX group at 3-8, and at 15 months of age, as well as compared to the CTRL/SED group at 3, and 5-8 months of age. There was also a significant increase in lean mass in the HFD/SED group compared to the CTRL/SED and the CTRL/EX groups at 4 and 11 months, respectively ($p<0.05$, Figure 1D). Food consumption measured per gram and per mouse was significantly increased in the HFD/SED and HFD/EX groups at 4 months of age compared to the CTRL/EX group and at 1, 4, and 8 months of age compared to the CTRL/SED group. Food consumption was also significantly increased in the CTRL/EX and HFD/SED groups at 3 months of age compared to the CTRL/SED group ($p<0.05$, Figure 1E). Caloric intake was significantly increased in the HFD/SED and HFD/EX groups at 1 and 3-11 months of age compared to the CTRL/SED group, and from 3-5 and 7-9 months of age compared to the CTRL/EX group. Caloric intake was also significantly increased in the HFD/EX group at 3 and 12 months of age compared to the CTRL/SED group, in the HFD/EX group at 6 months of age compared to the CTRL/EX group, and in the HFD/SED group at 10 and 11 months of age compared to the CTRL/EX group ($p<0.05$,

Figure 1F). Data after 16 months are not shown as there were not enough mice per group for sufficient statistical power.

HFD-induced obesity alters substrate utilization with no effect of exercise after radiation exposure.

Figure 2 (A-D) shows the average measure of RER, VO2, VCO2, and energy expenditure over one 12-hour light (white bar) and one 12-hour dark cycle (black bar). Figure 2 (E-G) shows the average measure of RER, VO2, VCO2, and energy expenditure per hour within an entire light and dark cycle. There was a significant decrease in RER in the HFD groups compared to the CTRL groups in the light and dark cycle, irrespective of exercise (both $p<0.0001$). There was no significant difference between groups during either the light or dark cycle for VO2, VCO2, and direct energy expenditure ($p>0.05$).

Lifelong exercise and HFD-induced obesity differentially influenced performance and behavioural healthspan outcomes after radiation exposure.

There was a significant increase in endurance performance in the CTRL/EX group compared to both SED groups 5 days pre- and 5 months post-radiation exposure, as well as between the HFD/EX group and the HFD/SED group at 5 days pre-radiation exposure ($p<0.05$, Figure 3A). Grip strength normalized to body weight was significantly reduced in the HFD mice compared to the CTRL mice regardless of exercise ($p<0.05$, Figure 3B). The beam break test revealed a trend towards decreased anxiety-like behaviour in the CTRL/EX group compared the CTRL/SED group during the light ($p=0.06$) and dark ($p=0.108$) cycles. Furthermore, there was a significant decrease in depression-like behaviour as assessed by the tail suspension test, in the HFD and EX mice compared to the CTRL and SED mice, respectively ($p<0.05$, main effects of diet and exercise).

HFD-induced obesity decreased overall healthspan index which was higher in lifelong exercise mice after radiation exposure.

A healthspan index was used to quantify overall healthspan benefits or detriments in each group relative to the CTRL/SED group (Table 1) (Schafer et al., 2019). The greatest benefit in anthropometric/metabolic outcomes were seen in the CTRL/EX group (score = 1), with no changes seen in the HFD/EX and HFD/SED groups (both score = 0). The CTRL/EX group also had the highest score for performance outcomes (score = 1), with no changes in the HFD/EX and HFD/SED groups (both scores = 0). For the behaviour outcomes, the HFD/EX group had the greatest improvement (score = 1) followed by the CTRL/EX group (score = 0.5) and then the HFD/SED group (score = 0). Lastly, the HFD/SED group had a slight detriment to morbidity (score = -0.5), but there were no changes in the CTRL/EX and HFD/EX group (both scores = 0). Cumulatively, the CTRL/EX group had the highest overall healthspan index (score = 2.5), followed by HFD/EX group (score = 1), and then the HFD/SED group (score = -0.5).

HFD-induced obesity and a sedentary lifestyle increased cancer incidence but was attenuated by lifelong exercise after radiation exposure.

Cancer incidence (Figure 4E) was significantly increased in the HFD/SED group (16/19), compared to the CTRL/EX group (9/18) ($p<0.05$). There was also a trend towards a significant increase in cancer incidence in the HFD/SED group compared to the CTRL/SED group (10/19; $p=0.079$) but no significant difference between the HFD/SED group compared to the HFD/EX group (10/16; $p=0.245$).

Lifelong exercise did not reduce all-cause mortality but reduced cancer-related mortality risk relative to mice with HFD-induced obesity after radiation exposure.

All-cause mortality was significantly decreased in the HFD/SED group compared to the CTRL/SED group with no effect of exercise ($p<0.05$, Figure 5A). There was no significant difference in the cumulative incidence (Figure 5B) or average incidence rate (Figure 5C) of

cancer-related mortality for each group (p>0.05). Risk of cancer-related mortality after radiation exposure, however, was significantly increased in the HFD/SED group compared to the CTRL/SED group (RR=1.60; 95% CI, 1.00-2.56; p=0.0495) (Table 3) and compared to the CTRL/EX group (RR=1.68; 95% CI, 1.02-2.78; p=0.0415) (Table 3). There was no significant difference in risk of cancer-related mortality in the HFD/SED group compared to the HFD/EX group (RR=1.35; 95% CI, 0.88-2.06, p=0.1707) (Table 3).

Discussion

Our overall objective was to evaluate the effects of lifelong exercise and HFD-induced obesity on healthspan, cancer incidence, and survival in a mouse model of radiation-induced cancer. Our results demonstrate that lifelong exercise improved overall healthspan as well as decreased cancer incidence (Figure 4E) and relative risk of cancer-related mortality. Importantly, our results also demonstrate that the majority of these outcomes were improved in exercise-trained mice but made worse in mice with HFD-induced obesity, when compared to the CTRL/SED group. Taken together, these data extend our previous findings indicating that exercise attenuated, and HFD-induced obesity exacerbated the short-term deleterious effects of radiation. In addition, these data suggest that exercise and dietary interventions may modulate secondary cancer risk in the rapidly growing population of cancer survivors.

Exercise training has been shown to enhance numerous components of quality of life, physical function, and behavioural outcomes for both patients with cancer and cancer survivors (Campbell et al., 2019; Dennett et al., 2016; Hilfiker et al., 2018; LaVoy et al., 2016; Schwartz et al., 2017). However, there is a lack of evidence examining the persistence of these factors across the lifespan. Our results are comparable with work using non-irradiated mice (Nilsson et al., 2019; Schafer et al., 2019), in that the greatest improvement in healthspan was seen in exercise-trained mice. We extend previous findings by demonstrating that after being exposed to radiation, the exercise-induced improvements persist across the lifespan, especially when adipose mass is maintained at relatively normal levels. Another important factor to consider is that these various

aspects of healthspan deteriorate with age but can be attenuated by exercise in both human (Garatachea et al., 2015; Langhammer et al., 2018) and murine populations (Nilsson et al., 2019; Schafer et al., 2019). The majority of our healthspan assessments were completed when the mice were 17 months of age, which would be roughly equivalent to humans in late adulthood. Given that the majority of cancer survivors will be over the age of 60 within the next 20 years (Shapiro, 2018), our results offer valuable insight into the use of lifelong exercise interventions as a means to improve physical and psychological well-being among this population. These findings are particularly relevant as cancer survivors tend to score lower in physical and mental health related quality of life measures compared to non-cancer survivors (Weaver et al., 2012). Taken together, these data suggest that exercise across the lifespan may extend the healthspan of cancer survivors.

Apart from improved healthspan, we also saw a significant increase in cancer incidence in the HFD/SED group compared to the CTRL/EX group, with a variety of cancer types identified. The model used in this study is the gold standard to investigate radiation-induced AML in rodents (Rivina et al., 2016). This is because it has a low spontaneous tumour rate (<1%) and a 3 Gy dose of ionizing radiation has been shown to induce AML in 25% of mice by 18 months after radiation that mimics the genetic and phenotypic characteristics of human AML (Rivina et al., 2014, 2016). Given that AML is the most common radiation induced cancer, it was expected that it would have been the predominant cancer observed in our mice; however, it was only confirmed in two HFD/SED mice at 16- and 18-months after radiation exposure. The most prevalent cancer detected in all four experimental groups was liver cancer; specifically hepatocellular adenoma which is a benign tumour that has been well documented in older mice belonging to the CBA strain (Strong & Smith, 1936). Despite this, we show that the incidence of this tumour is largely reduced in exercise-trained mice and those without HFD-induced obesity. Moreover, histological analysis revealed that these tumours lead to gross hepatic damage and were the probable cause of death. As a result, these tumours were still considered in the cancer incidence and cancer-

related mortality analyses. Radiation-induced hepatic cancers are not well documented but radiation therapy has been shown to increase liver disease which in turn can increase liver cancer risk (Kim & Jung, 2017).

A recent systematic review indicated a 28-44% reduction in risk of cancer-related mortality after cancer diagnosis among those who exercised compared to those who did not (Cormie et al., 2017). Interestingly, this effect was found almost exclusively in studies that looked at patients with either breast, colorectal, or prostate cancer (Cormie et al., 2017). There was also no evidence of any study showing that exercise reduced cancer-related mortality among those with hematological cancers and more importantly, the study did not consider treatment regimen in their analysis (Cormie et al., 2017). Therefore, our data provides novel insight suggesting that lifelong exercise training can reduce the risk of cancer-related mortality following radiation exposure compared to sedentary counterparts with HFD-induced obesity. Despite reduced cancer incidence and risk of cancer-related mortality, we saw no exercise effects in terms of overall survival. This appears to be consistent among the limited clinical studies evaluating the effects of exercise on survival in cancer. For instance, there was no significant difference in overall survival or disease-free survival among patients with breast cancer who exercised during chemotherapy treatment compared to those who did not exercise. There was, however, a greater trend towards an exercise effect in disease-free survival compared to overall survival in that study (Courneya et al., 2014). Together, these findings suggest that exercise may have a more potent effect on disease-free survival rather than on overall survival in the context of cancer. As such, a lack of an exercise effect on overall survival in this study is not necessarily surprising.

Apart from the main outcomes of this study, it is worth noting an interesting trend in the incidence rates of cancer-related mortality presented in Figure 5C. While not statistically significant compared to the other groups, the drastic increase in incidence rate between 15 and 18 months after radiation in the HFD/SED group is certainly meaningful. For instance, it is 5.8-fold greater than the incidence rate of the CTRL/EX group and 2.9-fold greater than the HFD/EX

group. The incidence rates are expressed as the proportion of new cancer cases over the amount of follow-time (100 mouse-months). This would mean if we followed 100 mice per group for 1 month, the number of mice with cancer in the HFD/SED group would be 5.8- and 2.9-times greater than the number of mice with cancer in the CTRL/EX and HFD/EX group, respectively. This therefore suggests that the incidence rate of cancer-related mortality induced by HFD-induced obesity at late timepoints after radiation exposure, can perhaps be attenuated by exercise-training adopted earlier on after radiation exposure. Interestingly, work from our group has shown a similar exercise effect in a model of colorectal cancer, where mice who previously had HFD-induced obesity and then exercise-trained for 22 weeks, showed decreased CRC incidence compared to mice who continued to have HFD-induced obesity (Emmons et al., 2019). Taken together, this finding suggests that at late timepoints after radiation exposure, HFD-induced obesity exacerbates the incidence rate of cancer-related mortality, which could potentially be attenuated with exercise training. As such, it would be interesting for future studies to explore the effects of introducing exercise-training to mice with HFD-induced obesity after radiation exposure.

In conclusion, it has been well established that exercise alone can have a positive effect on hematopoiesis, whereas both radiation and obesity can individually be detrimental to hematopoiesis. In addition, our previous work showed that at an acute time point after radiation exposure, exercise training mitigated some of the deleterious effects of radiation and obesity on hematopoiesis. Our present study extends previous work by showing that lifelong exercise training improved healthspan, decreased cancer incidence, and risk of cancer-related mortality in a mouse model of radiation-induced cancer. Importantly, we also show these effects are largely opposed among mice with HFD-induced obesity that remained sedentary. Given that the number of cancer survivors exposed to radiation is increasing, and that there is substantial evidence highlighting the increased cancer risk associated with radiation exposure, our findings support the need for studies to investigate the long-term effects of radiation, exercise, and obesity in a clinical setting of cancer survivors.

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Author Contributions

EF, and MD contributed to study design and direction. EF, DB and MN primarily performed experiments with significant contributions from PM, JL, and JL. JK processed and analyzed all histological samples. EF, DB, and MD contributed to analysis. EF and MD wrote the manuscript. All authors edited the manuscript and approved final submission.

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The authors have no financial conflicts of interest to disclose.

Figure Captions

Figure 1. Lifelong exercise and HFD-induced obesity differentially influenced body composition throughout the lifespan after radiation exposure.

A schematic of the experimental timeline (A). Body weight (B), body fat percentage (C) absolute lean body mass (D) food intake measured per gram (E) and kilocalories (F) until 16 months of age. The dotted grey line represents the age at which mice were radiated. Data are presented as mean ± SEM (n=4-20/group/time point). *$p<0.05$ significant difference between CTRL/SED compared with HFD/SED and HFD/EX. $^{\beta}p<0.05$ significant difference between CTRL/EX compared with HFD/SED and HFD/EX, $^{\alpha}$significant difference between CTRL/SED compared with HFD/SED, $^{\eta}$significant difference between CTRL/EX compared with HFD/SED, $^{\varepsilon}$significant difference between CTRL/SED compared with HFD/EX, ‡significant difference between CTRL/EX compared with HFD/EX, †significant difference between CTRL/SED compared with CTRL/EX and HFD/SED.

Figure 2. HFD-induced obesity alters substrate utilization with no effect of exercise after radiation exposure.

(A) Average respiratory exchange ratio (RER), (B) oxygen consumption (VO2), (C) carbon dioxide production (VCO2), and (D) energy expenditure over one 12-hour light (white bar) and one 12-hour dark cycle (black bar). (E) average RER, (F) VO2, (G) VCO2, and (H) energy expenditure during the light and dark cycle. Data are presented as mean ± SEM (n = 3-8/group). $^{\#}p<0.05$ main effect of diet.

Figure 3. Lifelong exercise and HFD-induced obesity differentially influenced performance and behavioural healthspan outcomes after radiation exposure.

(A) Endurance performance for each group at baseline, 5 days pre-IR, 5 days post-IR, and 5 months post-IR (IR; ionizing radiation). Data are presented as mean ± SEM (n = 17-20/group/time point). (B) Forelimb grip strength normalized to body weight, (C) anxiety-like behaviour as

measured by the mean number of beams broken per group over an entire light and dark cycle (D), and depression-like activity as measured by time spent immobilized during a six-minute tail suspension test. (B-D) were all assessed at 17 months of age. Data are presented as mean ± SEM (n = 3-8/group). $^{\beta}p<0.05$ significant difference between CTRL/EX compared with HFD/SED and HFD/EX, $^{\S}p<0.05$ significant difference between HFD/EX compared with HFD/SED and HFD/SED, $^{\#}p<0.05$ main effect of diet, *p<0.05 main effect of exercise.

Figure 4. HFD-induced obesity and a sedentary lifestyle increased cancer incidence but was attenuated by lifelong exercise after radiation exposure.

Representative images of various tumours identified. (A) Sheets of AML cells in the bone marrow (60x), (B) a mass of AML cells (AML) is delineated with black arrowheads from the liver (L) that is infiltrated by small clusters of AML cells in the sinusoids (some indicated by full black arrows) (20x), (C) hepatocellular adenoma (HepA) is delineated by arrowheads from liver (L) (20x), and (D) hepatic adenocarcinoma (HepAc) is delineated by arrowheads from the liver (L) (20x). H&E stain. (E) Pie charts highlighting the distribution of various tumour types per group identified through histological analysis (n=16-19 per group). *p<0.05 compared to the HFD/SED group.

Figure 5. Lifelong exercise did not reduce all-cause mortality but reduced cancer-related mortality risk relative to mice with HFD-induced obesity after radiation exposure.

(A) Overall survival across the entire study (n = 20 mice/group). The dotted grey line represents the age at which mice were irradiated. (B) The cumulative incidence of cancer-related mortality for each group (n = 19-20 mice/group). (C) The cancer-related mortality incidence rate per 100 mouse-months divided into 3-month intervals across the entire study (n = 19-20 mice/group). In (B & C), one mouse from the HFD/EX group was excluded as it died prior to being exposed to radiation. Survival was examined by Kaplan-Meier analysis. *p<0.05 CTRL/EX vs. HFD/SED group.

Figure 1

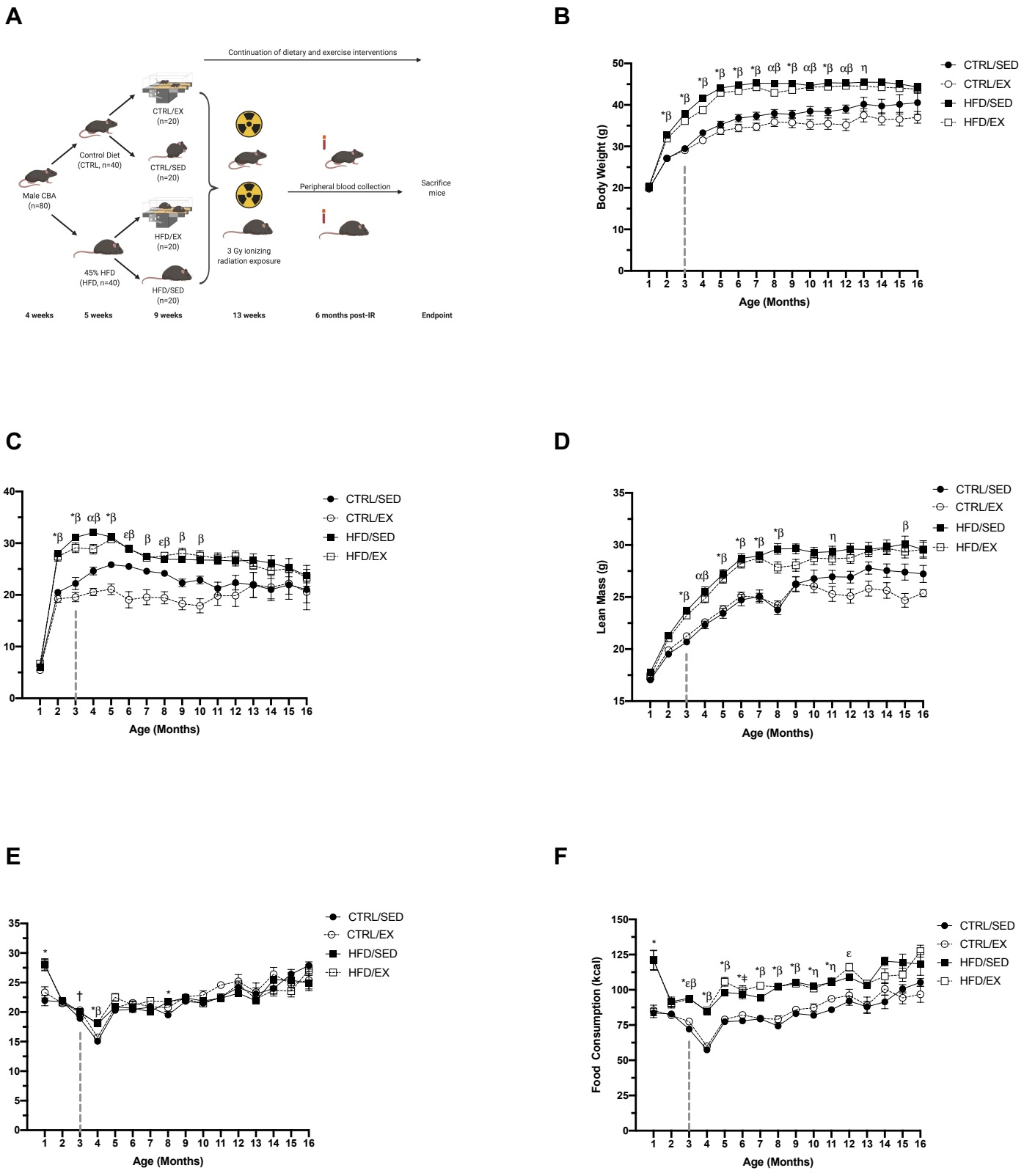

A
Continuation of dietary and exercise interventions
Male CBA (n=80)
Control Diet (CTRL, n=40)
45% HFD (HFD, n=40)
CTRL/EX (n=20)
CTRL/SED (n=20)
HFD/EX (n=20)
HFD/SED (n=20)
3 Gy ionizing radiation exposure
Peripheral blood collection
Sacrifice mice
4 weeks
5 weeks
9 weeks
13 weeks
6 months post-IR
Endpoint
B
Body Weight (g)
Age (Months)
CTRL/SED
CTRL/EX
HFD/SED
HFD/EX
C
Body Fat %
Age (Months)
D
Lean Mass (g)
Age (Months)
E
Food Consumption (g)
Age (Months)
F
Food Consumption (kcal)
Age (Months)

Figure 2

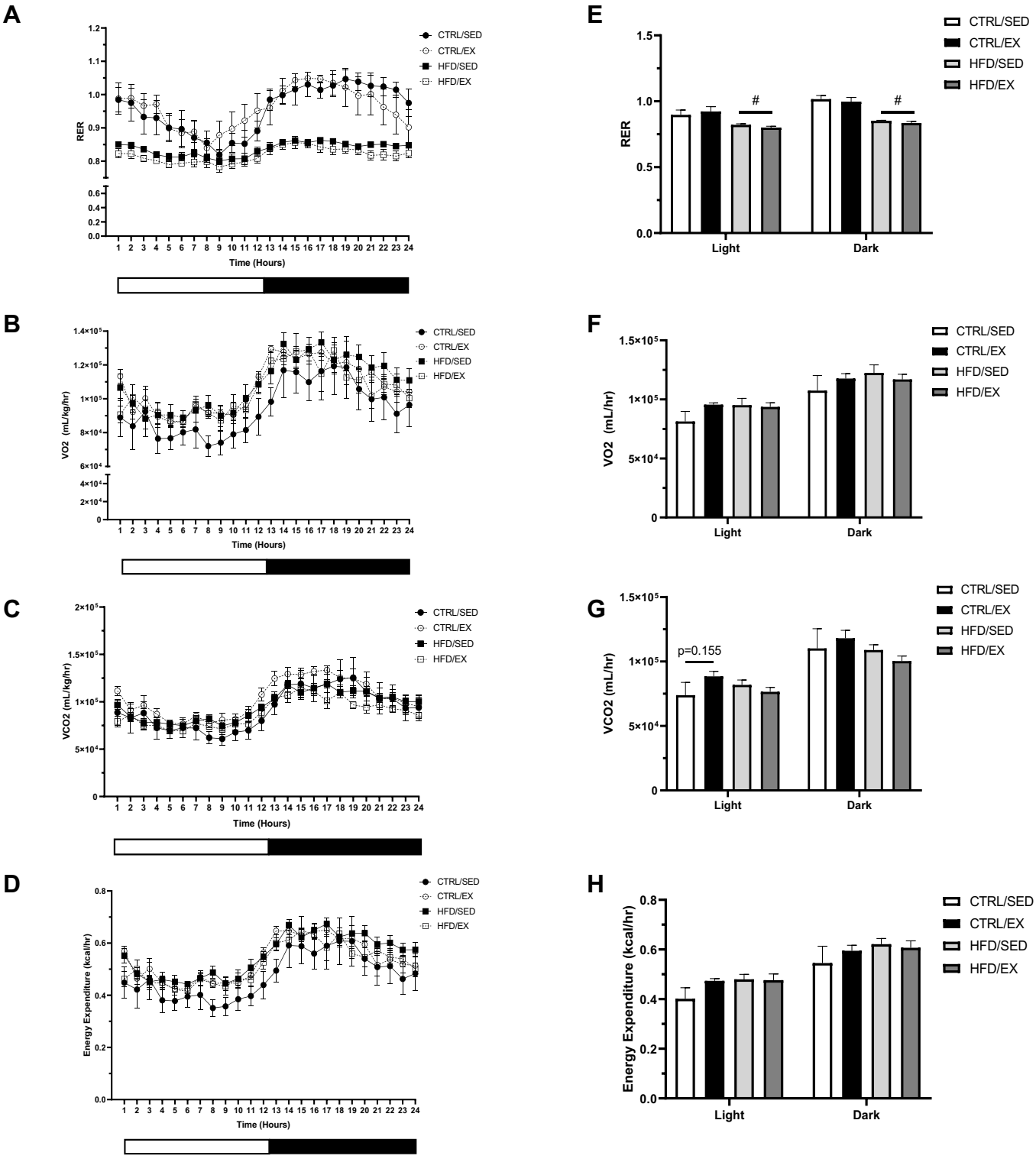

A
RER
Time (Hours)
CTRL/SED
CTRL/EX
HFD/SED
HFD/EX
B
VO2 (mL/kg/hr)
Time (Hours)
C
VCO2 (mL/kg/hr)
Time (Hours)
D
Energy Expenditure (kcal/hr)
Time (Hours)
E
RER
#
Light
Dark
F
VO2 (mL/hr)
Light
Dark
G
VCO2 (mL/hr)
p=0.155
Light
Dark
H
Energy Expenditure (kcal/hr)
Light
Dark

Figure 3

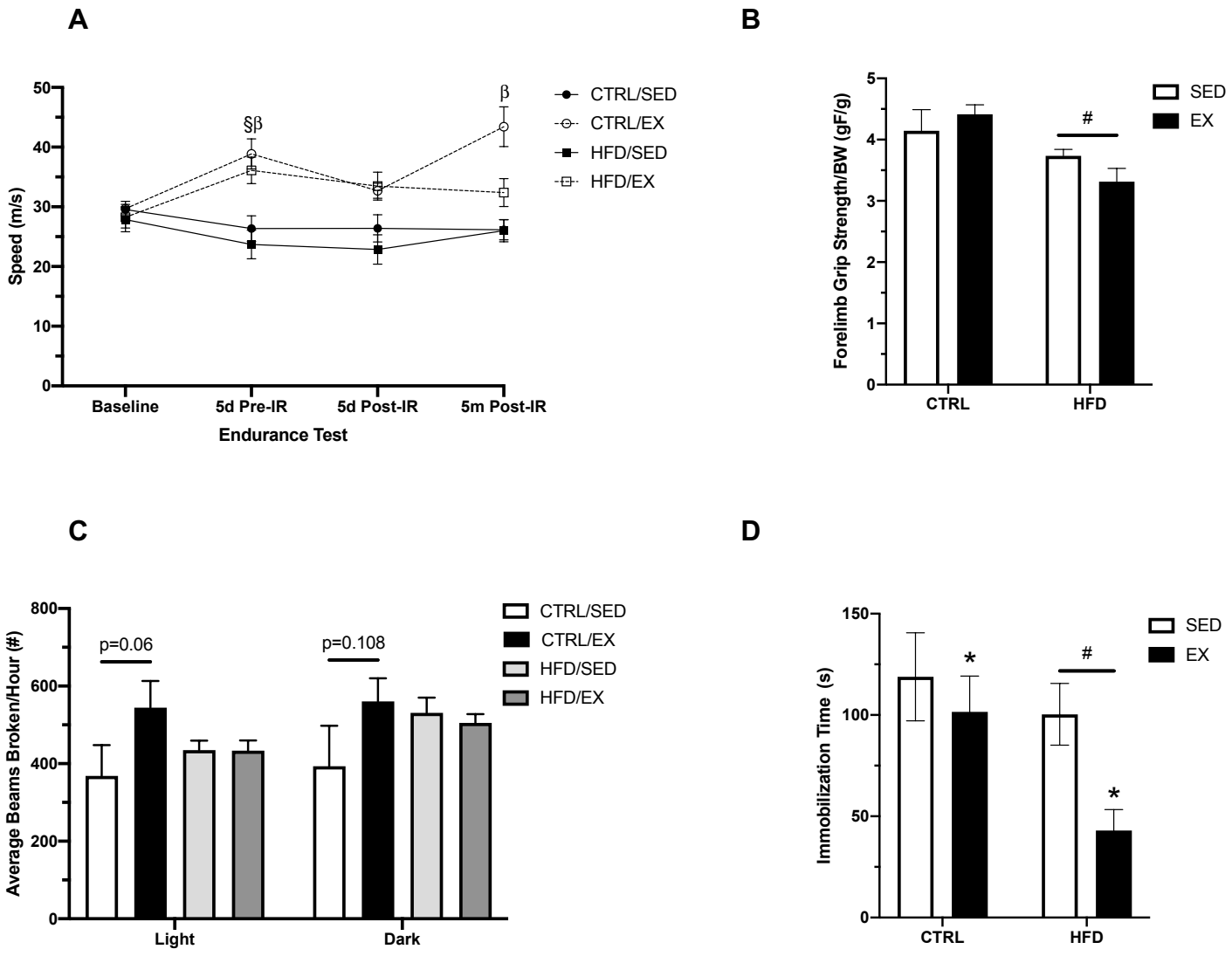

A
Speed (m/s)
50
40
30
20
10
0
§β
β
CTRL/SED
CTRL/EX
HFD/SED
HFD/EX
Baseline
5d Pre-IR
5d Post-IR
5m Post-IR
Endurance Test
B
Forelimb Grip Strength/BW (gF/g)
5
4
3
2
1
0
#
SED
EX
CTRL
HFD
C
Average Beams Broken/Hour (#)
800
600
400
200
0
p=0.06
p=0.108
CTRL/SED
CTRL/EX
HFD/SED
HFD/EX
Light
Dark
D
Immobilization Time (s)
150
100
50
0
*
#
*
SED
EX
CTRL
HFD

Table 1. HFD-induced obesity decreased overall healthspan index which was reversed by lifelong exercise after radiation exposure.

Outcomes	Parameters	Summary data: Mean ± SEM or Number (%)				p-value			[a]Score (-1 to +1)		
		CTRL/SED	CTRL/EX	HFD/SED	HFD/EX	CTRL/EX	HFD/SED	HFD/EX	CTRL/EX	HFD/SED	HFD/EX
Anthropometric/ Metabolic	[b]Body fat (%)	23.9 ± 0.5	20.39 ± 1.0	27.90 ± 0.5	28.13 ± 0.5	**0.0007**	**<0.0001**	**<0.0001**	1	-1	-1
	[b]Lean mass (g)	24.9 ± 0.5	24.9 ± 0.3	28.8 ± 0.4	27.6 ± 0.5	>0.9999	**<0.0001**	**0.0004**	0	1	1
	Metabolism	VO2: 94390 ± 10637.6 mL	VO2: 106618 ± 2311.9 mL	VO2: 108770 ± 6149.1 mL	VO2: 105271 ± 3638.6 mL	0.4306	0.2499	0.4667	0	0	0
		VCO2: 92015 ± 12584.6 mL	VCO2: 103249 ± 3368.8 mL	VCO2: 95368 ± 3894.0 mL	VCO2: 88416 ± 3392.9 mL	0.4268	0.9441	0.9366	0	0	0
		Heat: 0.47 ± 0.06 kcal	Heat: 0.53 ± 0.01 kcal	Heat: 0.55 ± 0.02 kcal	Heat: 0.52 ± 0.02 kcal	0.3421	0.1339	0.4966	0	0	0
	HDL:LDL ratio (A.U.)	16.17 ± 4.4	19.98 ± 4.9	12.6 ± 3.2	18.22 ± 5.2	0.9156	0.9283	0.9816	0	0	0
Performance	[c]Endurance (m/min)	26.3 ± 1.6	37.33 ± 1.6	24.43 ± 1.4	33.0 ± 1.4	**<0.0001**	>0.9999	**0.0064**	1	0	1
	Grip Strength/BW (F/g)	4.1 ± 0.3	4.4 ± 0.2	3.7 ± 0.1	3.3 ± 0.2	0.6722	0.3599	**0.032**	0	0	-1
Behaviour	Anxiety (beams broken)	380.8 ± 91.8	552.6 ± 57.7	482.9 ± 26.3	469.1 ± 21.8	**0.0612**	0.3035	0.4259	0.5	0	0
	Depression (s)	118.9 ± 21.7	101.5 ± 17.7	100.4 ± 15.2	42.94 ± 10.4	0.8185	0.7711	0.0228	0	0	1
Morbidity	Tumour burden	10/19 (53%)	9/18 (50%)	16/19 (84%)	10/16 (63%)	>0.9999	**0.079**	0.734	0	-0.5	0
	Seizure burden	2/11 (18%)	7/16 (44%)	1/20 (6%)	2/20 (13%)	0.231	0.549	0.9999	0	0	0
Total									**2.5**	**-0.5**	**1**

[a]**P value: 0** = p>0.1 = 0; **±0.5** = 0.05<p<0.01; **±1** = p<0.05
[b]2-Factor ANOVA of average per group from 1st EchoMRI after radiation to endpoint for each mouse; CTRL/EX, HFD/SED, and HFD/EX with CTRL/SED as control.
[c]2-Factor ANOVA of average per group after radiation; CTRL/EX, HFD/SED, and HFD/EX with CTRL/SED as control.

Data for the metabolic, performance and behaviour outcomes are presented as mean ± SEM (n=4-20/group/outcome). Body fat and lean mass were measured from the time of radiation exposure (13 weeks) until endpoint. Tumour and seizure burden were assessed across the lifespan after radiation exposure. All other outcomes were measured at 17 months of age. Healthspan score was assigned based on the p-value as determined by 2-Way ANOVA or Fisher's Exact test comparing the CTRL/EX, HFD/SED and HFD/EX groups to the CTRL/SED group.

Figure 4

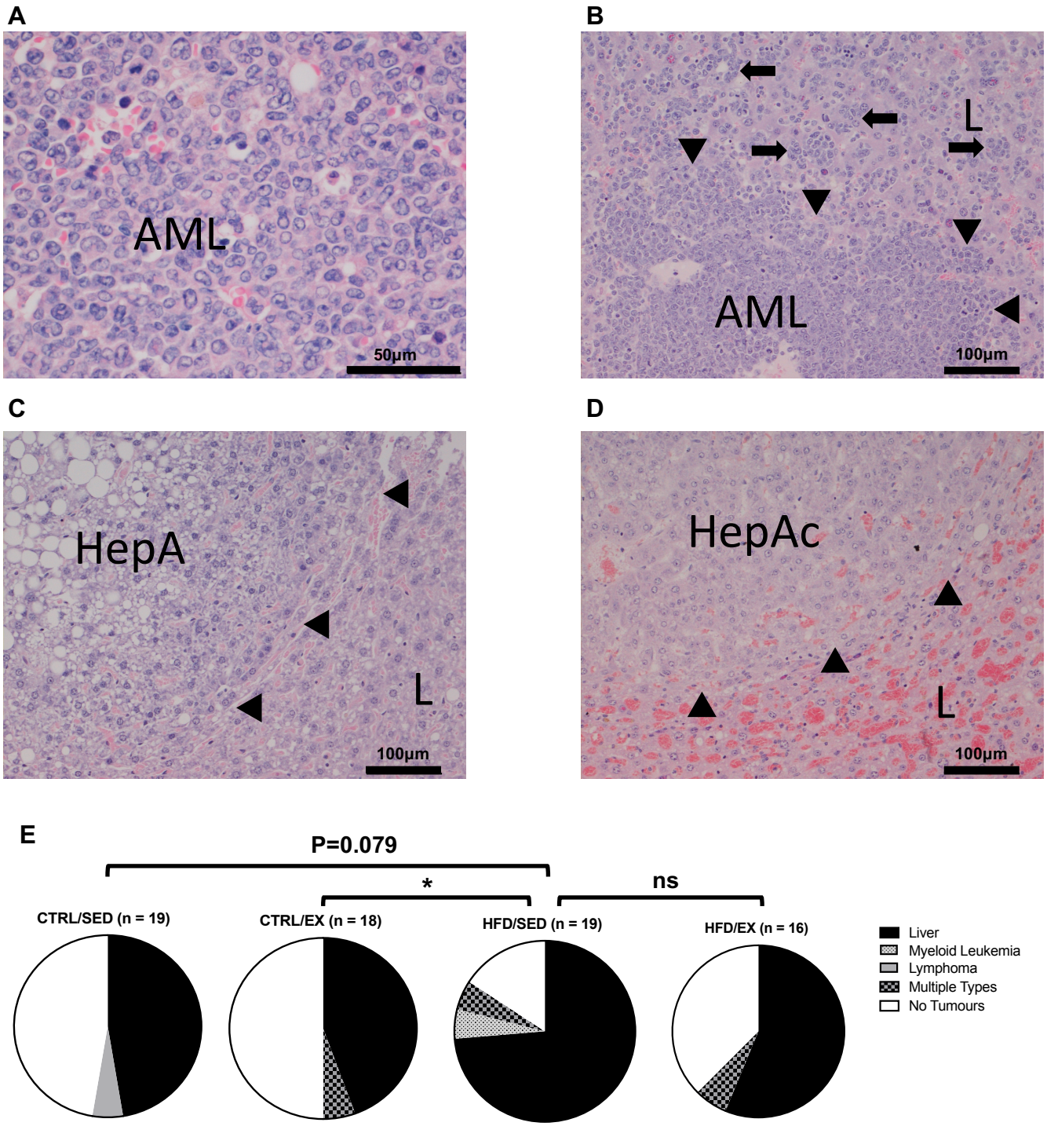
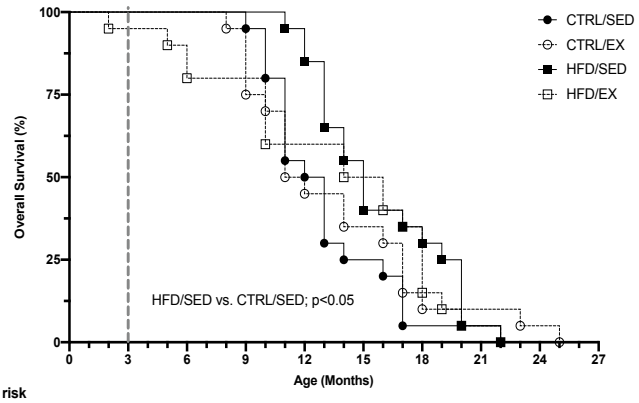

A
AML
50µm
B
L
AML
100µm
C
HepA
L
100µm
D
HepAc
L
100µm
E
P=0.079
*
ns
CTRL/SED (n = 19)
CTRL/EX (n = 18)
HFD/SED (n = 19)
HFD/EX (n = 16)
Liver
Myeloid Leukemia
Lymphoma
Multiple Types
No Tumours

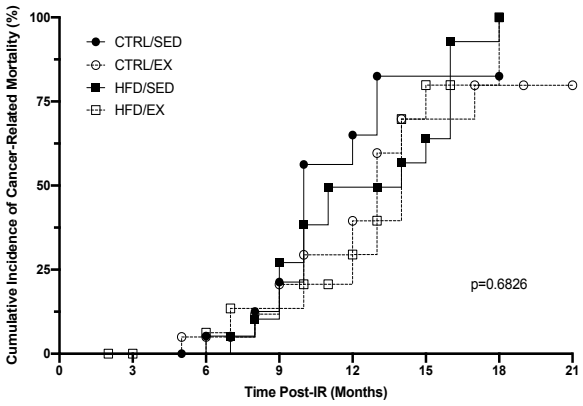
Figure 5

A



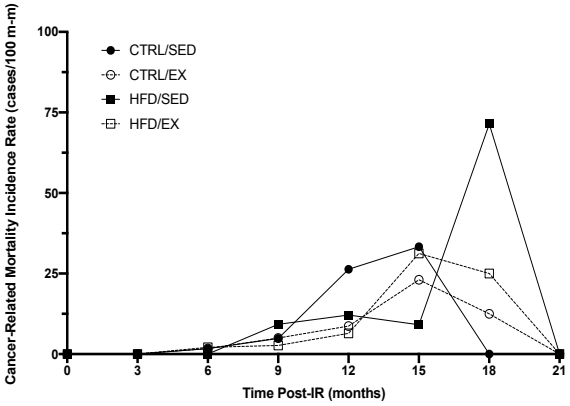
# at risk										
CTRL/SED	20	20	20	20	11	5	1	1	0	0
CTRL/EX	20	20	20	19	10	7	3	2	1	0
HFD/SED	20	20	20	20	19	11	7	1	0	0
HFD/EX	20	19	18	16	12	10	7	3	0	0

B



# at risk								
CTRL/SED	20	20	19	10	5	1	1	0
CTRL/EX	20	20	17	10	7	3	2	1
HFD/SED	20	20	20	16	9	6	1	0
HFD/EX	19	18	16	12	9	6	1	0

C



Follow-time (Incidence Rate)								Total
CTRL/SED	60 (0)	59 (1.7)	41 (4.9)	19 (26.3)	6 (33.3)	3 (0)	0 (0)	188 (5.3)
CTRL/EX	60 (0)	57 (1.8)	40 (5.0)	23 (8.7)	13 (23.1)	8 (12.5)	4 (0)	205 (4.4)
HFD/SED	60 (0)	60 (0)	54 (9.3)	33 (12.1)	22 (9.1)	7 (71.4)	0 (0)	236 (6.8)
HFD/EX	56 (0)	48 (2.1)	37 (2.7)	31 (6.5)	15 (31.3)	4 (25.0)	0 (0)	192 (5.2)

Table 2. Exercise-training did not improve the average incidence rate of cancer-related mortality relative to mice with HFD-induced obesity, after radiation exposure.

	CTRL/SED (n=19)	CTRL/EX (n=18)	HFD/SED (n=19)	HFD/EX (n=16)
Cases (%)	10 (53)	9 (50)	16 (84)	10 (63)
Follow time (m-m)	188	205	236	192
Incidence Rate (cases/100 m-m)	5.3	4.4	6.8	5.2
Incidence Rate Ratio (95% CI)	---	0.83 (0.30-2.26)	1.27 (0.54-3.14)	0.98 (0.37-2.62)

Incidence rate was determined per group as the proportion of confirmed cancer cases over the total follow-time contributed by the mice at risk in each group. The incidence rate ratio was calculated by diving the incidence rate of each group by the incidence rate of the CTRL/SED group. The 95% confidence interval (95% CI) was calculated for each comparison using the epi.2by2 package in RSudio.

Table 3. After radiation exposure, HFD-induced obesity increased the relative risk of cancer-related mortality compared to mice without HFD-induced obesity and those who engaged in lifelong exercise-training.

Absolute risk				
	CTRL/SED (n=19)	**CTRL/EX (n=18)**	**HFD/SED (n=19)**	**HFD/EX (n=16)**
	0.53	0.50	0.84	0.63

Relative Risk (95% CI)				
	CTRL/SED (n=19)	**CTRL/EX (n=18)**	**HFD/SED (n=19)**	**HFD/EX (n=16)**
CTRL/SED	1	0.95 (0.51-1.78)	**1.60 (1.00-2.56)**	1.19 (0.67-2.10)
CTRL/EX	1.05 (0.56-1.97)	1	**1.68 (1.02-2.78)**	1.25 (0.69-2.27)
HFD/SED	0.62 (0.39-1.00)	**0.59 (0.36-0.98)**	1	0.74 (0.48-1.14)
HFD/EX	0.84 (0.48-1.49)	0.80 (0.44-1.45)	1.35 (0.88-2.06)	1

Absolute risk was determined per group as the proportion of confirmed cancer cases over the number of mice at risk per group. Relative risk was determined as the proportion of the absolute risk per group from left to right in the table, over the absolute risk per group from top to bottom in the table. The 95% confidence interval (95% CI) was calculated for each comparison using the epi.2by2 package in RSudio. Statistically significant results are indicated in bold.

Chapter III: Global Discussion

By 2040, the number of cancer survivors in the United States will be close to 24 million, most of whom will be 60 years of age or older (Shapiro, 2018), and who would have received radiation therapy as part of their treatment (American Cancer Society, 2010). This is problematic as studies have shown that the late effects of radiation exposure can lead to the development of secondary cancers (Kleinerman et al., 1995; Nguyen & Wu, 2011), of which AML is most common (Richardson et al., 2009). Addressing the radiation delivery side of therapy, however, is just one factor in addressing the challenges facing this rapidly growing cancer survivor population. For instance, among this population, there are also increases in obesity and physical inactivity, both of which have been linked to increased cancer risk in general (Clague & Bernstein, 2012; Renehan et al., 2008), but also AML (Castillo et al., 2012). Moreover, psychological and physical well-being are reported to be lower among cancer survivors than non-cancer survivors (Weaver et al., 2012). Unfortunately, with a decrease in healthspan and increased risk of secondary cancer development, it can be expected that cancer survivors will have an increased risk of mortality. It is therefore imperative to develop strategies such as exercise training to potentially mitigate the late effects that radiation and obesity can have on healthspan, cancer incidence, and lifespan. This in turn, would provide valuable new insights into cancer treatment and lifestyle modifications to improve healthspan, decrease secondary cancer incidence, and promote longevity among cancer survivors exposed to radiation during treatment (Figure 6).

Until recently, literature exploring the combinatory effects of radiation, exercise, and obesity had not been studied. In the recent work from our lab, analysis of the cellular and proteomic changes in the bone marrow at an acute timepoint after radiation exposure suggested that obesity negatively affected hematopoiesis, with exercise having the opposite effect (Emmons et al., 2019). Moreover, bone marrow supernatant extracted from irradiated mice with obesity increased leukemic cell viability *in vitro* compared to irradiated mice without obesity (Emmons et al., 2019).

This provided promising evidence to support exercise as a strategy to mitigate the long-term effects of radiation, specifically in reducing cancer incidence and improving survival *in vivo*, which was the aim of the present study. Using an established model of radiation induced cancer (Rivina et al., 2016), our results demonstrate that CTRL/EX mice had the greatest improvements in healthspan compared to all other groups, as well as significantly lower cancer incidence and risk of cancer-related mortality compared to the HFD/SED group. Taken together, our findings are the first to show that lifelong exercise was able to improve healthspan, decrease cancer incidence, and ultimately decrease the risk of cancer-related mortality in a mouse model of radiation-induced cancer. While this study analyzed more global outcomes, the following discussion will explore potential cellular and molecular targets that may explain some of these findings and guide future work in this field.

With respect to healthspan, we found that the CTRL/EX mice had greater improvements than the HFD/EX and HFD/SED groups when normalized to the CTRL/SED group. The overall healthspan score was composed of metabolic, behaviour, performance, and morbidity outcomes. The majority of these findings align with the existing literature showing that exercise can improve several aspects of healthspan in both human (Burke et al., 2017; Campbell et al., 2019) and murine populations (Nilsson et al., 2019; Schafer et al., 2019). With that said, it was surprising to see that mice fed a HFD had significantly decreased depression-like behaviour compared to those fed a CTRL diet ($p<0.05$), given that numerous studies suggest a positive correlation between obesity and depression in humans (Faith et al., 2002; Luppino et al., 2010; Mulugeta et al., 2018). Among laboratory mice, however, the findings are controversial as some studies using both diet-induced and genetic models of obesity have shown increased depression-like behaviour (Vagena et al., 2019), while other studies using similar models have shown reduced depression-like behaviour (Del Rosario et al., 2012; Finger et al., 2011; Karth et al., 2019). One reason for this discrepancy could be due to the testing modality used to assess depression-like behaviour (Bai et al., 2001; Cryan et al., 2005). Another reason could be that none of these studies tested mice

at nearly the same age as those tested in our current study, nor did they include radiation as a confounder. There is extremely limited evidence examining the link between radiation exposure and depression-like behaviour, although one study did show that mice who received increasing doses of radiation to the brain (between 0.25-1Gy) had greater depression-like behaviour in a tail suspension test compared to non-irradiated controls (Dos Santos et al., 2018). Therefore, the novel results seen in our study may suggest that the HFD offered a greater anti-depressive effect compared to CTRL-diet fed mice following whole body radiation. With that said, depression was just one of several components used to assess healthspan in this study, rather than the sole focus of this study. Therefore, the use of the tail suspension was sufficient to assess depression in our mice as the analysis into the molecular changes in the brain are beyond the scope of this study. Future studies, with depression as their primary outcome, however, should use the tail suspension test in combination with the analysis of anti-depressive markers in the brain such as serotonin and/or brain-derived neurotrophic factor (Martinowich & Lu, 2008), in order to gain further insight into whether diet reduces depression-like behaviour in irradiated mice.

With regards to cancer incidence, our study showed that the HFD/SED group had the highest cancer incidence compared to all other groups. Notably, the most common group of cancers identified in our mice were adenomas, not leukemias. Early work on the CBA strain showed that adenomas are common (Strong & Smith, 1936), although there is a lack of evidence analyzing the effect of radiation on liver cancer incidence in this strain. The low incidence of AML in this study, with it only identified in two mice in the HFD/SED group, was still surprising given that it is the most common radiation-induced cancer. Some possible reasons for this can be found in work from our group's previous study. Specifically, proteomics analysis revealed that one month after radiation exposure, exercise-trained mice had increased pro-hematopoietic factors (i.e. M-CSF, MIP-2α and CXCL12), decreased expression of pro-inflammatory cytokines (i.e. TNFα and IL-1β), and increased expression of cytokines that inhibit AML viability (i.e. IGFBP-3), compared to

mice who were sedentary and fed a HFD (Emmons, Ngu, et al., 2019). In addition, we also found that hematopoietic stem and progenitor cells (HSPCs) in exercise-trained mice experienced less oxidative stress compared to sedentary mice which could result in a decreased likelihood of these cells becoming oncogenic (Emmons, Ngu, et al., 2019). While these data provide some insight into some of the HSC-intrinsic changes that may contribute to the decreased cancer incidence with exercise-training observed in our study, they have yet to be evaluated long-term after radiation exposure and therefore, should be the focus of future research.

Importantly, our previous study also highlighted important changes that occurred in non-hematopoietic cells which could provide more explanations for the results found in our study (Emmons et al., 2019). Specifically, we previously showed that exercise-trained mice had an increase in endothelial progenitors and MSCs compared to the mice who remained sedentary. Maintenance of these cell types in the HSC niche is vital for steady state hematopoiesis and in the context of AML. Both radiation and AML have been shown to be damaging to the vessels within the HSC niche (Cao et al., 2011; Duarte et al., 2018). This is problematic given that endothelial cells play vital roles in HSC proliferation and differentiation, release CXCL12 and SCF to improve HSC maintenance, and can even decrease reactive oxygen species in different regions on the niche (Asada et al., 2017; Ding et al., 2012). Notably, increasing the number of vessels that supply the HSC niche has been shown to decrease the number of AML cells and increase survival in mice following chemotherapy (Duarte et al., 2018). Taken together, this suggests that preserving or increasing the vasculature in the HSC niche may be a mechanism through which AML incidence is attenuated. Therefore, future work should explore the long-term vasculature responses to lifelong exercise, obesity, and radiation exposure in the bone marrow.

MSCs on the other hand have been suggested to be relatively radiation-resistant (Singh et al., 2012; Sugrue et al., 2013), up until recently where a study showed that this was only the case for a few MSC subsets (Severe et al., 2019). $LepR^{+}$-MSCs are one of the various subtypes of MSCs that regulate hematopoiesis by being sources of CXCL12 and SCF, and have been shown

to be depleted after radiation exposure (Severe et al., 2019). Radiation-induced depletion of LepR$^+$-MSCs may be due to enhanced adipogenic differentiation (Zhou et al., 2017). These LepR$^+$-MSC-derived adipocytes then become important sources of SCF and aid in HSC recovery after injury (Zhou et al., 2017). It has also been suggested that this acute increase in MAT can decrease AML burden in mice with active AML (Boyd et al., 2017). Unfortunately, as would be the case among cancer survivors, the long-term accumulation of MAT in a disease-free context can have detrimental effects on HSCs long-term (Naveiras et al., 2009), and support AML recurrence by causing myeloid skewing (Patel et al., 2018). Notably, however, exercise has been shown to decrease MAT and increase osteoblast number (Baker et al., 2011), even after radiation exposure (Emmons, Ngu, et al., 2019). This suggests that LepR$^+$-MSCs and their progeny may play an important role in AML suppression or development, respectively. As such, future work should explore the long-term changes to LepR$^+$-MSCs, MAT, and osteoblasts in the HSC niche in combination with lifelong exercise, obesity, and radiation exposure.

Ultimately, with improvements in healthspan and lower cancer incidence in the exercise groups, it was expected that these effects would result in improved longevity. Our results demonstrate that despite seeing no significant differences in overall survival, mice who were exercise-trained had lower risk of cancer-related mortality compared to the HFD/SED group. The reason for this is likely multi-factorial, and certainly the reasons presented above and in chapter II, likely also contribute to this difference. It is also worth noting that the CBA strain is highly susceptible to seizures after 6 months of age and this contributed to 15% of the total deaths in this study (see Table 1). Another important factor to consider is that both radiation and a HFD produce an aging phenotype at the cellular level. Indeed, sub-lethal radiation doses have been shown to induce premature upregulation of senescence markers *p16*^{Ink4a} and SA-β-gal, which are similarly expressed in aged HSC populations (C. Wang et al., 2013, 2015; Y. Wang et al., 2006). Moreover, radiation has been shown to downregulate expression of Meis1 which reduces the self-renewal capacity of HSCs (Unnisa et al., 2012). Lastly, a 3 Gy dose of radiation has been shown

to increase HSC divisions by nearly 10 times more than HSCs not exposed to radiation, increasing the risk of early senescence (Ban & Kai, 2009). A HFD on the other hand, through its induction of myeloid skewing, prematurely exhausts the pool of the more quiescent HSCs and is characteristic of aging HSCs (Lee et al., 2018; Rimmelé et al., 2014; Van Den Berg et al., 2016). Furthermore, a HFD has been shown to increase pro-inflammatory cytokines which in turn leads to greater oxidative stress and DNA damage in HSCs (Emmons, Xu, et al., 2019; Mangialardi et al., 2014). This suggests that combined with radiation, a HFD prematurely induces an aging phenotype that can inevitably result in increased cancer risk and therefore increased risk of cancer-related mortality. Importantly, most of these factors have been shown to be reduced with exercise, highlighting possible explanations for the decreased risk of cancer-related mortality compared to the HFD/SED group (Nilsson et al., 2019; Rebelo-Marques et al., 2018). Therefore, investigation into these targets in lifelong exercise-trained mice with and without HFD-induced obesity after radiation exposure is warranted.

Apart from the main outcomes in this study, it is worth discussing some of the statistical methods used in this study especially with regards to cancer incidence and cancer-related mortality. As mentioned in Chapter II, cancer incidence is the amount of new cancer cases over the number of mice who were at risk (i.e. exposed to radiation). In this study we show this in two ways. The first is by determining the incidence over the entire 18-month timeframe (Figure 4E) and the second was by determining the cumulative incidence (Figure 5B) which shows the cancer incidence at various timepoints throughout the study. The first method is beneficial because it is less impacted by competing events (i.e. mice dying from other causes besides cancer). The second method, however, is more sensitive to competing events. It is based on survival analysis which allows for censoring of mice that died due to competing events. Censoring does not cause any change to the curve, but it does decrease the number of mice that are at risk of developing cancer in the next month. Therefore, the more mice there are that are censored, especially early on, the more variable the end of these curves become, typically resulting in an overestimation of

incidence. Furthermore, in survival analysis, one of the key assumptions is that the risk reduction does not change with time. While typically the log-rank test is not overly affected by slight deviations in this assumption, a clear violation is when curves cross which increases the likelihood of developing a type II error (Bouliotis & Billingham, 2011). With that said, the curve still provides valuable insight into how incidence changes at different times throughout the study. It does not, however, indicate the rate at which these cancer-related deaths occur (also known as incidence rates). Information about incidence rates is particularly important because it provides a way to most appropriately deal with competing events. Incidence rates provide information about the number of new cases over the number of mice that were at risk and for how long they were at risk for. Even if a mouse died from competing events, the duration of time it was at risk for is incorporated into the data rather than being censored. As such, by providing all three methods, this study provides a more comprehensive view into the effects of radiation, HFD-induced obesity and exercise-training on cancer incidence and cancer-related mortality overall and at different timepoints in the study.

Strengths

This study presented various strengths that allowed for a more accurate interpretation of the data and better guide future work in the field in both pre-clinical and clinical settings. This study used the gold-standard murine model of radiation-induced cancer with a pathology of AML that closely resembles that in humans, and a low spontaneous AML rate (Rivina et al., 2016). Moreover, the number of potential biases were carefully considered and eliminated to the best of our ability. For instance, all histological samples were analyzed by a trained veterinary pathologist blinded to the experimental conditions. CBC and lipoprotein analyses were also conducted by investigators blinded to the experimental conditions. During health checks, while carried out by the primary researchers involved in this study, all decisions made around implementing treatments or euthanasia required secondary confirmation by veterinary technicians or veterinarians who were also blinded to the experimental conditions. With regards to the exercise

program implemented, it was designed to closely resemble the recent guidelines established for the cancer survivor population (Campbell et al., 2019). Lastly, the 45% HFD used also closely mimics the composition of a western diet and has been shown to exhibit various hallmarks of metabolic syndrome and obesity, such as elevated HbA1c and pro-inflammatory cytokines (Emmons et al., 2019).

Limitations

Despite the various strengths in the study, it is important to also consider a number of limitations when interpreting the data in this study. As is the case with all animal models, clinical studies need to be conducted to confirm the translatability of this work to humans. One important difference to note is that hematopoietic neoplasms in mice can originate in both the spleen and bone marrow, whereas in humans they are mostly found in the bone marrow (Kogan et al., 2002). When this is the case, however, typically blast cells are more prominent in the bone marrow. As such it is recommended to make the diagnosis of leukemia from bone marrow tissue rather than splenic tissue (Kogan et al., 2002). In this study, splenic involvement was only present in three cases of lymphoma and both cases of AML (11% of total cancer cases). In addition, as mentioned earlier, there is a high incidence of liver cancer and seizures in the CBA strain, which may have contributed to earlier mortality and thus, preventing AML development in some of the mice. Another limitation to consider is that we did not include a non-irradiated group. While the goal of this study was to look at the effects of exercise and/or HFD-induced obesity after radiation exposure, and using an animal model that would be most representative of the cancer survivor population, we are not able to conclude to what extent the damaging effects of radiation alone contributed to these results. Current studies are ongoing in the lab that include this group.

Taken together, this study is the first to combine several long-term behaviour and disease-relevant outcomes to directly evaluate how lifelong exercise training and HFD-induced obesity can impact healthspan, cancer incidence, and cancer-related mortality in a mouse model of radiation-induced cancer (Figure 6). Moving forward, we aim for this work to be foundational in

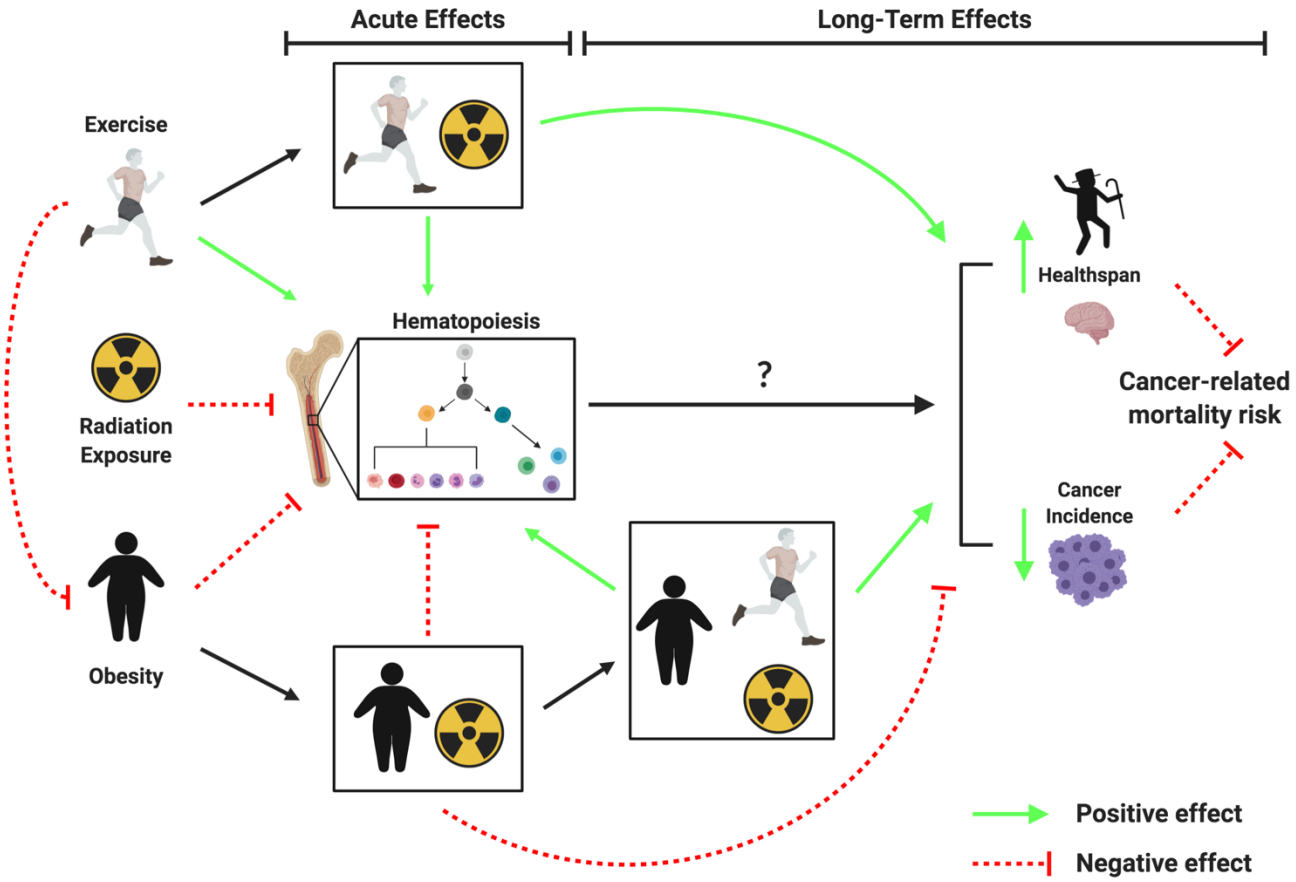
motivating more clinical work to focus on the damaging late effects of radiation among cancer survivors and the implementation of exercise and dietary interventions to improve their overall health while simultaneously protect against treatment-related cancer. This work also provides preclinical support for increased long-term cancer surveillance in cancer survivors with obesity who were exposed to radiation therapy.

Figure Caption

Figure 6. The effects of radiation, exercise, obesity or their combination on hematopoiesis, healthspan, cancer incidence and cancer-related mortality.

Exercise alone can have a positive effect (green arrows) on hematopoiesis, whereas both radiation and obesity can individually have a negative effect (red dashed arrows). At an acute timepoint after radiation, our previous work demonstrated that exercise training appears to mitigate some of the deleterious effects of radiation and obesity on hematopoiesis and has been suggested to potentially decrease the incidence of radiation-induced cancer. The present work expands on these findings by being the first to show that lifelong exercise training improved healthspan, decreased cancer incidence, and cancer-related mortality in a mouse model of radiation-induced cancer. We also show these effects are largely reversed among mice with HFD-induced obesity and who remained sedentary. Lastly, this study highlights the need for future investigation into the long-term cellular and molecular changes within the bone marrow that could account for the results shown in the present study. Created with BioRender.com.

Figure 6


Acute Effects
Long-Term Effects
Exercise
Radiation Exposure
Obesity
Hematopoiesis
?
Healthspan
Cancer-related mortality risk
Cancer Incidence
Positive effect
Negative effect

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APPENDIX A – SUPPLEMENTAL FIGURES AND TABLES

Figure S1. After radiation exposure, HFD-induced obesity, independent of exercise, increased total cholesterol levels but did not increase cardiovascular disease risk compared to mice fed a control diet.

(A) Total cholesterol levels in plasma, (B) HDL:LDL ratio, (C) HDL-cholesterol, and (D) LDL-cholesterol measured in plasma at 17 months of age. Data are presented as mean ± SEM (n = 3-8/group). $^{\#}p<0.05$ main effect of diet.

Figure S2. After radiation exposure, HFD-induced obesity and exercise-training differentially influenced absolute and relative liver and spleen weights.

(A) Absolute liver weight, (B) liver weight normalized to body weight, (C) absolute spleen weight, and (D) spleen weight normalized to body weight. Data are presented as mean ± SEM (n = 16-19/group). $^{\#}p<0.05$ main effect of diet, *P<0.05 main effect of exercise.

Figure S1.

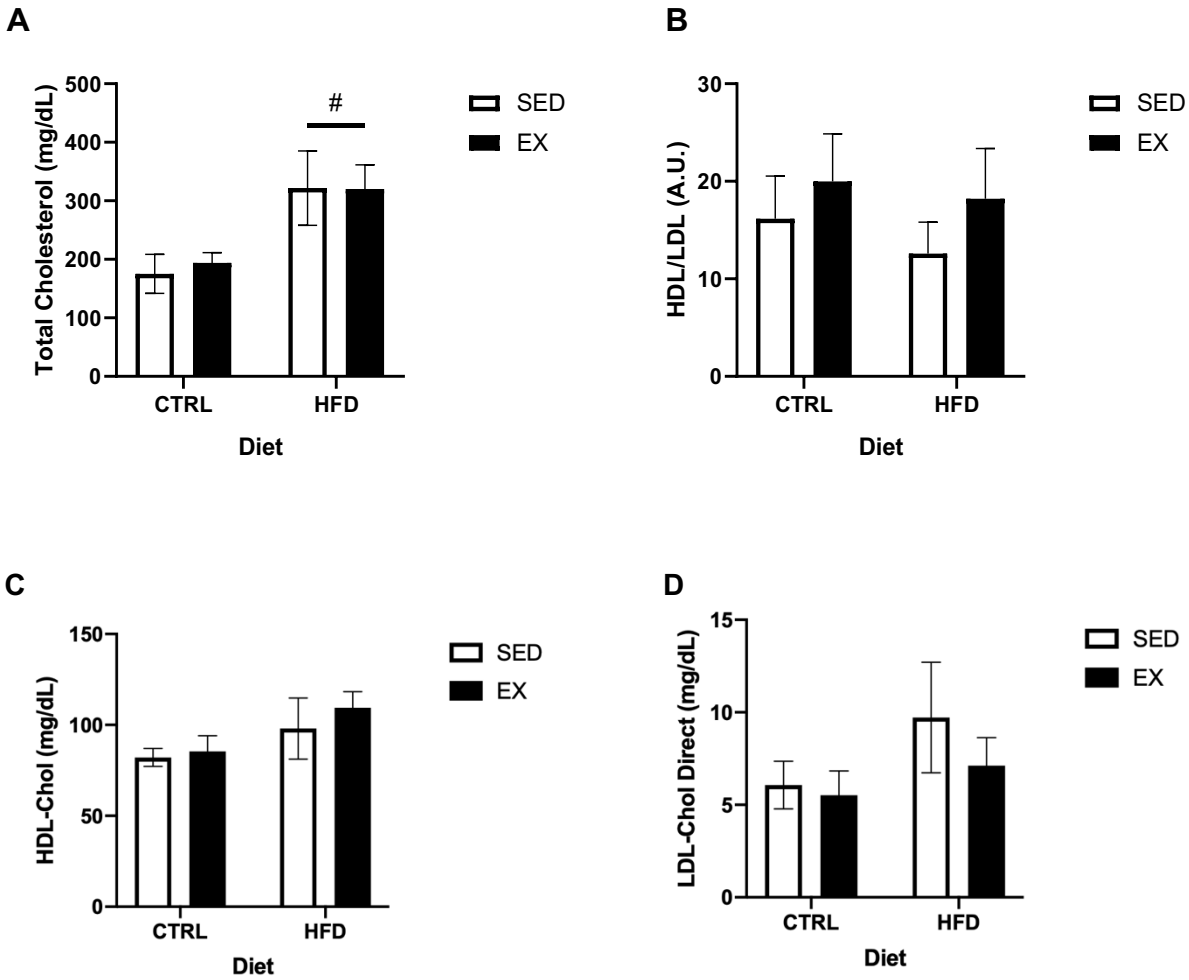
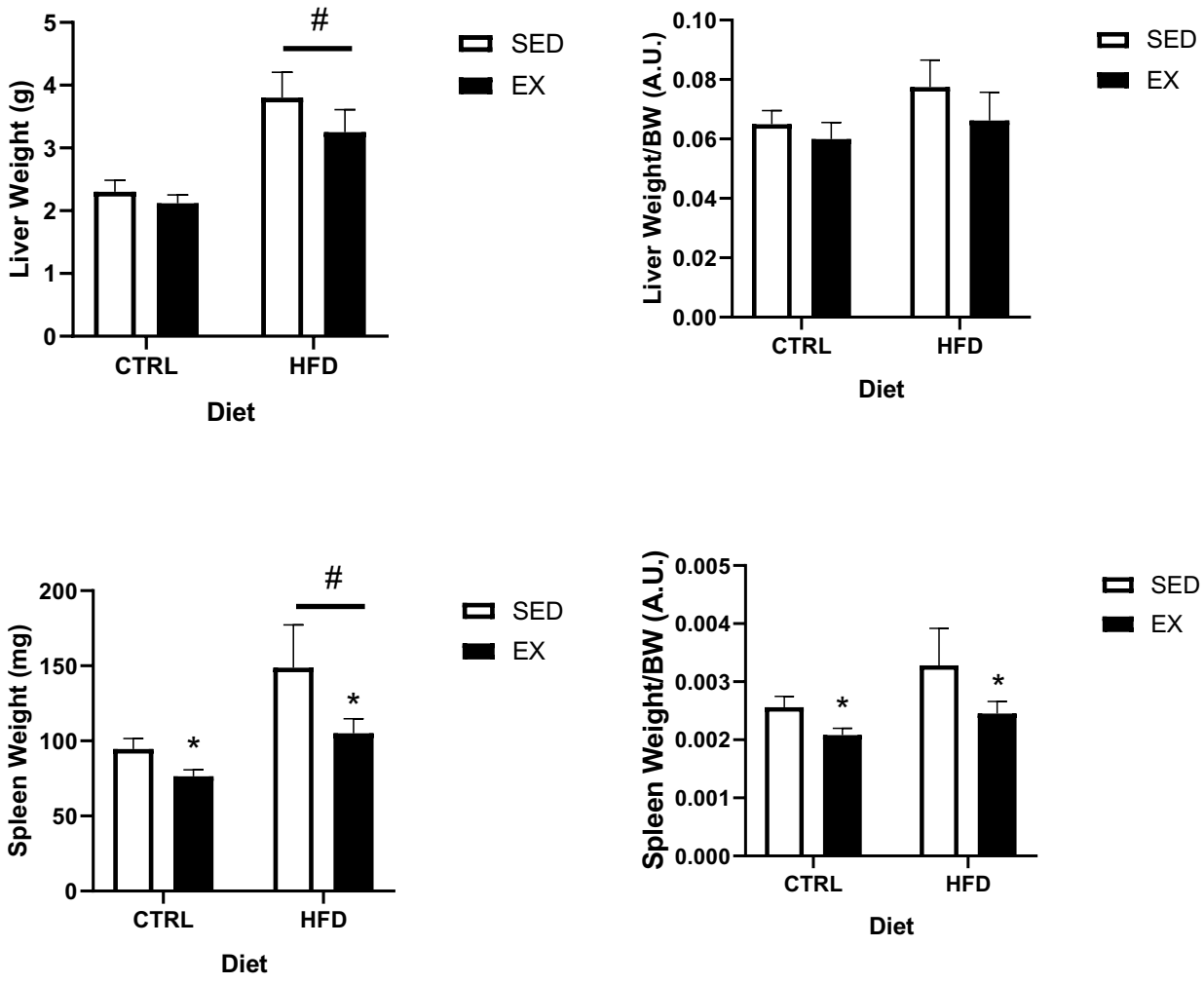

A
Total Cholesterol (mg/dL)
500
400
300
200
100
0
#
SED
EX
CTRL
HFD
Diet
B
HDL/LDL (A.U.)
30
20
10
0
SED
EX
CTRL
HFD
Diet
C
HDL-Chol (mg/dL)
150
100
50
0
SED
EX
CTRL
HFD
Diet
D
LDL-Chol Direct (mg/dL)
15
10
5
0
SED
EX
CTRL
HFD
Diet

Figure S2.


Liver Weight (g)
5
4
3
2
1
0
#
CTRL
HFD
Diet
SED
EX
Liver Weight/BW (A.U.)
0.10
0.08
0.06
0.04
0.02
0.00
CTRL
HFD
Diet
SED
EX
Spleen Weight (mg)
200
150
100
50
0
#
*
*
CTRL
HFD
Diet
SED
EX
Spleen Weight/BW (A.U.)
0.005
0.004
0.003
0.002
0.001
0.000
*
*
CTRL
HFD
Diet
SED
EX

S Table 1. HFD-induced obesity decreased overall healthspan index which was reversed by lifelong exercise after radiation exposure.

Outcomes	Parameters	Summary data: Mean ± SEM or Number (%)				Effect Size (95% CI)		
		CTRL/SED	CTRL/EX	HFD/SED	HFD/EX	CTRL/EX	HFD/SED	HFD/EX
Metabolic	[a]Body fat (%)	23.9 ± 0.5	20.39 ± 1.0	27.90 ± 0.5	28.13 ± 0.5	**-1.02 (-1.66, -0.35)**	**1.92 (1.14, 2.62)**	**1.99 (1.19, 2.71)**
	[a]Lean mass (g)	24.9 ± 0.5	24.9 ± 0.3	28.8 ± 0.4	27.6 ± 0.5	0.00 (-0.62, 0.62)	**2.50 (1.63, 3.28)**	**1.55 (0.81, 2.23)**
	Metabolism	VO2: 94390 ± 10637.6 mL	VO2: 106618 ± 2311.9 mL	VO2: 108770 ± 6149.1 mL	VO2: 105271 ± 3638.6 mL	0.85 (-0.61, 2.11)	0.77 (-0.52, 1.95)	0.75 (-0.58, 1.94)
		VCO2: 92015 ± 12584.6 mL	VCO2: 103249 ± 3368.8 mL	VCO2: 95368 ± 3894.0 mL	VCO2: 88416 ± 3392.9 mL	0.64 (-0.77, 1.91)	0.20 (-1.02, 1.39)	-0.22 (-1.43, 1.03)
		Heat: 0.47 ± 0.06 kcal	Heat: 0.53 ± 0.01 kcal	Heat: 0.55 ± 0.02 kcal	Heat: 0.52 ± 0.02 kcal	0.80 (-0.64, 2.07)	0.98 (-0.35, 2.16)	0.60 (-0.70, 1.80)
	HDL:LDL ratio (A.U.)	16.17 ± 4.4	19.98 ± 4.9	12.6 ± 3.2	18.22 ± 5.2	0.38 (-1.11, 1.77)	-0.49 (-1.87, 1.02)	0.17 (-1.20, 1.50)
Performance	[b]Endurance (m/min)	26.3 ± 1.6	37.33 ± 1.6	24.43 ± 1.4	33.0 ± 1.4	**1.58 (0.84, 2.26)**	-0.28 (-0.90, 0.35)	**1.03 (0.34, 1.67)**
	Grip Strength/BW (F/g)	4.1 ± 0.3	4.4 ± 0.2	3.7 ± 0.1	3.3 ± 0.2	0.59 (-0.88, 1.93)	-1.05 (-2.35, 0.43)	-1.44 (-2.76, 0.17)
Behaviour	Anxiety (beams broken)	380.8 ± 91.8	552.6 ± 57.7	482.9 ± 26.3	469.1 ± 21.8	1.08 (-0.36, 2.31)	0.86 (-0.44, 2.04)	0.76 (-0.57, 1.96)
	Depression (s)	118.9 ± 21.7	101.5 ± 17.7	100.4 ± 15.2	42.94 ± 10.4	-0.42 (-1.77, 1.03)	-0.44 (-1.74, 0.93)	**-2.50 (-3.93, -0.58)**
Morbidity	Tumour burden	10/19 (53%)	9/18 (50%)	16/19 (84%)	10/16 (63%)	-	-	-
	Seizure burden	2/11 (18%)	7/16 (44%)	1/20 (6%)	2/20 (13%)	-	-	-

[a]2-Factor ANOVA of average per group from 1st EchoMRI after radiation to endpoint for each mouse; CTRL/EX, HFD/SED, and HFD/EX with CTRL/SED as control.
[b]2-Factor ANOVA of average per group after radiation; CTRL/EX, HFD/SED, and HFD/EX with CTRL/SED as control.

Data for the metabolic, performance and behaviour outcomes are presented as mean ± SEM (n=4-20/group/outcome). Body fat and lean mass were measured from the time of radiation exposure (13 weeks) until endpoint. Tumour and seizure burden were assessed across the lifespan after radiation exposure. All other outcomes were measured at 17 months of age. The effect size and 95% confidence interval (95% CI) were calculated comparing the means of the CTRL/EX, HFD/SED and HFD/EX groups to the CTRL/SED group.

APPENDIX B - DIET INFORMATION

D12451 – Rodent Diet with 45 kcal% Fat

Data obtained from: https://researchdiets.com/formulas/d12451

Formulation:

Class Description	Ingredients	Grams
Protein	Casein, Lactic, 30 Mesh	200.00 g
Protein	Cystine, L	3.00 g
Carbohydrate	Sucrose, Fine Granulated	176.80 g
Carbohydrate	Lodex 10	100.00 g
Carbohydrate	Starch, Corn	72.80 g
Fiber	Solka Floc, FCC200	50.00 g
Fat	Lard	177.50 g
Fat	Soybean Oil, USP	25.00 g
Mineral	S10026B (RD-96 Mineral Mix)	50.00 g
Vitamin	Choline Bitartrate	2.00 g
Vitamin	V10001C (AIN-76A Vitamin Mix, 10X Concentration)	1.00 g
Dye	Dye, Red FD&C #40, Alum. Lake 35-42%	0.05 g
Total		858.15 g

Caloric Information (Physiological Fuel Values):

Protein: 20% Kcal
Fat: 45% Kcal
Carbohydrate: 35% Kcal
Energy Density: 4.7 Kcal/g

D10012M – AIN-93M Mature Rodent Diet

Data obtained from: https://researchdiets.com/formulas/d10012m

Formulation:

Class Description	Ingredients	Grams
Protein	Casein, Lactic, 30 Mesh	140.00 g
Protein	Cystine, L	1.80 g
Carbohydrate	Sucrose, Fine Granulated	100.00 g
Carbohydrate	Lodex 10	125.00 g
Carbohydrate	Starch, Corn	495.69 g
Fiber	Solka Floc, FCC200	50.00 g
Fat	Soybean Oil, USP	40.00 g
Mineral	S10022M (AIN-93 Mineral Mix for Mature Rodents)	35.00 g
Vitamin	Choline Bitartrate	10.00 g
Vitamin	V10037 (Vitamin Mix for AIN-93 Rodent Diets)	2.50 g
Anti-oxidant	Tert-Butylhydroquinone	0.01 g
Total		1000.00 g

Caloric Information (Physiological Fuel Values):

Protein: 15% Kcal
Fat: 9.4% Kcal
Carbohydrate: 76% Kcal
Energy Density: 3.81 Kcal/g

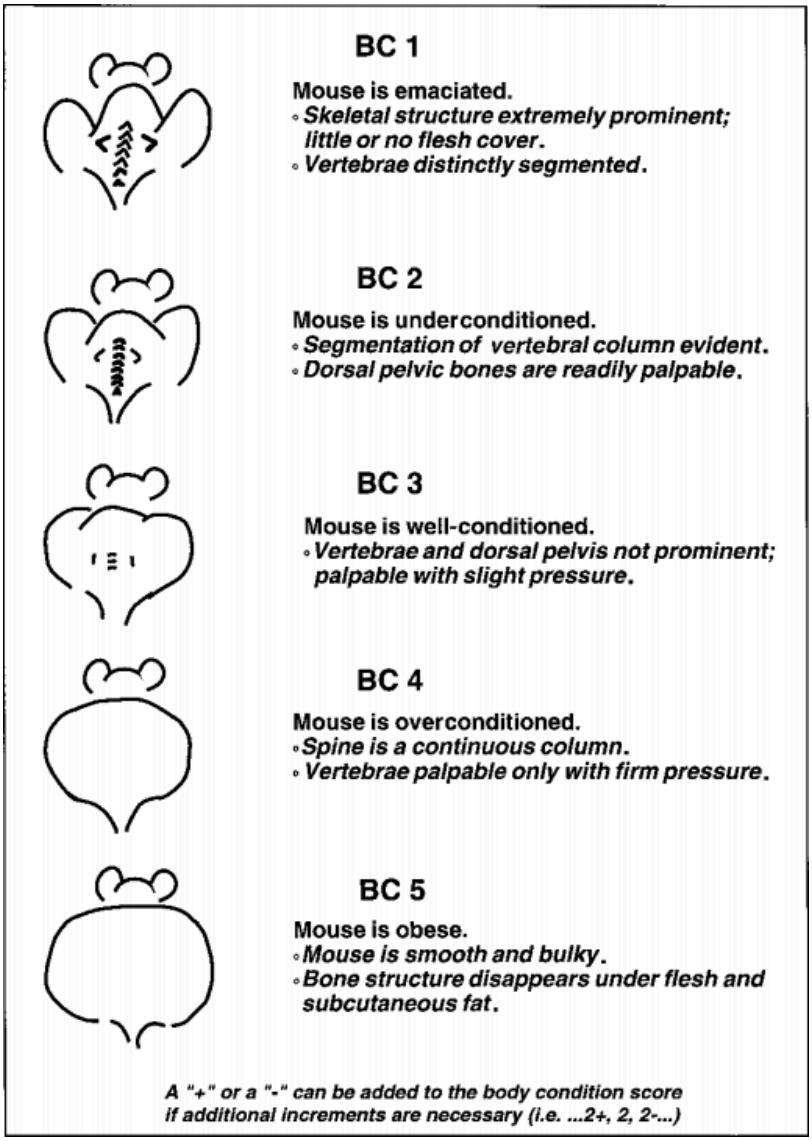
APPENDIX C – ENDPOINT MONITORING SYSTEM

Figure 8. Endpoint Monitoring Form

Date	Mouse #	A	B	C	D	E	F	G	H	I	J	K	Initials	Comments

Legend

A	Normal body condition	G	Lethargic
B	Body condition <3	H	Pale mucous membrane
C	Hunched	I	Masses
D	Ruffled coat	J	Moribund
E	Skin lesions	K	Euthanized
F	Dehydrated	**L**	**Seizure**



APPENDIX D – BEHAVIOUR TEST PROTOCOLS

The following protocols were originally created by the University of Ottawa Behavioural Core and have been included with their permission. The protocols have been adapted to reflect this specific study.

Body Composition – EchoMRI

Prior to Day of Testing

1. Ensure the machine is turned on at least one day before testing is scheduled.

Day of Testing

SETUP

1. Log on to the computer.
2. Open the Echo software program on the desktop.
3. Select Folder > Browse/New and choose the appropriate experimental folder.
    - If creating a new folder, it must be located within the EchoScans folder.
4. Select the species (Mice) from the drop down list on the right hand side of the screen.
5. Set the Primary Accumulation to 1.
6. Set Water Stage to No.

System Test

1. Insert the test probe with the oil-filled bottle into the MRI. Put the shorter end in first, then push it in as far as possible.
2. Select System Test > Yes. When finished, you will see System Test Completed Successfully.
    - Run a system test at the beginning of every experiment.
3. If the test was not successful, the machine will automatically calibrate itself and re-run the system test.

BEGIN TESTING

1. Place the animal in the appropriately sized red plastic tube (maximum weights are printed on the tubes; for mice, a size 40 tube is acceptable for mice 20-60g).
    - Place the animal into the open end of the tube. If you are having difficulties getting a mouse into the tube, hold the tube horizontally and hold the mouse by the base of its tail near the opening of the tube to encourage hiding behavior.
    - Gently push the animal to the end of the tube with the plastic insert.
    - Leave enough space for the length of the animal's body, not including the tail.
    - Ensure the animal is NOT compressed in the tube. Too little space can result in suffocation. It is better to give the animal too much space than too little as animal movement will not affect EchoMRI readings.
    - Use the Velcro to secure the insert in place.
2. Place the tube inside the machine, with the end of the tube holding the animal going in first. Push the tube in as far as possible, being careful not to push the insert and injure the animal.
3. Select Start Scan.

4. Input the mouse ID (and any other data/comments) in the dialog box.
    - These can be added or edited later if necessary.
    - NOTE: the placement of the plastic insert MUST NOT be adjusted while the tube is inside the machine. The tube MUST be removed from the machine and the mouse fully visible in the tube for any adjustments to the placement of the plastic insert to be made.
5. When finished, you will see Scan ALL DONE in the dialog box.
    - Each scan takes roughly two minutes, meaning the mouse will spend less than three minutes in the test tube. If technical issues are encountered that require troubleshooting, remove the mouse from the tube and replace it in its home cage. Do not leave the mouse in the sample tube for more than five minutes.
6. Remove the mouse from the machine and place it back in its home cage.
7. Clean the tube. Do not use alcohol or alcohol-based products.
8. Repeat for all mice.

EXPORT
1. Highlight the desired trials from the list.
2. Select Review > Extract Table.
3. Choose the destination folder and name the file.

Metabolic Activity - CLAMS

Prior to Day of Testing

1. Ensure the apparatus is turned on at least one day before testing is scheduled.

Day of Testing

SETUP
Prepare chambers.
1. Assemble as many housing chambers as needed for your experiment; one per mouse, to a maximum of 12.
2. Fill the food chambers with a powdered variety of their normal diet.
    i. Fill the clear tube 1/3 full with powdered food.
    ii. Invert the tube (so the grate is facing down) and insert the metal plunger.
    iii. Place the spring on top of the plunger.
    iv. Using the red-handled tool, feed the tool through the bottom of the black holder and through the spring. The metal post fits into the post on the feeder. You should not have to hold both in place.
    v. Set the feeder down so the grate is on a flat surface (some food will fall out). Push the black holder into the clear tube and turn to lock it into place.
    vi. Remove the red-handled tool and flip the feeder chamber over.
    vii. Top up the feeder if any food was lost in the “locking” process. Compress a small mound of food on the top of the feeder, over the metal grate, to facilitate food access for the mice.
    viii. It is ok if there is food in the black holder- it does not need to be completely clean.
3. Fill the water bottles all the way with clean water. Make sure to use the water from pre-filled ACVS bottles, not tap water. Screw the sipper on tightly and shake upside-down until bubbles no longer rise to ensure water has filled the sipper and there are no blockages.

Assemble chamber components within the temperature-controlled unit.

1. Gently place the filled food chambers on the black load cell. The load cells is very sensitive and could be damages so avoid pushing down very hard.
2. Place the assembled cage chamber into position, carefully lining up the food access tube.
3. Secure the IR framework to the cage chamber using the clips on the front and back of the chamber.
4. Insert the water bottle into the hole at the back, right side on the top of the cage. Push it down as far as possible- the black base of the sipper should be touching the top of the cage. Screw the bottle into place using the white plastic nut attached to the cage top.

Computer Setup

7. Open the Oxymax software program on the desktop. Note: The software must be open for 1 hour before calibrating the system.
8. Select Use Default Hardware Configuration (green checkmark) option.
9. Select File > Open Experiment Configuration.
10. Select File > Save Experiment Configuration As. Choose a name (usually the lab and date) and destination folder for the experiment such as C/BData/*Your lab/your folder*/CLAMS *date*
11. Select Experiment > Properties. Select the Environment tab. Under the Temperature Control tab, change the temperature in both the Start and End columns to the desired temperature. The Duration should be 4320 minutes (72 hours). Select Save to Controller. Ensure the Loop Schedule box is selected.
    i. Select Add Step to add another row to the schedule. Insert the desired temperature and duration in minutes.
    ii. When the Loop Schedule box is selected, the schedule will repeat every 24 hours. If you would like a different schedule each day, uncheck that box and calculate the minutes appropriately. White lines will appear in the bottom purple box where steps have been added, to give a visual representation of where the steps take place throughout the experiment.
    iii. Select Save to Controller when finished.
12. The Lighting Control (Experiment > Properties) is set up similar to the Temperature Control detailed above. The light schedule is set to change at 7am and 7pm, with each phase being 12 hours (720 minutes) in duration. Scheduled steps can also be added similar to the Temperature steps described above.
13. Select Experiment > Setup. Select the appropriate Data Filename by selecting Browse. Save the file under C/BData/*Your lab/your folder*. Enter the Subject IDs into the table. Make sure each chamber that you will be using is checked. Enter the Mass of the animals as 1.
    - Either before or immediately after your CLAMS experiment, put the mice through the EchoMRI to get a more accurate measurement of body mass composition. Do not enter 0. Select Done when finished.

Calibration

1. Make sure the software has been open for at least 1 hour before beginning this procedure.
2. Select Experiment > Calibrate.
3. Ensure both the O2 (%) and CO2 (%) boxes are checked. Also ensure the values entered under the Span column match the values on the mixed gas tank. The values will be printed on the card attached to the tank.
4. Select Start. A pop up box will ask you to open both the nitrogen and mixed gas tanks. You should feel the gas flowing through the yellow and clear tubes on the bottom shelf of the table.

- The left-most gauge should read 5-7 PSI on both the nitrogen and mixed gas tanks.

5. Follow the prompts on the screen. The progress bar shows how long each step will take.
6. When prompted (the status bar will say “Waiting on User”) to adjust the O2 offset, use the fine and coarse adjustment knobs to set the value on the screen as close to 0 as possible. The value on the screen turns from orange to green when in the proper range.
    - The left-most knob is the coarse adjustment, the center knob is the fine adjustment.
7. Continue with the calibration.
8. When prompted (the status bar will say “Waiting on User”) to adjust the O2, use the Span knob to set the value as close to 20.5 as possible. The value on the screen turns from orange to green when in the proper range.
    - The right-most knob is the Span knob.
9. Once completed, a pop up box will indicate to close the gas tanks.

BEGIN TESTING

1. Place each mouse into its corresponding cage chamber. Unlatch the IR framework and lift it slightly to allow enough space to place the mouse inside. Latch the framework back into place.
2. Select Experiment > Run. The program will run indefinitely.
3. The mice should be given at least 24 hours to acclimate to the chambers. Any data collected during this time should not be used. It is recommend collecting data for 72 hours to ensure 2 full light cycle changes worth of data are collected.
    - It is not recommended that experiments go for more than 7 days without cleaning the cages.
4. If feeding chambers need to be refilled, select Tools > Feeding > Refill Feeders. Chambers indicated in orange/red are getting low and need to be refilled. It can be very difficult to visually tell if the feeders are low on food since the IR framework blocks the top of the feeder. To refill the feeder:
    - Ensure the software is not measuring the desired chamber by checking the blue lights on the top of the unit.
    - Unselect the Enable box for the feeder you will be refilling.
    - Open the fridge unit and remove the feeder tube.
    - Refill the tube as quickly as possible.
    - Gently place the feeder chamber back in the appropriate position on the load cell.
    - Check the box to enable that feeder once again. The cell should turn green and go back to 0.
5. Fill out the wellness sheet and place on the outside of the door. The mice need to be visually checked every day.
    - Do not open the fridge unit when checking the mice. Look through the glass doors. The doors have blackout panels Velcro-ed to the outside that can be removed.
    - Put the panels back on the doors when finished.

WHEN TESTING IS COMPLETE

1. Select Experiment > Stop.
2. Remove the mice from the chambers and put back in their home cages.
3. Select File > Export > Export Parameters. Check the desired metrics in the Available box and select >> to add them to the Selected box. Select the desired Parameter Type. All parameters from each Parameter Type can be added to the same file (no need to export three times for each of the three Parameter Types).
4. Select Browse Path to select a save location. Create a new folder to save all exports in-each mouse is exported in its own CSV file.

Beam Break (BBK)

Prior to Day of Testing

1. If mice are grouped housed:
    - One day prior to testing, mark the tails of the mice with a permanent marker to easily identify the mice without handling.

   If mice are individually housed:
    - They should be individually housed for ~ 1 week prior to testing to ensure they have habituated to individual housing conditions

Day of Testing

SETUP

1. Set the light in the room to the desired brightness using a Lux-meter.
2. If testing overnight, ensure the lighting schedule is set so that the lights will turn on and off at 7AM and 7PM, respectively.
3. Place a clean shoebox mouse cage into each of the metal frames, one cage per mouse being tested. The metal frames are numbered.
    - Dump out excess bedding. In each cage you want just enough bedding to cover the floor of the cage, so as to not interfere with the sensors.
    - Do not place house or nesting square in the test cage.
    - If testing overnight, add the specific mouse diets and water to the metal hopper.
    - Only use metal hoppers which do not rock when placed in a cage.
    - Ensure each cage is positioned so that it does not rock or wobble from side to side when in the frame.
4. Bring your mice to the testing room and leave them to habituate for 30-60 minutes.
    - Close the door and turn over the “Do Not Disturb” sign hanging outside of the door to ensure that no one will enter during testing.
    - If testing overnight, fill out the “Temporary Housing Notes” document and leave on the outside of the door.
5. Log on to the computer.
6. Turn on the Micromax analyzer.
    - Make sure it is connected to the laptop via USB
7. Open Fusion 5.3 for Micromax on the desktop.
    - If you see the message “The nodes connected to the system have changed”, click OK. A second message may pop up regarding the node assignment; select Accept.
8. Under Tools, select Experiment settings.
    - Global:
        - Experiment Directory: Select “BData/*your lab/your folder*”.
        - Sample Duration (in seconds): For sampling every 5 minutes, enter 300 seconds and for sampling every hour, enter 3600 seconds. The minimum interval for collecting the data is 10 sec.
        - Locomotor Variables:
            - Pre-Check. Do not select.
            - Insertion Auto start. If selected, the recording will start for automatically for each cage when the animal is detected in that cage.
            - Tail length /Tail Fade and Tail thickness are not to be used.

- Click Apply, and then OK. You will return to Fusion program screen.

9. Create a new experiment by selecting File > New experiment.
    - An experiment properties dialog box will open.
        - On the left side of the screen select the cages you want to use for the experiment. There are 24 cages available for the test.
        - Click OK
    - The experimental details box will open. Fill in the following details.
        - Duration (min). For a 24hr experiment, enter 1440 (24 hr x 60 min).
        - Fill in the ID of the mouse in each cage. (You can also record this on a piece of tape affixed to the lid of each cage)
        - Fill in any other desired details.
    - Select OK when finished.

NOTE: Never unplug the cords going into the metal frames if a box is not working. If a box is not working and you do not need all 24 boxes, then just use another box and ask for a Behavioural Core technician to fix the box.

BEGIN TESTING

1. On the Fusion page, the list of cages and mouse numbers will be displayed in the top left portion of the screen. All of the cages should say “Ready”.
2. Click on the first cage so it is highlighted in blue. Click “Record Selected” above the cage list.
    - The cage will now say “Insert Animal”.
3. Place the mouse in the cage and cover with a cage lid.
    - It will now say “Recording” in green and be counting down the time of the test.
4. Repeat for all mice. Move as quickly and quietly as possible to minimize disruption to the mice already recording.
5. When all mice are loaded, leave the room and close the door.

WHEN TESTING IS COMPLETE

NOTE: Ensure the Micromax box is turned off **before** unloading mice or cages.

1. The test will automatically stop once the time set for the test has expired.
2. The word “Complete” will appear in each of the boxes when the test is finished.
3. Exit the Fusion program.
4. Reopen the Fusion program.
    - You must close, then re-open the program in order to export the data.
5. Select File > Open Experiment.
    - Your file is located on the C drive in your lab folder.
6. Select File > Export.
7. Select Comprehensive Output Data. Fill the field in the bottom left corner to specify the bin interval for the exported data (1 hour bin or 5 min bin) then select “Export”. Your files will automatically be saved in the BData folder.
    - The export file is in .csv format. It is recommended you open the file and save it again as an excel file.
8. Turn off the Micromax analyzer **before** unloading mice or used cages from the BBK system.
9. Log off the computer.
10. Place the mice back in their home cages and return them to their regular housing room.
11. Clean up the room and remove the dirty cages.

DATA ANALYSIS

1. For data analysis, use the column: “Ambulatory Activity Count” which represents the number of beam breaks while the animal is ambulating. This parameter will not include the stereotypic behaviour.
2. Any parameter such as Distance Traveled, and Movement Time would **only** report movements travelling in the **x-axis** direction (i.e. movements across the width of the cage, not the length). Y-axis data is not detected or reported with this system.
3. **Do not** use the stereotypy measurements (i.e. rearing), **including jumping**, as it does not always agree with what we’ve scored by hand. The outputs Stereotype Count, Number of Stereotypy, and Stereotype Time are **NOT** valid outcomes with the Behaviour Core’s current BBK system.
4. For data analysis using parameters other than “Ambulatory Activity Count, please consult with Behaviour Core staff, as many of the parameters given in the BBK output are not valid outcomes with this specific BBK system and setup.

Forelimb Grip Strength

Day of Testing

SETUP

1. Bring your mice to the testing room and leave to habituate for 30-60 minutes.
    - Turn over the “Do Not Disturb” sign hanging outside of the door to ensure that no one will enter during testing.

BEGIN TESTING

1. Always weigh each mouse before taking grip strength measurements, as grip data can be normalized using animal body weight.
2. Press the power button to turn on the instrument.
    - The instrument is ready to use when a numerical value is displayed on the screen.
3. Press the Zero key to reset the value to 0.
4. Hold the mouse (see below for holding techniques) and gently move it close to the grid until the mouse grabs it with its front paws. Gently pull the mouse along a horizontal plane relative to the grid at a steady speed of ~ 2.5 cm/sec, until the animal releases the grid.
    - Pulling the mice away more slowly results in many mice prematurely releasing the bar with one or both paws.
5. Record the value of force that is displayed on the screen in gram force (GF).
    - The instrument only displays the maximal value.
6. Press the Zero key to clear the screen.
    - You may have to press it multiple times.
7. Repeat for a maximum of 8 consecutive measurements for one animal.
    - Allow mice to rest for roughly 10 seconds between trials.
8. Place the mouse back in its home cage.
9. Repeat for all mice.
10. When finished, log off of the computer, return the mice to their regular housing room, and clean the testing room.

Holding Techniques

The method used for holding the mouse for the forepaw force measurement is very important for an accurate measurement. The results are highly repeatable if the mouse is handled in a consistent manner.

There are 3 recommended methods:

1. Hold the mouse around the midsection with the fingers and thumb curling under the body and the index finger on top, over the back and shoulders, with the tip of the finger by the mouse's neck.
2. Grasp the mouse by the scruff of the neck with one hand, and at the base of the tail with the other hand and bring it close to the grid until it can grip the bar.
3. Hold the mouse from the base of its tail and lower it toward the base of the bar until it grips the bar with both forepaws.

Tail Suspension

Day of Testing

SETUP

1. Bring your mice to the testing room and leave to habituate for 30-60 minutes.
    - Turn over the "Do Not Disturb" sign hanging outside of the door to ensure that no one will enter during testing.
2. Log on to the computer.
3. Turn on the Tail Suspension Interface cabinet and ENV-505TS Load Cell Amplifier.
4. Turn on the integration knob on the Tail suspension Interface cabinet DIG-735. The default position for the switch of the High pass filter on DIG-735 cabinet is 1 Hz.
5. Open the Tail Suspension software program.
6. Select File > Preferences. Set the following values:
    - Select the number of chambers to 4
    - Resolution to 200 msec and Graph history to 40 sec
    - Check "Save CSV Text summary files" to save a text summary file for your experiment
    - Check "CSV Batch Summary file" to save a CSV batch summary file
    - Check "Save CSV Raw data files"
    - Check "Automatic file Naming" to automatically name the data file based on Experiment, group and subject
    - Use the Browse button to select the directory where data files are stored: c/BData/Your Lab/TS/your name
    - Select "By Exp" the Data Subdirectory Options to create a subdirectory with the test name. When set to None, no subdirectory is created, and all data files are saved into the data directory.
7. Select Experiment > Start experiment.
    - Click "Add experiment" and name a new experiment in Experiment ID field
    - Enter the experiment title then click OK to save the name
    - Fill the following values for each field:
        - Experiment time: 6 minutes
        - Upper Threshold: 50
        - Lower Threshold: 3

- Gain: 4
- Block time: selected automatically when selected Number of Blocks
- Number of blocks 12 (each block will be 30 sec allowing for a 6 min total testing time)
- Start Trigger: 50. The experiment will automatically start when activity measured by the load meet the Start trigger value.
- Press “Copy Data to all chambers” to have the same experimental setting for all the chambers
- Enter the info for the Group, Subject ID, Subject info, Comment for each chamber

BEGIN TESTING

1. Clean the chambers with the solution provided.
2. Make sure you have new tape available and already cut to an appropriate size for each of the mice.
3. Press Start experiment.
    - Press Select all Chambers if you are using all 4; otherwise select the appropriate chambers.
4. Click Start button.
    - The test will not start until the mouse is taped to the metal bar.
5. Thread the mouse’s tail through a clear tube, then secure the end of the tail to the metal bar with tape. The ventral side of the mouse is facing the back wall of the chamber.
    - Position the clear tube so that there is a small gap between the tape and the tube. The tube should not be pressing into the mouse’s body.
    - Be VERY gentle when taping the mouse’s tail to the bar as it is very sensitive and can be damaged if too much force is applied.
6. Tape all mice to the bars as quickly as possible. Leave the room and close the door when finished.
7. The software will start automatically recording the data.
    - You should see the time counting down in the top corner of each chamber.
8. After 6 minutes, the software will stop.
    - The software may ask you to save the trial once all chambers are stopped. In this case, name the file as per the trial number and save in the appropriate folder.
9. Carefully take the mouse off the metal bar. Remove the tape and plastic tube from their tail and return the mice to their home cage.
    - Remove the mice in the same order that they were first put on to minimize the total amount of time each mouse is suspended.
10. Repeat for all mice, cleaning between trials.
11. To add another trial to the same experiment, select Experiment > Start Experiment. Select the experiment name for the next group of 4 mice. Fill in the mouse numbers, etc as described above.

WHEN TESTING IS COMPLETE

1. View > View raw data file to see the raw data or View CSV Batch Summary file to see the CSV data
2. Select the test you want to see and click Open.
3. Save CSV Batch Summary file as an Excel file in your labs folder – C:\BData\Lab Folder
4. When finished, log off of the computer, return the mice to their regular housing room, and sweep the testing room.

APPENDIX E – OTHER PROTOCOLS USED IN THE THESIS

Exercise Training

Acclimation

1. On the 1st day of testing, allow mice to habituate to the testing room for a minimum of 30 minutes.
2. Gently place each mouse in the treadmill, one mouse per lane.
3. Turn the speed to a maximum of 10m/min and allow the mice to run for no longer than 10 minutes.
4. Repeat steps 2-3 until all mice have been trained. Between each round, make sure to clean the lanes and the dividers with an anti-septic solution (70% ethanol works well).

Training – Week 1

Frequency: 3x/week
Intensity:
- Warm up: 8 m/min
- Training: 10 m/min
- Cool down: 8 m/min

Time:
- Warm up: 10 min
- Training: 25 min
- Cool down: 8 min

Type: Endurance Exercise

Training – Week 2

Frequency: 3x/week
Intensity:
- Warm up: 8 m/min
- Training: 10 m/min
- Cool down: 8 m/min

Time:
- Warm up: 10 min
- Training: 35 min
- Cool down: 5 min

Type: Endurance Exercise

Training – Week 3

Frequency: 3x/week
Intensity:
- Warm up: 8 m/min
- Training: 10 m/min
- Cool down: 8 m/min

Time:
- Warm up: 10 min
- Training: 45 min

- Cool down: 5 min

Type: Endurance Exercise

Training – Week 4

Frequency: 3x/week
Intensity:
- Warm up: 10 m/min
- Training: 10 m/min
- Cool down: 8 m/min

Time:
- Warm up: 10 min
- Training: 45 min
- Cool down: 5 min

Type: Endurance Exercise

Training – Weeks 5 - 18

Frequency: 3x/week
Intensity:
- Warm up: 10 m/min
- Training: Increase speed by 1 m/min (starting at 11 m/min on week 5) until you reach 24 m/min (week 18)
- Cool down: 8 m/min

Time:
- Warm up: 10 min
- Training: 45 min
- Cool down: 5 min

Type: Endurance Exercise

Training – Weeks 18 – 56

Frequency: 3x/week
Intensity:
- Warm up: 10 m/min
- Training: 24 m/min
- Cool down: 8 m/min

Time:
- Warm up: 10 min
- Training: 45 min
- Cool down: 5 min

Type: Endurance Exercise

Training – Weeks 56 – End of study

Frequency: 2x/week
Intensity:
- Warm up: 10 m/min
- Training: 24 m/min
- Cool down: 8 m/min

Time:

- Warm up: 10 min
- Training: 45 min
- Cool down: 5 min

Type: Endurance Exercise

Necropsy

Materials

- 5, 50 mL conical tubes (label each one with the mouse ID, organ and fixative) for the following organs:
    - Spleen
    - Liver
    - Heart
    - Lung
    - Brain
- 1, 15 mL conical tube (labelled with the mouse ID and fixative)
- ~100 mL of 10% NB formalin
    - Note: hematological tissues like spleen and liver require ~30-40mL of formalin to be properly fixed and prevent autolysis
- Dissection tools (Do not need to be sterile but should be cleaned well with ethanol or other anti-septic solution after use)
    - Sharp scissors
    - Tweezers
    - Scoopula
- KimWipes
- Scale (ACVS Procedure Room has a more accurate scale)

Organ Dissection

1. Thoroughly spray the dead mouse with ethanol or anti-septic solution to prevent the fur from sticking to the scissors.
2. Staring from the bottom and making your way up, cut away the skin and muscle layers in the abdomen all the way up to the ribs. Alternatively, you can continue cutting through the ribs up to the chin.
3. With your tweezers, carefully pull the intestines out of the way and push the stomach towards your left to reveal the spleen. It is generally tucked behind the stomach and appears dark red and oblong. To remove, cut on either side of the spleen.
4. Weigh and document the weight of the spleen and transfer to labelled 50mL conical tube.
5. To remove the liver, cut away the diaphragm (thin transparent sheet between liver and lungs). It is often easiest to carefully push the liver to one side to reveal more of the diaphragm on the lateral aspects of the liver. **Note:** the liver is very delicate and can be damaged quite easily.
6. Weigh and document the weight of the liver and transfer to labelled 50mL conical tube.
7. Cut through the ribs and either with your hands or tools, spread the rib cage apart.
8. Continue to cut up to the chin until you can see the trachea (tube with ring-like appearance).

9. With your tweezers, pull up from the trachea to remove the heart and lungs together. If this doesn't work, carefully cut away the connective tissue below and surrounding the heart and lungs.
10. Separate the heart from the lungs.
11. Weigh and document the weights of the heart and lungs and transfer each to their respective labelled 50mL conical tube.
12. Decapitate the mouse at the cervical spine. Note: to collect brain, it is important **not to** cervically dislocate the mice after asphyxiation as this can damage the brain.
13. Cut away the skin and muscle on the top of the head to reveal the skull.
14. **VERY** carefully, hold your scissors parallel to the table and superficially cut around the skull, starting from the back and cutting around the sides of the skull. Note: Going too deep or having your scissors perpendicular to the floor can potentially damage the brain.
15. Use your tweezers to grab the back of the skull and pull up to remove it.
16. With a scoopula, gently slide it along the later border of the skull and underneath the brain.
17. Pull up and back to dislodge the brain.
18. Weigh and document the weight of the brain and transfer to labelled 50mL conical tube.

Femur Dissection

1. Run your fingers gently up the femur until you get to the hip. With your scissors, cut around the hip to ensure you don't break the femur head.
2. Once removed from the body, begin cutting away the hip bone and any excess muscle/connective tissue. **Note:** Using a KimWipe makes cleaning the bone a lot easier. It is almost impossible to remove everything from the bone but you should be able to at least see the femoral head. At the distal end of the femur, there is a piece of cartilage that should be removed also to clean the bone further.
3. Repeat 1-2 with the other leg.
4. You can place both femurs in the same 15 mL conical tube but ensure there is at least 5-6 mL of 10% NB Formalin.
5. Place the tube with both femurs on the shaker at room temperature with gentle agitation for 48 hours.
6. After 48 hours, place both femurs in a cassette and rinse them under cool running water for 1 hour.
7. For histology, the femurs should be placed immediately in 14% EDTA solution for 14 days.
8. After 14 days, place the femurs in a cassette and rinse them under cool running water for 1 hour.
9. Store the femurs in a 2mL Eppendorf tube filled with 70% ethanol at 4^{o}C until ready for processing, embedding and sectioning.

APPENDIX F – STATISTICAL ANALYSES USING RSTUDIO

```
library(epiR)

## Loading required package: survival

## Package epiR 1.0-14 is loaded

## Type help(epi.about) for summary information

## Type browseVignettes(package = 'epiR') to learn how to use epiR for applie
d epidemiological analyses

##

## for incidence rate ratio calculations

dat1 <- matrix(c(9,205,10,188),ncol=2,byrow = TRUE)
colnames(dat1) <- c("Disease +","Time at Risk")
rownames(dat1) <- c("Expose +","Expose -")
pred1 <- epi.2by2(dat1,method = "cohort.time",conf.level = 0.95,units = 100,o
utcome = "as.columns")
dat2 <- matrix(c(16,236,10,188),ncol=2,byrow = TRUE)
colnames(dat2) <- c("Disease +","Time at Risk")
rownames(dat2) <- c("Expose +","Expose -")
pred2 <- epi.2by2(dat2,method = "cohort.time",conf.level = 0.95,units = 100,o
utcome = "as.columns")
dat3 <- matrix(c(10,192,10,188),ncol=2,byrow = TRUE)
colnames(dat3) <- c("Disease +","Time at Risk")
rownames(dat3) <- c("Expose +","Expose -")
pred3 <- epi.2by2(dat3,method = "cohort.time",conf.level = 0.95,units = 100,o
utcome = "as.columns")
pred1

##              Outcome +    Time at risk        Inc rate *
## Exposed +            9             205             4.39
## Exposed -           10             188             5.32
## Total               19             393             4.83
##
## Point estimates and 95% CIs:
## -------------------------------------------------------------------
## Inc rate ratio                                0.83 (0.30, 2.26)
## Attrib rate *                                 -0.93 (-5.30, 3.44)
## Attrib rate in population *                   -0.48 (-4.43, 3.46)
## Attrib fraction in exposed (%)                -21.16 (-236.99, 55.75)
## Attrib fraction in population (%)             -10.02 (-29.57, 12.37)
## -------------------------------------------------------------------
##  Wald confidence limits
##  CI: confidence interval
##  * Outcomes per 100 units of population time at risk

pred2
```

```
##              Outcome +    Time at risk        Inc rate *
## Exposed +           16             236             6.78
## Exposed -           10             188             5.32
## Total               26             424             6.13
##
## Point estimates and 95% CIs:
## -------------------------------------------------------------------
## Inc rate ratio                                 1.27 (0.54, 3.14)
## Attrib rate *                                  1.46 (-3.22, 6.14)
## Attrib rate in population *                    0.81 (-3.24, 4.87)
## Attrib fraction in exposed (%)                 21.54 (-83.88, 68.17)
## Attrib fraction in population (%)              13.26 (-8.87, 36.32)
## -------------------------------------------------------------------
##  Wald confidence limits
##  CI: confidence interval
##  * Outcomes per 100 units of population time at risk

pred3

##              Outcome +    Time at risk        Inc rate *
## Exposed +           10             192             5.21
## Exposed -           10             188             5.32
## Total               20             380             5.26
##
## Point estimates and 95% CIs:
## -------------------------------------------------------------------
## Inc rate ratio                                 0.98 (0.37, 2.62)
## Attrib rate *                                  -0.11 (-4.72, 4.50)
## Attrib rate in population *                    -0.06 (-4.08, 3.97)
## Attrib fraction in exposed (%)                 -2.13 (-173.40, 61.85)
## Attrib fraction in population (%)              -1.06 (-20.34, 20.66)
## -------------------------------------------------------------------
##  Wald confidence limits
##  CI: confidence interval
##  * Outcomes per 100 units of population time at risk

## for relative risk

##comparing to CTRL-SED
dat4 <- matrix(c(9,9,10,9),ncol=2,byrow = TRUE)
colnames(dat4) <- c("Disease +","Disease -")
rownames(dat4) <- c("Expose +","Expose -")
pred4 <- epi.2by2(dat4,method = "cohort.count",conf.level = 0.95,units = 100,
outcome = "as.columns")
dat5 <- matrix(c(16,3,10,9),ncol=2,byrow = TRUE)
colnames(dat5) <- c("Disease +","Disease -")
rownames(dat5) <- c("Expose +","Expose -")
pred5 <- epi.2by2(dat5,method = "cohort.count",conf.level = 0.95,units = 100,
outcome = "as.columns")
dat6 <- matrix(c(10,6,10,9),ncol=2,byrow = TRUE)
```

```
colnames(dat6) <- c("Disease +","Disease -")
rownames(dat6) <- c("Expose +","Expose -")
pred6 <- epi.2by2(dat6,method = "cohort.count",conf.level = 0.95,units = 100,
outcome = "as.columns")
##comparing to CTRL-EX
dat7 <- matrix(c(10,9,9,9),ncol=2,byrow = TRUE)
colnames(dat7) <- c("Disease +","Disease -")
rownames(dat7) <- c("Expose +","Expose -")
pred7 <- epi.2by2(dat7,method = "cohort.count",conf.level = 0.95,units = 100,
outcome = "as.columns")
dat8 <- matrix(c(16,3,9,9),ncol=2,byrow = TRUE)
colnames(dat8) <- c("Disease +","Disease -")
rownames(dat8) <- c("Expose +","Expose -")
pred8 <- epi.2by2(dat8,method = "cohort.count",conf.level = 0.95,units = 100,
outcome = "as.columns")
dat9 <- matrix(c(10,6,9,9),ncol=2,byrow = TRUE)
colnames(dat9) <- c("Disease +","Disease -")
rownames(dat9) <- c("Expose +","Expose -")
pred9 <- epi.2by2(dat9,method = "cohort.count",conf.level = 0.95,units = 100,
outcome = "as.columns")
##comparing to HFD-SED
dat10 <- matrix(c(10,9,16,3),ncol=2,byrow = TRUE)
colnames(dat10) <- c("Disease +","Disease -")
rownames(dat10) <- c("Expose +","Expose -")
pred10 <- epi.2by2(dat10,method = "cohort.count",conf.level = 0.95,units = 10
0,outcome = "as.columns")
dat11 <- matrix(c(9,9,16,3),ncol=2,byrow = TRUE)
colnames(dat11) <- c("Disease +","Disease -")
rownames(dat11) <- c("Expose +","Expose -")
pred11 <- epi.2by2(dat11,method = "cohort.count",conf.level = 0.95,units = 10
0,outcome = "as.columns")
dat12 <- matrix(c(10,6,16,3),ncol=2,byrow = TRUE)
colnames(dat12) <- c("Disease +","Disease -")
rownames(dat12) <- c("Expose +","Expose -")
pred12 <- epi.2by2(dat12,method = "cohort.count",conf.level = 0.95,units = 10
0,outcome = "as.columns")
##comparing to HFD-EX
dat13 <- matrix(c(10,9,10,6),ncol=2,byrow = TRUE)
colnames(dat13) <- c("Disease +","Disease -")
rownames(dat13) <- c("Expose +","Expose -")
pred13 <- epi.2by2(dat13,method = "cohort.count",conf.level = 0.95,units = 10
0,outcome = "as.columns")
dat14 <- matrix(c(9,9,10,6),ncol=2,byrow = TRUE)
colnames(dat14) <- c("Disease +","Disease -")
rownames(dat14) <- c("Expose +","Expose -")
pred14 <- epi.2by2(dat14,method = "cohort.count",conf.level = 0.95,units = 10
0,outcome = "as.columns")
dat15 <- matrix(c(16,3,10,6),ncol=2,byrow = TRUE)
colnames(dat15) <- c("Disease +","Disease -")
rownames(dat15) <- c("Expose +","Expose -")
```

```
pred15 <- epi.2by2(dat15,method = "cohort.count",conf.level = 0.95,units = 10
0,outcome = "as.columns")
pred4
```

```
##              Outcome +    Outcome -      Total        Inc risk *
## Exposed +            9            9         18              50.0
## Exposed -           10            9         19              52.6
## Total               19           18         37              51.4
##                  Odds
## Exposed +        1.00
## Exposed -        1.11
## Total            1.06
##
## Point estimates and 95% CIs:
## -------------------------------------------------------------------
## Inc risk ratio                                0.95 (0.51, 1.78)
## Odds ratio                                    0.90 (0.25, 3.27)
## Attrib risk *                                 -2.63 (-34.84, 29.58)
## Attrib risk in population *                   -1.28 (-28.91, 26.35)
## Attrib fraction in exposed (%)                -5.26 (-97.40, 43.87)
## Attrib fraction in population (%)             -2.49 (-38.07, 23.92)
## -------------------------------------------------------------------
##  Test that OR = 1: chi2(1) = 0.026 Pr>chi2 = 0.87
##  Wald confidence limits
##  CI: confidence interval
##  * Outcomes per 100 population units
```

```
pred5
```

```
##              Outcome +    Outcome -      Total        Inc risk *
## Exposed +           16            3         19              84.2
## Exposed -           10            9         19              52.6
## Total               26           12         38              68.4
##                  Odds
## Exposed +        5.33
## Exposed -        1.11
## Total            2.17
##
## Point estimates and 95% CIs:
## -------------------------------------------------------------------
## Inc risk ratio                                1.60 (1.00, 2.56)
## Odds ratio                                    4.80 (1.04, 22.10)
## Attrib risk *                                 31.58 (3.78, 59.38)
## Attrib risk in population *                   15.79 (-11.09, 42.67)
## Attrib fraction in exposed (%)                37.50 (0.11, 60.89)
## Attrib fraction in population (%)             23.08 (-3.60, 42.89)
## -------------------------------------------------------------------
##  Test that OR = 1: chi2(1) = 4.385 Pr>chi2 = 0.04
##  Wald confidence limits
```

```
##  CI: confidence interval
##  * Outcomes per 100 population units

pred6

##              Outcome +    Outcome -      Total        Inc risk *
## Exposed +           10            6         16              62.5
## Exposed -           10            9         19              52.6
## Total               20           15         35              57.1
##                  Odds
## Exposed +        1.67
## Exposed -        1.11
## Total            1.33
##
## Point estimates and 95% CIs:
## -------------------------------------------------------------------
## Inc risk ratio                                1.19 (0.67, 2.10)
## Odds ratio                                    1.50 (0.39, 5.81)
## Attrib risk *                                 9.87 (-22.79, 42.53)
## Attrib risk in population *                   4.51 (-23.29, 32.31)
## Attrib fraction in exposed (%)                15.79 (-49.05, 52.42)
## Attrib fraction in population (%)             7.89 (-22.71, 30.87)
## -------------------------------------------------------------------
##  Test that OR = 1: chi2(1) = 0.345 Pr>chi2 = 0.56
##  Wald confidence limits
##  CI: confidence interval
##  * Outcomes per 100 population units

pred7

##              Outcome +    Outcome -      Total        Inc risk *
## Exposed +           10            9         19              52.6
## Exposed -            9            9         18              50.0
## Total               19           18         37              51.4
##                  Odds
## Exposed +        1.11
## Exposed -        1.00
## Total            1.06
##
## Point estimates and 95% CIs:
## -------------------------------------------------------------------
## Inc risk ratio                                1.05 (0.56, 1.97)
## Odds ratio                                    1.11 (0.31, 4.04)
## Attrib risk *                                 2.63 (-29.58, 34.84)
## Attrib risk in population *                   1.35 (-26.81, 29.51)
## Attrib fraction in exposed (%)                5.00 (-78.16, 49.34)
## Attrib fraction in population (%)             2.63 (-35.58, 30.07)
## -------------------------------------------------------------------
##  Test that OR = 1: chi2(1) = 0.026 Pr>chi2 = 0.87
##  Wald confidence limits
```

```
##  CI: confidence interval
##  * Outcomes per 100 population units

pred8

##              Outcome +    Outcome -      Total        Inc risk *
## Exposed +           16            3         19              84.2
## Exposed -            9            9         18              50.0
## Total               25           12         37              67.6
##                  Odds
## Exposed +        5.33
## Exposed -        1.00
## Total            2.08
##
## Point estimates and 95% CIs:
## -------------------------------------------------------------------
## Inc risk ratio                                1.68 (1.02, 2.78)
## Odds ratio                                    5.33 (1.14, 24.90)
## Attrib risk *                                 34.21 (5.88, 62.54)
## Attrib risk in population *                   17.57 (-10.02, 45.15)
## Attrib fraction in exposed (%)                40.62 (1.98, 64.03)
## Attrib fraction in population (%)             26.00 (-3.04, 46.86)
## -------------------------------------------------------------------
##  Test that OR = 1: chi2(1) = 4.937 Pr>chi2 = 0.03
##  Wald confidence limits
##  CI: confidence interval
##  * Outcomes per 100 population units

pred9

##              Outcome +    Outcome -      Total        Inc risk *
## Exposed +           10            6         16              62.5
## Exposed -            9            9         18              50.0
## Total               19           15         34              55.9
##                  Odds
## Exposed +        1.67
## Exposed -        1.00
## Total            1.27
##
## Point estimates and 95% CIs:
## -------------------------------------------------------------------
## Inc risk ratio                                1.25 (0.69, 2.27)
## Odds ratio                                    1.67 (0.42, 6.56)
## Attrib risk *                                 12.50 (-20.61, 45.61)
## Attrib risk in population *                   5.88 (-22.61, 34.38)
## Attrib fraction in exposed (%)                20.00 (-45.46, 56.00)
## Attrib fraction in population (%)             10.53 (-22.84, 34.83)
## -------------------------------------------------------------------
##  Test that OR = 1: chi2(1) = 0.537 Pr>chi2 = 0.46
##  Wald confidence limits
```

```
##  CI: confidence interval
##  * Outcomes per 100 population units

pred10

##              Outcome +    Outcome -      Total        Inc risk *
## Exposed +           10            9         19              52.6
## Exposed -           16            3         19              84.2
## Total               26           12         38              68.4
##                  Odds
## Exposed +        1.11
## Exposed -        5.33
## Total            2.17
##
## Point estimates and 95% CIs:
## -------------------------------------------------------------------
## Inc risk ratio                                0.62 (0.39, 1.00)
## Odds ratio                                    0.21 (0.05, 0.96)
## Attrib risk *                                 -31.58 (-59.38, -3.78)
## Attrib risk in population *                   -15.79 (-37.86, 6.28)
## Attrib fraction in exposed (%)                -60.00 (-155.72, -0.11)
## Attrib fraction in population (%)             -23.08 (-49.53, -1.30)
## -------------------------------------------------------------------
##  Test that OR = 1: chi2(1) = 4.385 Pr>chi2 = 0.04
##  Wald confidence limits
##  CI: confidence interval
##  * Outcomes per 100 population units

pred11

##              Outcome +    Outcome -      Total        Inc risk *
## Exposed +            9            9         18              50.0
## Exposed -           16            3         19              84.2
## Total               25           12         37              67.6
##                  Odds
## Exposed +        1.00
## Exposed -        5.33
## Total            2.08
##
## Point estimates and 95% CIs:
## -------------------------------------------------------------------
## Inc risk ratio                                0.59 (0.36, 0.98)
## Odds ratio                                    0.19 (0.04, 0.88)
## Attrib risk *                                 -34.21 (-62.54, -5.88)
## Attrib risk in population *                   -16.64 (-38.92, 5.64)
## Attrib fraction in exposed (%)                -68.42 (-178.05, -2.02)
## Attrib fraction in population (%)             -24.63 (-51.93, -2.24)
## -------------------------------------------------------------------
##  Test that OR = 1: chi2(1) = 4.937 Pr>chi2 = 0.03
##  Wald confidence limits
```

```
##  CI: confidence interval
##  * Outcomes per 100 population units

pred12

##              Outcome +    Outcome -      Total        Inc risk *
## Exposed +           10            6         16              62.5
## Exposed -           16            3         19              84.2
## Total               26            9         35              74.3
##                  Odds
## Exposed +        1.67
## Exposed -        5.33
## Total            2.89
##
## Point estimates and 95% CIs:
## -------------------------------------------------------------------
## Inc risk ratio                                0.74 (0.48, 1.14)
## Odds ratio                                    0.31 (0.06, 1.54)
## Attrib risk *                                 -21.71 (-50.55, 7.13)
## Attrib risk in population *                   -9.92 (-31.80, 11.95)
## Attrib fraction in exposed (%)                -34.74 (-106.42, 12.05)
## Attrib fraction in population (%)             -13.36 (-34.50, 4.46)
## -------------------------------------------------------------------
##  Test that OR = 1: chi2(1) = 2.143 Pr>chi2 = 0.14
##  Wald confidence limits
##  CI: confidence interval
##  * Outcomes per 100 population units

pred13

##              Outcome +    Outcome -      Total        Inc risk *
## Exposed +           10            9         19              52.6
## Exposed -           10            6         16              62.5
## Total               20           15         35              57.1
##                  Odds
## Exposed +        1.11
## Exposed -        1.67
## Total            1.33
##
## Point estimates and 95% CIs:
## -------------------------------------------------------------------
## Inc risk ratio                                0.84 (0.48, 1.49)
## Odds ratio                                    0.67 (0.17, 2.58)
## Attrib risk *                                 -9.87 (-42.53, 22.79)
## Attrib risk in population *                   -5.36 (-34.19, 23.48)
## Attrib fraction in exposed (%)                -18.75 (-110.19, 32.91)
## Attrib fraction in population (%)             -9.38 (-45.72, 17.91)
## -------------------------------------------------------------------
##  Test that OR = 1: chi2(1) = 0.345 Pr>chi2 = 0.56
##  Wald confidence limits
```

```
##  CI: confidence interval
##  * Outcomes per 100 population units

pred14

##              Outcome +    Outcome -      Total        Inc risk *
## Exposed +            9            9         18              50.0
## Exposed -           10            6         16              62.5
## Total               19           15         34              55.9
##                  Odds
## Exposed +        1.00
## Exposed -        1.67
## Total            1.27
##
## Point estimates and 95% CIs:
## -------------------------------------------------------------------
## Inc risk ratio                                0.80 (0.44, 1.45)
## Odds ratio                                    0.60 (0.15, 2.36)
## Attrib risk *                                 -12.50 (-45.61, 20.61)
## Attrib risk in population *                   -6.62 (-35.62, 22.39)
## Attrib fraction in exposed (%)                -25.00 (-127.28, 31.25)
## Attrib fraction in population (%)             -11.84 (-48.83, 15.95)
## -------------------------------------------------------------------
##  Test that OR = 1: chi2(1) = 0.537 Pr>chi2 = 0.46
##  Wald confidence limits
##  CI: confidence interval
##  * Outcomes per 100 population units

pred15

##              Outcome +    Outcome -      Total        Inc risk *
## Exposed +           16            3         19              84.2
## Exposed -           10            6         16              62.5
## Total               26            9         35              74.3
##                  Odds
## Exposed +        5.33
## Exposed -        1.67
## Total            2.89
##
## Point estimates and 95% CIs:
## -------------------------------------------------------------------
## Inc risk ratio                                1.35 (0.88, 2.06)
## Odds ratio                                    3.20 (0.65, 15.78)
## Attrib risk *                                 21.71 (-7.13, 50.55)
## Attrib risk in population *                   11.79 (-16.01, 39.58)
## Attrib fraction in exposed (%)                25.78 (-13.70, 51.55)
## Attrib fraction in population (%)             15.87 (-9.87, 35.57)
## -------------------------------------------------------------------
##  Test that OR = 1: chi2(1) = 2.143 Pr>chi2 = 0.14
##  Wald confidence limits
```

```
##  CI: confidence interval
##  * Outcomes per 100 population units
```