Exercise Prevents Diet-induced Cellular Senescence in Adipose Tissue

Running Title: Senescence Prevention through Exercise

Marissa J. Schafer¹,², Thomas A. White¹, Glenda Evans¹, Jason M. Tonne³, Grace C. Verzosa⁴, Michael B. Stout¹,⁵, Daniel L. Mazula¹, Allyson K. Palmer¹, Darren J. Baker¹,⁶, Michael D. Jensen⁷, Michael S. Torbenson⁸, Jordan D. Miller¹,⁴, Yasuhiro Ikeda³, Tamara Tchkonia¹, Jan M. van Deursen¹,⁹, James L. Kirkland¹,⁵, Nathan K. LeBrasseur¹,²

¹Robert and Arlene Kogod Center on Aging, Departments of ²Physical Medicine and Rehabilitation, ³Molecular Medicine, ⁴Surgery, ⁵Internal Medicine, ⁶Pediatric and Adolescent Medicine, ⁷Medicine, Division of Endocrinology, ⁸Laboratory Medicine and Pathology, and ⁹Biochemistry and Molecular Biology, Mayo Clinic, Rochester, MN USA

Correspondence: Nathan K. LeBrasseur, Ph.D.
Robert and Arlene Kogod Center on Aging
Mayo Clinic
200 First Street SW
Rochester, MN 55905

Phone: 507.266.0727
Fax: 507.293.3853
Email: lebrasseur.nathan@mayo.edu

Conflicts of Interest: Mayo Clinic, J.L.K., and T.T. have a financial interest related to this research with intellectual property licensed to a commercial entity. This research has been reviewed by the Mayo Clinic Conflict of Interest Review Board and is being conducted in compliance with Mayo Clinic Conflict of Interest policies.

Key Words: obesity, diabetes, aging, inflammation, physical activity
Abstract

Considerable evidence implicates cellular senescence in the biology of aging and chronic disease. Diet and exercise are determinants of healthy aging; however, the extent to which they affect the behavior and accretion of senescent cells within distinct tissues is not clear. Here we tested the hypothesis that exercise prevents premature senescent cell accumulation and systemic metabolic dysfunction induced by a fast food diet (FFD). Using transgenic mice that express enhanced green fluorescent protein (EGFP) in response to activation of the senescence-associated p16\textsuperscript{INK4a} promoter, we demonstrate that FFD consumption causes deleterious changes in body weight and composition, as well as measures of physical, cardiac, and metabolic health. The harmful effects of the FFD were associated with dramatic increases in several markers of senescence, including p16, EGFP, senescence-associated β-galactosidase, and the senescence-associated secretory phenotype (SASP), specifically in visceral adipose tissue. We show that exercise prevents both the accumulation of senescent cells and the expression of the SASP, while nullifying the damaging effects of the FFD on parameters of health. We also demonstrate that exercise initiated following long-term FFD-feeding reduces senescent phenotype makers in visceral adipose while attenuating physical impairments, suggesting that exercise may provide restorative benefit by mitigating accrued senescent burden. These findings highlight a novel mechanism by which exercise mediates its beneficial effects and reinforce the impact of modifiable lifestyle choices on healthspan.
Introduction

Unhealthy diets and sedentary lifestyles are factors fueling the obesity epidemic, wherein approximately 35% of middle aged Americans are obese (1). Heavily implicated in this public health issue is routine consumption of calorie-dense, nutrient-poor fast foods and sugar-sweetened beverages, akin to a fast food diet (2). Nutrient excess leading to metabolic dysfunction increases the risk for and accelerates the onset of numerous age-related conditions, including diabetes, cardiovascular disease, Alzheimer’s disease, and cancer (3; 4). Fat mass distribution further influences chronic disease risk, with visceral adiposity serving as a stronger predictor of all-cause mortality, relative to subcutaneous adiposity (5). In contrast, exercise positively affects body composition, enhances physical fitness, and is protective against numerous age-related diseases (6). Despite the widely-recognized effects of diet and exercise on healthspan, the fundamental mechanisms by which they influence the biology of aging and chronic disease remain elusive.

Cellular senescence is a state of stable growth arrest triggered by telomere erosion, DNA lesions, reactive oxygen species (ROS), and other mitogenic and metabolic stressors. It is mediated by the inhibition of cell cycle progression through p16^INK4a/retinoblastoma protein (Rb) and/or the activation of cell cycle arrest through p53/p21. Characteristic gene expression signature and morphological shifts define the transition into a senescence state, but the functional role of senescent cells within a given tissue milieu is highly cell-type, concentration, and context dependent (7). Multiple lines of evidence implicate cellular senescence in the biology of aging and the genesis of age-related conditions (8; 9). In particular, biomarkers of senescent cells, including p16 and
senescence-associated β-galactosidase (SA-β-gal) levels, increase in multiple tissues with advancing age and in the context of chronic disease (10). Senescent cells actively secrete a broad repertoire of cytokines, chemokines, matrix remodeling proteases, and growth factors, collectively referred to as the senescence-associated secretory phenotype (SASP) (11). Despite their cell-autonomous role in prevention of malignant transformation, through the SASP, senescent cells damage neighboring cells, paradoxically fuel the aberrant growth and invasion of malignant cells, and promote inflammation (7; 8). Senescent cells and the SASP are thus believed to drive degenerative, hyperproliferative, and inflammatory conditions of aging (12). This premise is further supported by recent studies demonstrating that targeted deletion of p16\textsuperscript{INK4a}-expressing senescent cells delays the onset of several age-related phenotypes including thymic involution (13) and, in a mouse model of accelerated aging, cataracts, lordokyphosis, and diminished exercise capacity (14). More recently, pharmacological agents selected for their ability to kill senescent cells, termed \textit{senolytics}, or inhibit the SASP, have shown therapeutic benefit on parameters of physical health and function when administered to chronologically aged, progeroid, and/or irradiated mice (15; 16).

Whether and how lifestyle choices in middle age influence the premature genesis of pro-aging senescent phenotypes in distinct tissues remains unclear. Accordingly, we sought to determine the extent to which nutrient excess and exercise affect the onset and progression of cellular senescence and the SASP using adult transgenic mice that express a construct harboring enhanced green fluorescent protein (EGFP) in response to the senescence-sensitive promoter, p16\textsuperscript{INK4a}.
Methods

Mice and Experimental Interventions. Mice harboring the p16<sup>INK4a</sup>-EGFP transgenic construct (14) were generated on a genetically heterogeneous background (4-strain cross, as detailed in (17)). For the prevention study, 8 month old male mice were divided into four groups of comparable mean body weights. The groups were then randomly assigned to one of the following 16-week interventions: normal diet (ND) (13% energy as fat, PicoLab Rodent Diet 20 (5053), Lab Diet, St. Louis, MO), fast food diet (FFD) (40% energy as fat (milk fat, 12% saturated) with 0.2% cholesterol (Western Diet (5342), Test Diet, St. Louis, MO), and high fructose corn syrup in the drinking water (42 g/l), see (18)), ND plus exercise, or FFD plus exercise. For the treatment study, 5-6 month old male mice were provided ND or FFD for 16 weeks. FFD mice were then randomized to sedentary or exercise groups based on body weight, for a total of 3 groups, which were followed for an additional 14 weeks. Thus, all mice in both the prevention and treatment studies were approximately 1 year old at necropsy. All mice were individually housed in ventilated cages and provided food and water ad libitum. Exercised mice were provided wireless running wheels, and exercise behavior was monitored using Wheel Manager Data Acquisition Software (Med Associates, St. Albans, VT). Experiments were performed under protocols approved by the Mayo Clinic IACUC.

Body Composition and Healthspan Measures. Body weight and food intake were measured weekly. Body composition (total body lean and fat mass) was assessed monthly in unanesthetized mice by quantitative magnetic resonance as previously described (19) (EchoMRI-100, Houston, TX). At the end of the study, subcutaneous and visceral fat in the lumbar region was quantified in anaesthetized mice using micro-computed
tomography (Scanco vivaCT 40, Wayne, PA). As a measure of physical function, exercise capacity was determined on a motorized treadmill (Columbus Instruments, Columbus, OH) as previously described (20). Cardiac function in mice under light isoflurane anaesthesia was assessed by echocardiography using the Vevo 2100 system (FUJIFILM VisualSonics, Inc. Toronto, CA) as recently described (21). For metabolic function, glucose and insulin concentrations and glucose tolerance following a 6 hour fast were assessed as previously described (22).

**Tissue Assessments.** Individual tissues were harvested, weighed, and processed for downstream analyses. Portions of individual adipose tissue depots were fixed in PBS containing 2.0% formaldehyde and 0.2% glutaraldehyde for cell size determination and SA-β-gal activity (see below). The sizes of adipocytes in fat tissue were determined using Metamorph software (Molecular Devices, Sunnyvale, CA). Liver tissue was fixed in 10% formalin, dehydrated and embedded in paraffin. Liver sections were stained with hematoxylin and eosin for overall morphology. A pathologist who was not aware of treatment assignments gave grades of 0, 1, 2, 3, and 4 to sections in which 0, 1-4, 5-30, 31-60, and 61-100% of hepatocytes, respectively, had lipid macrovesicles. Grades of 0, 1, 2, and 3 were assigned to liver sections with 0, 1-30, 31-60, and 61-100% of hepatocytes containing lipid microvesicles. Liver ceramides were quantified using ultra-performance liquid chromatography/tandem mass spectrometry as recently described (23). Pancreata were embedded and frozen in OCT Compound (Sakura Finetek USA, Inc., Torrance, CA). Immunostaining of cryosections and quantification of insulin-positive mass was performed as previously described (24).
Markers of Cellular Senescence and the SASP. For transcriptional analysis, Trizol-based extraction was used to isolate RNA from whole mouse tissues, which were subjected to nanodrop concentration and purity analysis prior to cDNA synthesis. TaqMan qPCR assays (Life Technologies, Carlsbad, CA) were used for detection of p16 (Mm00494449_m1), Mcp1 (Mm00441243_g1), and Igf1 (Mm00439561_m1). PrimeTime 5’ nuclease qPCR assays (Integrated DNA Technologies, Coralville, IA) were used for detection of p21 (Mm.PT.56a.17125846), p53 (Mm.PT.56a.44013092), Il6 (Mm.PT.56a.10005566), Pai1 (Mm.PT.58.6413525), Mmp3 (Mm.PT.58.9719290), CD68 (Mm.PT.58.32698807), and Tbp (Mm.PT.39a.22214839). A SYBR qPCR assay (Integrated DNA Technologies) was used for detection of EGFP (FW 5’-CAA CTA CAA CAG CCA CAA CG-3’; RV 5’-GGT CAC GAA CTC CAG CAG-3’). Adipose tissue depots were stained for SA-β-gal activity as previously described (25).

Statistical Analysis. Significant differences between groups for dependent variables (diet (ND and FFD) and behavior (sedentary and exercise)) were tested using two-way analysis of variance. Tukey’s multiple comparisons test was used for post hoc analyses for between-group comparisons. Analyses were conducted using GraphPad Prism Statistical Software Version 6.0 (San Diego, CA).
Results

Exercise prevents multiple indices of diet-induced metabolic dysfunction. To investigate the potential role of cellular senescence in diet-induced dyshomeostasis, which may be attenuated by exercise, we provided eight-month-old male mice harboring an EGFP transgene driven by the p16^{INK4a} promoter with either a normal diet (ND) or a high fat diet enriched with saturated fat, cholesterol, and high fructose corn syrup, equivalent to a fast food diet (FFD), for 4 months. Subsets of ND- and FFD-fed mice were provided with running wheels. Mice provided with the FFD consumed more total calories within the first weeks of the study, but within one month, total calorie intake was not different among any of the groups (Sup. Fig. 1A). Increased energy intake in exercising FFD mice corresponded to elevated average daily running distances, albeit non-statistically significant, within the first 2 months of the study (Sup. Fig 1B).

We assessed whether clinically relevant health indices, including body weight, adipose mass, physical activity, circulating insulin and glucose concentrations, cardiac function, and liver health, were altered by diet and exercise. At study onset, average body weight and fat mass were equivalent among ND- and FFD-fed mice, but following 4 months, FFD-fed mice weighed 31% more and accumulated twice the total fat of ND-fed mice (Figure 1A and B). Differences between ND- and FFD-fed mice in subcutaneous and visceral fat were evident by weight (Sup. Fig. 2A) and in micro-computed tomography scans of the lumbar region (Figure 1C). The visceral fat of FFD-fed mice was composed of significantly larger adipocytes and a greater percentage of large adipocytes than that of ND-fed mice (Sup. Fig. 2B). Exercise blunted the ND- and, more dramatically, the FFD-induced accretion of body weight and fat mass (Figure 1A-1C,
Sup. Fig. 2A). In fact, exercised FFD-fed mice had visceral and subcutaneous fat weights that were not statistically different from those of ND-fed mice (Sup. Fig. 2A). The FFD-induced hypertrophy of adipocytes was also prevented by exercise (Sup. Fig. 2B).

Assessment of physical function using a treadmill test revealed that FFD diet alone caused a modest but non-significant decrease in physical performance, while exercised mice on both ND and FFD ran a significantly greater distance to exhaustion than sedentary peers (Figure 1D). Exercised mice also had positive cardiac adaptations relative to sedentary peers, including increased heart weight to body weight ratios, indicative of physiological hypertrophy (Sup. Fig. 3A), and improved ejection fractions measured by echocardiography (Sup. Fig. 3B). Sedentary mice on FFD exhibited poorer values for both of these parameters of cardiac health (Sup. Fig. 3A and 3B), as well as a deleterious increase in left ventricular end diastolic dimension (LVEDD) relative to mice on ND and exercised FFD-fed peers (Sup. Fig. 3C).

With respect to metabolic function, no differences in fasting glucose were observed between groups (data not shown); however, sedentary mice on FFD had dramatically increased insulin concentrations. This hallmark of diet-induced insulin resistance was robustly reduced by exercise (Figure 2A). Correspondingly, we observed grossly enlarged beta cell masses and insulin positive areas in the pancreata of sedentary, but not exercised, FFD-fed mice (Figure 2B and 2C). Further evidence of preserved insulin action in exercised mice on FFD was apparent in a glucose tolerance test, in which they were indistinguishable from mice on ND (Figure 2D). In contrast, sedentary mice on FFD had more pronounced excursions and impaired clearance of circulating glucose.
Nutrient excess can lead to adipocyte dysfunction, reflected in impaired triglyceride deposition, increased lipolysis, and lipotoxicity, or the accumulation of lipids in peripheral tissues (26). As expected, liver weights of sedentary FFD-fed mice were significantly greater than ND-fed mice (Sup. Fig. 4A). Longer chain ceramides, C16 and C24:1, and liver triglycerides, which are associated with insulin resistance (27), were also significantly elevated in the livers of sedentary mice (Sup. Fig. 4B and 4C). These markers of hepatic lipotoxicity were prevented by exercise (Sup. Fig. 4B and 4C), as were select FFD-induced gross morphological changes (Sup. Fig. 4D). Compared to ND-fed mice, livers of sedentary and exercised FFD-fed mice demonstrated increases in the percentage of hepatocytes with lipid macrovesicles in their cytoplasm, an early event in the pathogenesis of steatosis (Sup. Fig. 4E). The majority of hepatocytes in the livers of sedentary mice on FFD also had highly abundant lipid microvesicles, a feature associated with mitochondrial injury or dysfunction (28). This consequence of the FFD was abrogated by exercise (Sup. Fig. 4F). Collectively, these data underscore the salutary influence of exercise on several parameters of health, and its ability to prevent multiple harmful effects of nutrient excess.

The detrimental effects of nutrient excess on senescent cell burden and the SASP in visceral fat are prevented by exercise. Although accumulation of senescent cells occurs with advancing age, prematurely elevated senescent cell burden may be both a cause and consequence of metabolic dysfunction (9; 29). We hypothesized that routine FFD consumption in middle age promotes accretion of senescent cells, and accordingly, we probed expression of senescent biomarkers p16, p21, and p53 within discrete tissues. Compared to ND-fed mice, visceral fat of sedentary FFD-fed mice contained
significantly higher mRNA levels of p16 (Figure 3A) and p53 (Figure 3B), as well as its downstream target, p21 (Figure 3C), indicating pronounced activation of pro-senescence markers. Exercise completely blocked FFD-induced increases in p53 and p21 (Figure 3B and 3C). The expression of p16 in subcutaneous adipose tissue, liver, skeletal muscle, pancreas, kidney, heart (left ventricle), and aorta was not altered in response to diet or exercise (Figure 3A). Similar outcomes were observed for the expression of p53, which, in addition, was higher in the subcutaneous fat of FFD-fed mice relative to that of exercised mice on ND (Figure 3B). p21 expression was significantly elevated in subcutaneous fat and liver of sedentary FFD-fed mice, an effect that was prevented by exercise (Figure 3C). These findings suggest that nutrient excess in middle age activates expression of pro-senescence markers in visceral adipose tissue, and this effect is robustly attenuated by exercise. To a lesser degree, senescent signaling may also occur in tissues other than visceral fat and may be prevented by exercise. However, this likely involves mechanisms other than p16, such as the p53/p21 pathway.

Elevated expression of senescence markers distinctly within visceral adipose lead us to further investigate the influence of nutrient excess and exercise on additional indicators of cellular senescence and the SASP in this tissue. Quantification of EGFP expression confirmed that FFD-feeding greatly enhanced p16\textsuperscript{INK4a} promoter activity, which was strongly prevented by exercise (Figure 4A). To validate expression-based data, we performed immunostaining for the classic biomarker of senescence, senescence-associated β-galactosidase (SA β-gal) in visceral adipose tissue. In sedentary and exercised mice fed the ND, approximately 2% of cells stained were positive for SA β-gal (Figure 4B and 4C). In comparison, sedentary mice fed the FFD had more than 12% of
cells stain positively. Strikingly, exercise nullified this effect of the FFD and, as a result, the percentage of SA β-gal positive cells in exercised FFD-fed mice was identical to that of ND-fed middle-aged mice (Figure 4B and 4C).

In part, senescent cells disrupt a tissue’s structure and function and affect the systemic environment through the factors they secrete. Indeed, FFD-fed mice demonstrated significant increases in the expression of pro-inflammatory SASP markers including interleukin 6 (Il6), plasminogen activator inhibitor-1 (Pai1), and monocyte chemoattractant protein-1 (Mcp1) (Figure 3D). We also observed significantly increased expression of matrix metalloprotease 3 (Mmp3), a matrix remodeling protein and SASP component, within visceral fat following FFD-feeding. No significant differences were found in insulin-like growth factor 1 (Igf1) (Figure 4D). With the exception of Pai1, exercise prevented the induction of the SASP by the FFD. SASP signaling may paracrinely instigate senescence while recruiting inflammatory cells (30), ultimately propagating sterile inflammation (31). Indeed, we observed a 7.5-fold increase in expression of the pan-macrophage marker, CD68, in visceral adipose (Figure 4D). Exercise completely abrogated CD68 induction (Figure 4D). These findings indicate that diet and exercise are potent mediators of cellular senescence and the SASP, which may be a central mediator of obesity-induced inflammation in visceral adipose tissue.

Exercise reduces markers of cellular senescence and the SASP in diet-induced obese mice. Given the efficacy of exercise at preventing the molecular phenotype of senescence in visceral fat of FFD-fed mice, we next sought to test whether exercise may be leveraged to revert these consequences. Accordingly, we provided mice with ND or FFD for 16 weeks, during which FFD led to gains in total body weight (Figure 5A) and fat mass as
measured by quantitative magnetic resonance (Figure 5B). FFD mice were then randomized to voluntary wheel running or sedentary groups. Over the subsequent 14 weeks, body weight (Figure 5A) and fat mass (Figure 5B) declined in exercising mice. Micro-computed tomography scans further showed that exercise-mediated fat mass reductions occurred in both visceral and subcutaneous adipose depots (Figure 5C). Exercise adaptations were corroborated by a forced-run treadmill test to exhaustion, during which, exercised mice ran 126% farther than sedentary FFD-fed peers (Figure 5D).

To explore whether exercise mitigates diet-induced cellular senescence, we conducted gene expression profiling on visceral adipose extracts. Similar to the prevention study, exposure to FFD for 30 weeks resulted in a significant >4-fold increase in p16 expression in visceral fat of sedentary mice (Figure 5E). Despite continued consumption of the FFD, the treatment of obese mice with exercise for 14 weeks reverted this effect, as evidenced by reduced expression of p16 to levels that were not different from ND-fed control mice. Expression of p53 and p21 did not significantly differ between ND- and FFD-fed mice (Figure 5E), suggesting that p16 rather than p53/p21 signaling may drive long-term senescence maintenance. Consistent with induction of the senescence biomarker p16, we observed significant increases in the SASP component, Pai1, in sedentary FFD-fed mice. Increases in Mcp1 were not significant (Figure 5F). CD68 transcriptional levels were also higher in sedentary FFD-fed mice than ND-fed peers (Figure 5F). Treatment of FFD-fed mice with exercise attenuated elevated Pai1 and CD68 levels (Figure 5F). Cumulatively, these results point to diminution of a diet-induced senescent phenotype in visceral adipose tissue as a means by which exercise
provides restorative benefit to abate chronic disease following long-term sedentary behavior and nutrient excess.
Discussion

The present study demonstrates the robust effects of modifiable lifestyle factors on the accumulation of senescent cells and the expression of the SASP in middle age. Our data highlight the harmful consequences of nutrient excess and the remarkably protective influence of exercise on this biological process and, in turn, fundamental measures of physical, cardiovascular, and metabolic function. In the face of population aging, an obesity epidemic, and global reductions in physical inactivity, these findings have significant implications for human health.

Obesity is, fundamentally, a condition of energy imbalance; the consumption of more energy than is expended. The increased storage demands placed on adipocytes in the face of nutrient excess ultimately compromise their function, reflected in impaired storage of lipids and augmented release of free fatty acids and inflammatory mediators (26). Our data support the premise that visceral adipose tissue dysfunction and its sequelae are partly mediated through cellular senescence (12). The stromal vascular fraction of adipose tissue is rich in progenitor cells, or preadipocytes, that are prone to senescence and exhibit a proinflammatory profile (12; 16; 32). We show that nutrient excess markedly increased the expression of p16 and other markers of senescence including p53 and p21 and the activity of SA-β-gal. These changes were associated with induction of pro-inflammatory cytokines, chemokines, and matrix remodeling proteins (e.g., Il6, Mcp1, Pai1, and Mmp3, respectively), collectively referred to as the SASP.

SASP factors mechanistically contribute to metabolic disease. Knockout of Pai1 abrogates insulin resistance and obesity brought on by high fat feeding (33). Similarly, blockade of adipose Mcp1 signaling exerts anti-inflammatory effects (34), and ablation of
the Mcp1 receptor (C-C motif chemokine receptor–2 (Ccr2^+/−)) increases adiponectin levels and improves glucose homeostasis following high fat feeding, relative to Ccr2^+/+ controls matched for adiposity (35). Furthermore, SASP signaling has been implicated as a means of senescence transmission to neighboring cells (30), suggesting that the SASP may be responsible for induction and amplification of inflammation arising from nutrient excess. We demonstrate that FFD-feeding strongly induces visceral adipose expression of Pai1, Mcp1, and CD68 coincident with increases in p16 expression and SA-β-gal-positive cells, which is prevented by exercise. Similarly, we show that treatment of obese mice with voluntary exercise is able to revert aspects of this molecular phenotype. Our data and prior evidence supports the premise that senescent cells may be a primary source of obesity-associated inflammation, which is central to the pathogenesis of type 2 diabetes and its complications (9) and highlights the potential of exercise as an effective intervention.

In agreement with our findings, Minamino et al. reported that senescent cells accumulate in the adipose tissue of younger mice with ectopic expression of agouti peptide, which leads to excessive nutrient intake, obesity and diabetes (36). However, a more recent study failed to show that high fat feeding accelerates age-related p16^{INK4a} expression as quantified by whole-body luciferase imaging or mRNA abundance in isolated livers or spleens (37). It is plausible that imaging was not adequately sensitive to detect diet-induced changes in the abundance of p16^{INK4a}-positive senescent cells in vivo. Furthermore, nutrient excess may more potently induce the accumulation of p16^{INK4a} and/or p53-positive senescent cells in adipose tissue compared to other organs. Our results show that a senescence phenotype is readily activated specifically within visceral
fat, and to a lesser degree within subcutaneous fat, in middle age by FFD. As visceral adipose is a stronger driver of metabolic-induced morbidity, relative to other depots (5), it is possible that senescent signaling may mediate this organ’s unique role in sensing and negatively affecting the body in response to nutrient excess, particularly regarding the inflammatory component of obesity-induced dyshomeostasis. The causal role of senescent cells and the SASP in the genesis of obesity associated conditions and the therapeutic efficacy of their removal, therefore, requires further examination.

The tissue specificity and temporal induction of cellular senescence warrants further consideration. Other groups have demonstrated increased senescence markers in variable tissues in response to nutrient excess, including elevated aortic p16 levels following 20 weeks of high fat feeding in 4-week-old C57BL/6 J mice (38), elevated hepatic p16 and p21 levels following 13 weeks of high fat feeding in 5-week-old rats (39), and elevated pancreatic p38 and SA-β-gal levels following 12 months of high fat feeding in 4-week-old C57BL/6 J mice (40). We conducted analyses in 12-month-old mice administered dietary and/or exercise intervention for the previous 16 or 30 weeks, and our experiments employed a diet that was high in sugar and fat. We observed robust induction of p16 and p53 in visceral adipose and induction of p21 in visceral and subcutaneous adipose and liver. Expression of these markers also appeared to increase in other tissues, including pancreas, but did not reach statistical significance. Given the differences in age, genetic background, and diet composition between our study and the noted reports, it is not surprising that others identified senescence signatures in tissues that were not prominent in our exploration. However, the composite results unanimously show that nutrient excess leads to induction of senescence in multiple tissues responsible
for coordinating metabolic health and cumulatively highlight the need for additional work to tease out the time course of this progression in uniform contexts.

The beneficial effects of exercise on healthspan are irrefutable; however, the biological mechanisms through which they act are not completely understood. Our findings confirm that exercise can positively affect multiple parameters of physical, cardiac, and metabolic health in middle-aged mice and, importantly, overcome the damaging effects of nutrient excess. Moreover, using established biomarkers, we show for the first time that exercise prevents and reduces indicators of cellular senescence in visceral adipose tissue induced by FFD. This is significant given the considerable evidence implicating senescent cells and the SASP in the biology of chronic diseases (8) and the beneficial effects of their removal on several parameters of health, at least in a model of accelerated aging (14). There are three possibilities by which exercise prevented the diet-induced accumulation of senescent cells. First, the increased energy demands and use of dietary macronutrients during bouts of exercise may have limited the metabolic and replicative stresses experienced by cells in adipose tissue and consequently, their transition into a senescent state. Smaller adipocytes and fat depots as well as lower liver weights and abundance of liver ceramides in exercised FFD-fed mice compared to their sedentary peers, may reflect this. Second, it is plausible that exercise may have augmented the clearance of senescent cells. Their fate is highly variable. In benign melanocytotic nevi, senescent cells can persist for decades (41), while senescent liver carcinoma cells acutely activate the innate immune system to mediate their clearance and limit tumor growth (42). This possibility is supported by our data showing exercise reduces p16 expression in mice even after obesity and its consequences have been
established. Third, exercise could have invoked protective responses against triggers of cellular senescence in the context of nutrient excess, as exercise counters DNA damage (43), telomere erosion (44), oxidative stress (45), protein aggregation (46), and mitochondrial dysfunction (47) in multiple cell types. We speculate that exercise may have induced such defense systems to prevent adipose tissue cells from senescing. Indeed, additional work is needed to better understand how and when to leverage exercise to impact the accumulation, behavior, and persistence of senescent cells in the context of nutrient excess.

Adipose tissue is a critically important tissue in organismal health and aging. In contrast to the associations between obesity and phenotypes suggestive of accelerated aging, reductions in fat mass through calorie restriction (48), surgery (49), mutations in the insulin signaling pathway (50), or exercise as shown here, enhance healthspan in various organisms. Our findings lend support to the concept that the SASP is a major determinant of the secretory profile, or endocrine function, of adipose tissue. In aging and obesity, adipose tissue is a primary source of inflammatory mediators implicated in the genesis of diabetes and other chronic diseases (12). Previously, we demonstrated that the expression of components of the SASP including Pai1 and Il6 were distinctly higher in p16^{INK4a}-positive senescent cells than non-senescent cells residing in adipose tissue (9). In the present study, our results show that exercise prevents the SASP within visceral adipose tissue, and remarkably, reduces aspects of the SASP when initiated after its accumulation. We propose this is an unappreciated mechanism through which physical activity interventions may impact healthspan, particularly in those who are overweight or obese. Of note, the association between obesity-associated subclinical inflammation and
the genesis of type 2 diabetes has been reported to be stronger in women than men (51). Additional work is needed to determine the extent to which senescent cell burden and the SASP may account for this sex difference, and whether exercise is as protective in females as we observed in males. Therapeutic approaches to suppress the SASP, such as exercise, may offer a means to negate the deleterious systemic effects of senescent cells in the genesis of obesity- and age-related chronic diseases.

In sum, our data highlight a novel and significant mechanism by which exercise positively affects organismal health. Given the considerable evidence that cellular senescence is a fundamental mechanism of aging and the genesis of chronic diseases, our findings reinforce the notion that lifestyle choices are powerful determinants of healthspan. Additional studies are necessary to determine the mechanisms by which exercise prevents and reverses cellular senescence and the SASP and at what ages and in what disease states it is most effective.
**Author Contributions**

MJS helped collect and analyze data, designed and implemented follow-up experiments, and drafted and revised the manuscript. TAW helped design the study, collected and analyzed data, and drafted the manuscript. GLE, JMT, GCV, MBS, DLM, AKP, MST, and YI collected and analyzed data. DJB and JMvD helped design the study and provided study resources. MDJ, JDM, and TT helped design the study and collected and analyzed data. JLK helped design study, interpreted data, and drafted the manuscript. NKL designed the study, interpreted data, and drafted and revised manuscript.
Acknowledgments

This work was supported by the Glenn Foundation for Medical Research (DJB, JMvD, JLK, NKl), NIH grant AG041122 (JMvD, JLK, NKl), the Pritzker Foundation (NKl), and a generous gift from Robert and Arlene Kogod. The authors greatly appreciate the technical expertise and support of Tamara Pirtskhalava, Nathan W. Werneburg, Carolyn M. Roos, Anthony J. Croatt, Xuan-Mai Persson, and all of Mayo Clinic. We also acknowledge the support of the Metabolic Studies Core of the Minnesota Obesity Center (DK50456). Nathan K. LeBrasseur is the guarantor of this study, has full access to all data, and takes full responsibility for the work as a whole, including the study design, access to data, and the decision to submit and publish the manuscript. TT, AKP, JLK, and NKl have a financial interest related to this research; all other authors have no conflicts of interest to declare.
References

1. Ogden CL, Carroll MD, Kit BK, Flegal KM: Prevalence of childhood and adult obesity in the United States, 2011-2012. Jama 2014;311:806-814

2. Pereira MA, Kartashov AI, Ebbeling CB, Van Horn L, Slattery ML, Jacobs DR, Jr., Ludwig DS: Fast-food habits, weight gain, and insulin resistance (the CARDIA study): 15-year prospective analysis. Lancet 2005;365:36-42

3. Must A, Spadano J, Coakley EH, Field AE, Colditz G, Dietz WH: The disease burden associated with overweight and obesity. JAMA 1999;282:1523-1529

4. Whitmer RA, Gunderson EP, Barrett-Connor E, Quesenberry CP, Jr., Yaffe K: Obesity in middle age and future risk of dementia: a 27 year longitudinal population based study. BMJ 2005;330:1360

5. Fox CS, Massaro JM, Hoffmann U, Pou KM, Maurovich-Horvat P, Liu CY, Vasan RS, Murabito JM, Meigs JB, Cupples LA, D'Agostino RB, Sr., O'Donnell CJ: Abdominal visceral and subcutaneous adipose tissue compartments: association with metabolic risk factors in the Framingham Heart Study. Circulation 2007;116:39-48

6. Blair SN, Kohl HW, 3rd, Barlow CE, Paffenbarger RS, Jr., Gibbons LW, Macera CA: Changes in physical fitness and all-cause mortality. A prospective study of healthy and unhealthy men. JAMA 1995;273:1093-1098

7. Campisi J: Senescent cells, tumor suppression, and organismal aging: good citizens, bad neighbors. Cell 2005;120:513-522

8. Tchkonia T, Zhu Y, van Deursen J, Campisi J, Kirkland JL: Cellular senescence and the senescent secretory phenotype: therapeutic opportunities. J Clin Invest 2013;123:966-972
9. Palmer AK, Tchkonia T, LeBrasseur NK, Chini EN, Xu M, Kirkland JL: Cellular Senescence in Type 2 Diabetes: A Therapeutic Opportunity. Diabetes 2015;64:2289-2298

10. Collado M, Blasco MA, Serrano M: Cellular senescence in cancer and aging. Cell 2007;130:223-233

11. Coppe JP, Desprez PY, Krtolica A, Campisi J: The senescence-associated secretory phenotype: the dark side of tumor suppression. Annu Rev Pathol 2010;5:99-118

12. Tchkonia T, Morbeck DE, Von Zglinicki T, Van Deursen J, Lustgarten J, Scrable H, Khosla S, Jensen MD, Kirkland JL: Fat tissue, aging, and cellular senescence. Aging Cell 2010;9:667-684

13. Liu Y, Johnson SM, Fedoriw Y, Rogers AB, Yuan H, Krishnamurthy J, Sharpless NE: Expression of p16(INK4a) prevents cancer and promotes aging in lymphocytes. Blood 2011;117:3257-3267

14. Baker DJ, Wijshake T, Tchkonia T, LeBrasseur NK, Childs BG, van de Sluis B, Kirkland JL, van Deursen JM: Clearance of p16Ink4a-positive senescent cells delays ageing-associated disorders. Nature 2011;479:232-236

15. Zhu Y, Tchkonia T, Pirtskhalava T, Gower AC, Ding H, Girogadze N, Palmer AK, Ikeno Y, Hubbard GB, Lenburg M, O'Hara SP, LaRusso NF, Miller JD, Roos CM, Verzosa GC, LeBrasseur NK, Wren JD, Farr JN, Khosla S, Stout MB, McGowan SJ, Fuhrmann-Stroissnigg H, Gurkar AU, Zhao J, Colangelo D, Dorronsoro A, Ling YY, Barghouthy AS, Navarro DC, Sano T, Robbins PD, Niedernhofer LJ, Kirkland JL: The Achilles’ heel of senescent cells: from transcriptome to senolytic drugs. Aging Cell 2015;
16. Xu M, Tchkonia T, Ding H, Ogrodnik M, Lubbers ER, Pirtskhalava T, White TA, Johnson KO, Stout MB, Mezera V, Giorgadze N, Jensen MD, LeBrasseur NK, Kirkland JL: JAK inhibition alleviates the cellular senescence-associated secretory phenotype and frailty in old age. Proc Natl Acad Sci U S A 2015;112:E6301-6310

17. Miller RA, Austad S, Burke D, Chrisp C, Dysko R, Galecki A, Jackson A, Monnier V: Exotic mice as models for aging research: polemic and prospectus. Neurobiol Aging 1999;20:217-231

18. Charlton M, Krishnan A, Viker K, Sanderson S, Cazanave S, McConico A, Masuoko H, Gores G: Fast food diet mouse: novel small animal model of NASH with ballooning, progressive fibrosis, and high physiological fidelity to the human condition. American journal of physiology Gastrointestinal and liver physiology 2011;301:G825-834

19. Akasaki Y, Ouchi N, Izumiya Y, Bernardo BL, Lebrasseur NK, Walsh K: Glycolytic fast-twitch muscle fiber restoration counters adverse age-related changes in body composition and metabolism. Aging Cell 2013;

20. LeBrasseur NK, Schelhorn TM, Bernardo BL, Cosgrove PG, Loria PM, Brown TA: Myostatin inhibition enhances the effects of exercise on performance and metabolic outcomes in aged mice. J Gerontol A Biol Sci Med Sci 2009;64:940-948

21. Roos CM, Hagler M, Zhang B, Oehler EA, Arghami A, Miller JD: Transcriptional and phenotypic changes in aorta and aortic valve with aging and MnSOD deficiency in mice. Am J Physiol Heart Circ Physiol 2013;305:H1428-1439

22. Bernardo BL, Wachtmann TS, Cosgrove PG, Kuhn M, Opsahl AC, Judkins KM, Freeman TB, Hadcock JR, LeBrasseur NK: Postnatal PPARdelta activation and
myostatin inhibition exert distinct yet complimentary effects on the metabolic profile of obese insulin-resistant mice. PLoS One 2010;5:e11307

23. Chow LS, Mashek DG, Austin E, Eberly LE, Persson XM, Mashek MT, Seaquist ER, Jensen MD: Training status diverges muscle diacylglycerol accumulation during free fatty acid elevation. Am J Physiol Endocrinol Metab 2014;307:E124-131

24. Tonne JM, Sakuma T, Deeds MC, Munoz-Gomez M, Barry MA, Kudva YC, Ikeda Y: Global gene expression profiling of pancreatic islets in mice during streptozotocin-induced beta-cell damage and pancreatic Glp-1 gene therapy. Disease models & mechanisms 2013;6:1236-1245

25. Villaret A, Galitzky J, Decaunes P, Esteve D, Marques MA, Sengenes C, Chiotasso P, Tchkonia T, Lafontan M, Kirkland JL, Bouleumie A: Adipose tissue endothelial cells from obese human subjects: differences among depots in angiogenic, metabolic, and inflammatory gene expression and cellular senescence. Diabetes 2010;59:2755-2763

26. Guilherme A, Virbasius JV, Puri V, Czech MP: Adipocyte dysfunctions linking obesity to insulin resistance and type 2 diabetes. Nat Rev Mol Cell Biol 2008;9:367-377

27. Haus JM, Kashyap SR, Kasumov T, Zhang R, Kelly KR, Defronzo RA, Kirwan JP: Plasma ceramides are elevated in obese subjects with type 2 diabetes and correlate with the severity of insulin resistance. Diabetes 2009;58:337-343

28. Fromenty B, Pessayre D: Inhibition of mitochondrial beta-oxidation as a mechanism of hepatotoxicity. Pharmacology & therapeutics 1995;67:101-154
29. Escande C, Nin V, Pirtskhalava T, Chini CC, Thereza Barbosa M, Mathison A, Urrutia R, Tchkonia T, Kirkland JL, Chini EN: Deleted in Breast Cancer 1 regulates cellular senescence during obesity. Aging Cell 2014;13:951-953

30. Acosta JC, Banito A, Wuestefeld T, Georgilis A, Janich P, Morton JP, Athineos D, Kang TW, Lasitschka F, Andrulis M, Pascual G, Morris KJ, Khan S, Jin H, Dharmalingam G, Snijders AP, Carroll T, Capper D, Pritchard C, Inman GJ, Longerich T, Sansom OJ, Benitah SA, Zender L, Gil J: A complex secretory program orchestrated by the inflammasome controls paracrine senescence. Nat Cell Biol 2013;15:978-990

31. Freund A, Orjalo AV, Desprez PY, Campisi J: Inflammatory networks during cellular senescence: causes and consequences. Trends Mol Med 2010;16:238-246

32. Escande C, Nin V, Pirtskhalava T, Chini CC, Thereza Barbosa M, Mathison A, Urrutia R, Tchkonia T, Kirkland JL, Chini EN: Deleted in Breast Cancer 1 regulates cellular senescence during obesity. Aging Cell 2014;

33. Ma LJ, Mao SL, Taylor KL, Kanjanabuch T, Guan Y, Zhang Y, Brown NJ, Swift LL, McGuinness OP, Wasserman DH, Vaughan DE, Fogo AB: Prevention of obesity and insulin resistance in mice lacking plasminogen activator inhibitor 1. Diabetes 2004;53:336-346

34. Yu R, Kim CS, Kwon BS, Kawada T: Mesenteric adipose tissue-derived monocyte chemoattractant protein-1 plays a crucial role in adipose tissue macrophage migration and activation in obese mice. Obesity (Silver Spring) 2006;14:1353-1362

35. Weisberg SP, Hunter D, Huber R, Lemieux J, Slaymaker S, Vaddi K, Charo I, Leibel RL, Ferrante AW, Jr.: CCR2 modulates inflammatory and metabolic effects of high-fat feeding. The Journal of clinical investigation 2006;116:115-124
36. Minamino T, Orimo M, Shimizu I, Kunieda T, Yokoyama M, Ito T, Nojima A, Nabetani A, Oike Y, Matsubara H, Ishikawa F, Komuro I: A crucial role for adipose tissue p53 in the regulation of insulin resistance. Nat Med 2009;15:1082-1087

37. Sorrentino JA, Krishnamurthy J, Tilley S, Alb JG, Jr., Burd CE, Sharpless NE: p16INK4a reporter mice reveal age-promoting effects of environmental toxicants. J Clin Invest 2014;124:169-173

38. Wang CY, Kim HH, Hiroi Y, Sawada N, Salomone S, Benjamin LE, Walsh K, Moskowitz MA, Liao JK: Obesity increases vascular senescence and susceptibility to ischemic injury through chronic activation of Akt and mTOR. Sci Signal 2009;2:ra11

39. Zhang X, Zhou D, Strakovsky R, Zhang Y, Pan YX: Hepatic cellular senescence pathway genes are induced through histone modifications in a diet-induced obese rat model. Am J Physiol Gastrointest Liver Physiol 2012;302:G558-564

40. Sone H, Kagawa Y: Pancreatic beta cell senescence contributes to the pathogenesis of type 2 diabetes in high-fat diet-induced diabetic mice. Diabetologia 2005;48:58-67

41. Michaloglou C, Vredeveld LC, Soengas MS, Denoyelle C, Kuilman T, van der Horst CM, Majoor DM, Shay JW, Mooi WJ, Peeper DS: BRAFE600-associated senescence-like cell cycle arrest of human naevi. Nature 2005;436:720-724

42. Xue W, Zender L, Miething C, Dickins RA, Hernando E, Krizhanovsky V, Cordon-Cardo C, Lowe SW: Senescence and tumour clearance is triggered by p53 restoration in murine liver carcinomas. Nature 2007;445:656-660

43. Radak Z, Naito H, Kaneko T, Tahara S, Nakamoto H, Takahashi R, Cardozo-Pelaez F, Goto S: Exercise training decreases DNA damage and increases DNA repair and
resistance against oxidative stress of proteins in aged rat skeletal muscle. Pflugers Arch 2002;445:273-278

44. Werner C, Hanhoun M, Widmann T, Kazakov A, Semenov A, Poss J, Bauersachs J, Thum T, Pfrenndschuh M, Muller P, Haendeler J, Bohm M, Laufs U: Effects of physical exercise on myocardial telomere-regulating proteins, survival pathways, and apoptosis. J Am Coll Cardiol 2008;52:470-482

45. Ji LL: Exercise-induced modulation of antioxidant defense. Ann N Y Acad Sci 2002;959:82-92

46. He C, Bassik MC, Moresi V, Sun K, Wei Y, Zou Z, An Z, Loh J, Fisher J, Sun Q, Korsmeyer S, Packer M, May HI, Hill JA, Virgin HW, Gilpin C, Xiao G, Bassel-Duby R, Scherer PE, Levine B: Exercise-induced BCL2-regulated autophagy is required for muscle glucose homeostasis. Nature 2012;481:511-515

47. Safdar A, Bourgeois JM, Ogborn DI, Little JP, Hettinga BP, Akhtar M, Thompson JE, Melov S, Mocellin NJ, Kujoth GC, Prolla TA, Tarnopolsky MA: Endurance exercise rescues progeroid aging and induces systemic mitochondrial rejuvenation in mtDNA mutator mice. Proceedings of the National Academy of Sciences 2011;108:4135-4140

48. Masoro EJ: Caloric restriction and aging: an update. Exp Gerontol 2000;35:299-305

49. Muzumdar R, Allison DB, Huffman DM, Ma X, Atzmon G, Einstein FH, Fishman S, Poduval AD, McVei T, Keith SW, Barzilai N: Visceral adipose tissue modulates mammalian longevity. Aging Cell 2008;7:438-440
50. Blüher M, Kahn BB, Kahn CR: Extended Longevity in Mice Lacking the Insulin Receptor in Adipose Tissue. Science 2003;299:572-574

51. Thorand B, Baumert J, Kolb H, Meisinger C, Chambless L, Koenig W, Herder C: Sex differences in the prediction of type 2 diabetes by inflammatory markers: results from the MONICA/KORA Augsburg case-cohort study, 1984-2002. Diabetes Care 2007;30:854-860
Figure Legends

Figure 1. Nutrient excess and exercise exert opposing effects on body composition and physical endurance. Compared to mice fed a normal diet (ND), mice fed a fast food diet (FFD) for 16 weeks exhibited marked gains in body weight (A) and fat mass as determined by quantitative magnetic resonance (B). FFD-induced obesity was further evident in volumes of visceral (pink) and subcutaneous (grey) fat in the lumbar region of mice as assessed by computed tomography (C). Exercised mice receiving either ND or FFD exhibited significantly greater distances run to exhaustion on a treadmill than sedentary mice on either diet (D). For all analyses, n = 6-7 mice/group; *, **, and *** denote $p < 0.05$, 0.01, and 0.001, respectively.

Figure 2. Diet-induced deterioration of metabolic health is attenuated by exercise. Compared to sedentary and exercised mice on normal diet, sedentary FFD-fed mice exhibited significantly higher circulating insulin concentrations (A). Correspondingly, cross-sections of the pancreata of sedentary FFD-fed mice exhibited markedly greater beta cell masses (B,C) and insulin positive areas (representative images, (B)). Remarkably, these features of insulin resistance in FFD-fed mice were abrogated by exercise (A-C). Compromised and improved insulin actions in sedentary and exercised FFD-fed groups, respectively, were apparent in a glucose tolerance test. Namely, the higher peak and greater excursions in glucose concentrations observed in sedentary FFD-fed mice relative to ND-fed mice during the measure were erased by exercise (D). For all analyses, n = 6-7 mice/group; *, **, and *** denote $p < 0.05$, 0.01, and 0.001, respectively.
Figure 3. The effects of diet and exercise on senescence markers in multiple tissues.

To determine the extent to which nutrient excess and exercise affected cellular senescence in middle-aged mice, we compared the expression of p16 (A), p53 (B), and p21 (C) by qPCR in multiple tissues, including visceral (Vis) fat, subcutaneous (SQ) fat, liver, gastrocnemius (gastroc), pancreas (panc), kidney, heart, and aorta. For all analyses, n = 6-7 mice/group; *, **, and *** denote $p < 0.05, 0.01,$ and 0.001, respectively.

Figure 4. Exercise prevents diet-induced cellular senescence and the SASP within visceral adipose tissue. Compared to ND, the FFD caused a marked increase in the activity of the senescence-associated p16$\text{^{INK4a}}$ promoter, as measured by EGFP expression (A). The abundance of SA-β-gal positive cells in harvested visceral adipose tissue further validated the pro- and anti-senescent effects of nutrient excess and exercise, respectively (representative images (B) and summary data (C)). The expression of SASP and inflammatory factors was also increased in response to FFD, and these increases were attenuated by exercise (D). For all analyses, n = 6-7 mice/group; *, **, and *** denote $p < 0.05, 0.01,$ and 0.001, respectively.

Figure 5. Exercise initiated after long-term FFD feeding improves physical parameters and attenuates markers of visceral adipose senescence. Following 16 weeks of FFD feeding and sedentary lifestyle, exercise “treatment” in the form of voluntary running wheels for a subsequent 14 weeks (FFD→FFD+Exercise) lead to reductions in total body weight (A) and fat mass (B). Computed tomography assessment
of the lumbar region at endpoint revealed that exercise-mediated reductions corresponded to both visceral (pink) and subcutaneous (grey) adipose depots (C). Exercise capacity determined by treadmill testing, dramatically increased in FFD-fed mice switched from sedentary to running wheel exercise, relative to both ND- and FFD-sedentary groups (D). qPCR was used to compare expression of senescence induction factors p16, p53, and p21 (E) and SASP and inflammatory factors Pai1, Mcp1, and CD68 (F). For all analyses, n = 5-7 mice/group; * and ** denote $p < 0.05$ and 0.01, respectively.
Figure 1. Nutrient excess and exercise exert opposing effects on body composition and physical endurance.

139x108mm (300 x 300 DPI)
Figure 2. Diet-induced deterioration of metabolic health is attenuated by exercise.
Figure 3. The effects of diet and exercise on senescence markers in multiple tissues.
Figure 4. Exercise prevents diet-induced cellular senescence and the SASP within visceral adipose tissue.
Figure 5. Exercise initiated after long-term FFD feeding improves physical parameters and attenuates markers of visceral adipose senescence.

213x252mm (300 x 300 DPI)
Supplementary Figure 1. Daily energy intake and running distance in experimental mice.
65x24mm (300 x 300 DPI)
Supplementary Figure 2. Exercise diminishes diet-induced adipose accumulation.

66x24mm (300 x 300 DPI)
Supplementary Figure 3. Exercise confers benefits upon cardiac health even in the context of nutrient excess.

115x74mm (300 x 300 DPI)
Supplementary Figure 4. The effects of nutrient excess and exercise on liver composition and pathology.

171x163mm (300 x 300 DPI)