EFFECT OF CURCUMIN

ON HEPATIC SENESCENCE IN AGED MICE

By

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Abstract: Aging is a physiological decrease in several biological activities of organs that all organisms go through. The gradual deterioration of cell functioning due to damage accumulation in metabolic organs accelerates biological aging. Recently, dietary interventions with bioactive compounds have been linked to suppressing the accumulation of senescent cells and senescence-associated secretory phenotype (SASP). Curcumin has potent biochemical and biological activities, including antioxidant and anti-inflammatory actions. However, it remains largely unclear how curcumin exhibits its anti-aging properties such as protection from DNA damage and cell survival/cell fate decisions. The objective of this study was to examine the regulatory effect of dietary curcumin on hepatic cellular senescence in an aged mouse model. Aged (18-20 months old) male C57BL/6 mice were fed a normal chow diet (NCD) or NCD containing 0.4% (w/w) curcumin (NCD+CUR), high-fat high sugar diet (HFHSD) or an HFHSD+CUR (N=7-9 per group) for 8 or 15 weeks. Mice given HFHSD+CUR displayed a different metabolic phenotype compared to mice given an HFHSD alone. To examine the phenotypic plasticity led by transcriptomic alteration, RNA-Seq was used and analyzed differential gene expression using Gene Ontology (GO) terms and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis. There were 1687 and 3794 genes that showed a significant change with curcumin in NCD and HFHSD groups compared to their respective control groups. Curcumin supplementation altered hepatic gene expression profiling, especially in senescence pathways and inflammatory pathways. Then this study mechanistically sought how curcumin regulates the hepatic senescence pathway. It has been found that curcumin supplementation decreased senescence effectors, specifically p38 protein expression levels in the liver. Also, we observed that curcumin modulates pro-inflammatory pathways: *Cxcl2*, *Cxcl10*, *FoxO3,* and *IL-6* genes and NF-*k*B, p65, protein expression levels. By following the results, this study suggests that the multifaceted therapeutic potential of curcumin can be used as a protective agent for age-induced metabolic diseases.

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CHAPTER I

INTRODUCTION

Aging in the United States

The population of elderly people is continuously increasing. This is shown in the demographic profile in the United States, of which 14% are elderly (Chiu & Pinto, 2018). The world's population of people aged 60 years and older will double to 2.1 billion by 2050, according to the World Health Organization (WHO) (Michel et al., 2021). These demographic profile changes have brought improved life expectancy, which has increased to 78 years. With the increasing age of the baby boomer generation, driving the first wave of this demographic shift, the estimation of older adults in the United States in 2030 will reach around 74 million (Chiu & Pinto, 2018).

Diabetes in the United States

Diabetes is a prevalent disease in the United States and one of the leading causes of death. As of 2020, 34.2 million people in the US, 10.5% of the population, have diabetes. Among these, 25% of the elderly population, ages more than 65 years old, has diabetes. Taking a large portion of the elderly population is a significant driver of the diabetes epidemic (Control & Prevention, 2011). Beyond that, 88 million American

adults, 34.5% of the population, have prediabetes (Association, 2021). Along with the negative health outcomes associated with diabetes, it also results in 2.3 times increase in healthcare costs for Americans with diabetes. This adds up to 327 billion dollars spent annually on diabetes-associated medical costs, including indirect costs such as reduced work productivity and absenteeism (Care, 2018). Although this burden is in terms of its impact on working-age people, diabetes in older people has higher mortality and their functional status is reduced with increased risk of hospitalization (California Healthcare Foundation/American Geriatrics Society Panel in Improving Care for Elders with Diabetes, 2003). Diabetes in older people has a higher risk than in working-aged people due to the acute and chronic complications driven by the disease.

Background of the study

Cellular senescence refers to a stable and mostly permanent cell cycle arrest characterized by the accumulation of severe cellular damage (Campisi & d'Adda di Fagagna, 2007; López-Otín et al., 2013). It has emerged as an important hallmark of aging and age-associated diseases such as obesity, type 2 diabetes, cardiovascular disease, and cancer because senescent cells can accumulate exponentially in aged tissues (Childs et al., 2015; Muñoz-Espín & Serrano, 2014; Paez-Ribes et al., 2019). These cells secrete proinflammatory cytokines, chemokines, and growth factors that are hallmarks of the senescence-associated secretory phenotypes (SASPs), which affect tissue structure and functions, inhibit the proper functioning of the immune system, and can cause systemic inflammation (Van Deursen, 2014). Moreover, recent studies have implicated that mitogen-activated protein kinases (MAPK) pathways play a key role in the development of senescence traits by suppressing growth, increasing apoptosis resistance, and regulating SASP

(Anerillas et al., 2020; Kumari & Jat, 2021). Thus, targeting senescent cells is a promising strategy to alleviate or prevent aging and age-related diseases.

As part of this approach, researchers have described chemicals called ‘senolytics’ that cause the selective clearance of senescent cells by inducing apoptosis (Kirkland & Tchkonia, 2017). Recent studies suggest that analytics can improve physical functions and extend healthy lifespans as well as attenuate various age-related chronic disorders in aged animal models (Lee et al., 2022; Palmer et al., 2019; Roos et al., 2016; Xu et al., 2018; Zhang et al., 2019). In some cases, these agents are derived from natural food resources, and consequently have low toxicities (Li, Qin, et al., 2019).

Curcumin is a bioactive polyphenolic compound extracted from the herb *Curcuma longa* (Sundar Dhilip Kumar et al., 2018). It has antioxidant, anti-inflammatory, and anti-diabetic properties which may mitigate age-associated diseases (Kotha & Luthria, 2019; Shen et al., 2013; Sundar Dhilip Kumar et al., 2018). However, it is still unknown whether curcumin can prevent or delay cellular senescence in the aged mouse liver. To fill this scientific gap, this study was to determine if curcumin supplementation in naturally aged mice protects hepatic senescence.

Purpose of this study

The main <u>purpose</u> of this study is to find a candidate for senolytic among food-bioactive compounds that contribute to preventing age-associated metabolic diseases.

The <u>objective</u> of this study is to examine the regulatory effect of dietary curcumin on hepatic cellular senescence in an aged mouse model.

Specific aims

The hypothesis will be tested by accomplishing the following aims:

Specific aim 1: To evaluate the effect of curcumin on obesity prevention.

Specific aim 2: To analyze the effect of curcumin on hepatic senescence and its molecular mechanism.

Specific aim 3: To assess the effect of curcumin on maintaining insulin homeostasis.

Research hypothesis

Based on this conceptualization, this study hypothesizes that curcumin may provide a senolytic-mimicking effect by regulating aging-related pathways in the liver due to its pleiotropic traits.

Limitations of this study

There are some limitations in this study. Considering that dietary curcumin has low aqueous solubility, and poor oral bioavailability (Anand et al., 2007), supplementation of curcumin by incorporating it into diets may result in less biological efficacy in preventing age-associated declines in hepatic senescence. However, the current study was focused on the effect of dietary curcumin on hepatic senescence**.** Even under these limitations, significant effects were found with the incorporation of curcumin directly into the diets. Other curcumin formulations might have resulted in even greater beneficial effects. Additionally, this has not yet been studied if these effects

are specific to hepatocytes or if they occur in other metabolic organs such as skeletal muscle and white adipose tissue where reduced cellular senescence is well-described from epidemiological studies to be a feature of age-associated physiological decline.

CHAPTER II

REVIEW OF LITERATURE

Pathophysiological perspective: Aging

Aging is defined as time-dependent functional decline, which is inevitable for most living organisms. From a pathophysiological perspective, the most prominent trait of aging is a gradual loss of function that occurs at the cellular and molecular level. This impaired function is the primary risk factor for major age-associated diseases such as diabetes, cancer, cardiovascular disorders, and neurodegenerative diseases.

At the cellular level, the hallmarks of aging have been attempted to be identified and categorized at both the cellular and molecular aspect **(Table 1)** (López-Otín et al., 2013). These hallmarks and the pathogenic mechanisms for age-associated diseases have a lot of overlapped similarities. Due to these similarities, recent studies suggest that targeting one of these aging hallmarks – cellular senescence – can significantly ameliorate age-associate disease and extend healthy lifespan (Campisi & d'Adda di Fagagna, 2007; Childs et al., 2015; Ho et al., 2020; Paez-Ribes et al., 2019; Palmer et al., 2019).

Table 1. The Hallmarks of Aging

The Hallmarks of aging	**Factors**
Genomic instability	Nuclear DNA, mitochondrial DNA, nuclear architecture
Telomere attrition	Replicative senescence (Hayflick limit)
Epigenetic alterations	Histone modifications, DNA methylation, chromatin remodeling, transcriptional alteration, reversion of epigenetic changes
Loss of proteostasis	Chaperone-mediated protein folding and stability, proteolytic systems
Deregulated nutrient sensing	The insulin- and IGF-1 signaling pathway, other nutrient-sensing systems: mTOR, AMPK, and Sirtuins
Mitochondrial dysfunction	Reactive oxygen species, mitochondrial integrity, biogenesis, mitohormesis
Stem cell exhaustion	The loss of stem cell quiescence, stem cell depletion,
Altered intercellular communication	Inflammation, the interorgan coordination of the aging phenotype
Cellular senescence	Senescence-associated secretory phenotype (SASP)

Cellular senescence

One of the hallmarks of aging is cellular senescence **(Table 1)**, a stress response resulting in the stable arrest of the cell cycle (Ellison-Hughes, 2020). At some point, cellular senescence was noted to function by blocking the propagation of damaged cells to maintain tissue homeostasis. However, when the body cannot replenish lost cells, cellular senescence accelerates, resulting in accelerated aging (López-Otín et al., 2013). Recent studies have asserted that the elimination of senescent cells can reduce the risk of age-dependent deterioration in tissues and organs.

There are several main features of senescent cells: (a) arrested growth with enlarged cell morphology, (b) increased cell-cycle inhibitors (p21, p16 tumor suppressor protein), (c) DNA damage response (DDR) by the stress-responsive p38-MAPK signaling pathway, (d) senescence-associated secretory phenotype (SASP). Other than those main features, one of the biomarkers of senescent cells for detection is senescence-associated β-galactosidase (SA-β-gal) activity. SA-β-gal is a common histochemical staining technique used as a marker of senescent cells. The acidic lysosomal β-galactosidase in senescent cells drives this activity and it is overexpressed in near-neutral pH, which dyes the senescent cells a distinct blue color (Dimri et al., 1995). SA-β-gal was the first marker developed that allowed the detection of senescent cells in a tissue sample, in the study that indicated that senescent cells increase with age in vivo (Dimri et al., 1995).

One critical role of senescent cells is the secretion of various kinds of proteins – interleukins, chemokines, and other pro-inflammatory cytokines, proteases, metalloproteinases, and growth factors (Coppé et al., 2010; Freund et al., 2010). This phenomenon, known as SASP, is involved in many processes such as inflammation, angiogenesis, extracellular matrix reorganization, proliferation stimulation, and immune system modulation. Depending on the physiological

context, this can either be beneficial or detrimental. SASP can act both in a paracrine and autocrine manner by promoting low-grade inflammation either directly or indirectly (Campisi & d'Adda di Fagagna, 2007; Kuilman et al., 2010). Senescent cells can also induce a “Bystander effect” in which they secrete proteins that promote senescence in adjacent cells. This eventually causes local chronic inflammation that can lead to systemic inflammation (Chen et al., 2003; Grigg & Sonnenberg, 2017; Turner et al., 2016).

Hepatic senescence

The liver is a vital metabolic organ for maintaining whole-body homeostasis by regulating energy metabolism and molecular biosynthesis (Rui, 2014). Thus, age-related changes in liver function increase the chances of age-associated diseases systemically. Systemic energy metabolism is regulated by the liver through hepatic glucose and lipid homeostasis, steroid biosynthesis and degradation, and insulin signaling (Rui, 2014). For this reason, the liver is the main metabolic organ to mediate nutritional statuses such as protein restriction and calorie restriction (CR) during aging and age-associated disease (Meydani, 2001; Roth et al., 2007). In addition to that, maintaining homeostasis in hepatic energy metabolism is important because dysregulation can increase age-related conditions and aging-associated diseases: insulin resistance, diabetes mellitus, and non-alcoholic fatty liver disease (NAFLD) (Bonomini et al., 2015; Kim et al., 2015). Four major liver cells are hepatocytes, Kupffer cells, hepatic stellate cells, and liver sinusoidal endothelial cells. The function of these cells is directly affected by aging hallmarks **(Table 1)**. These liver cells have been thoroughly studied in a cellular aging model (Hunt et al., 2019) **(Figure 1).**

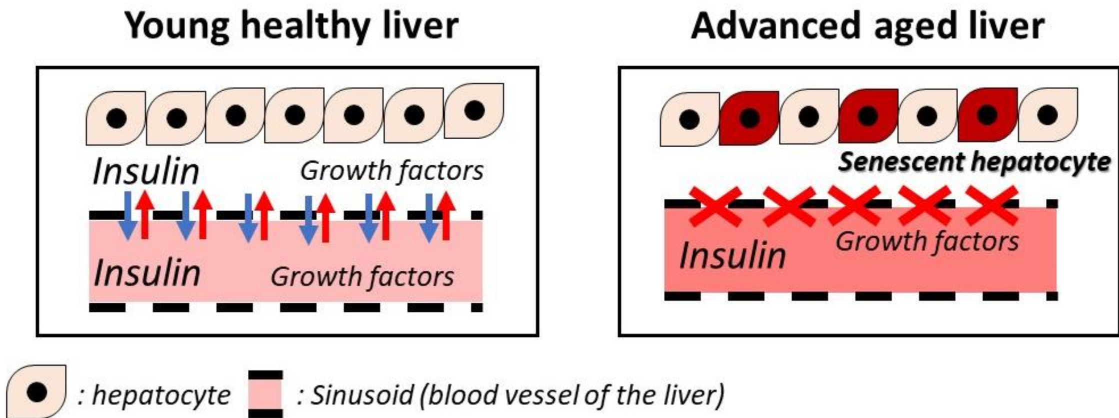

Figure 1. The aging liver

The biological function and mechanism of cellular senescence in liver disease are still not fully elucidated in detailed mechanisms and their biological function. However, recent studies have made meaningful progress, indicating that senescence can have adverse effects on the liver including cellular viability, and tissue regeneration with pathological circumstances (Ferreira-Gonzalez et al., 2018). Various genetically modified mouse models were developed to study senescence-associated liver disease including p16$^{INK4A-3MR}$mice, AhCre^{+}Mdm2$^{fl/fl}$mice, and senescence-accelerated mouse-prone8 (SAMP8). These mouse models were used to determine certain markers (p16, Mouse double minute 2 homolog (MDM2), p53, p21) of senescent cells *in vivo* (Bird et al., 2018; Clouston et al., 2005; Demaria et al., 2014; Liu et al., 2008).

Senolytics

Senolytics are molecules that selectively kill senescent cells without depending on transgenes. The term “senolytic” comes from the words “senescence” and “lytic”, a suffix meaning to destroy. Senolytics selectively terminate senescent cells by destroying their defenses and exposing them to an apoptotic environment (Hickson et al., 2019). Quercetin and Dasatinib,

two tyrosine kinase inhibitors, are senolytic candidates (Thoppil & Riabowol, 2020). Other natural compounds are receiving attention as potentially effective senolytic agents, such as quercetin (Justice et al., 2019; Zhu et al., 2017), fisetin (Yousefzadeh et al., 2018; Zhu et al., 2017), piperlongumine (Wang et al., 2016; Zhang et al., 2018) and curcumin analog (Li, He, et al., 2019). These natural senolytic agents have less toxicity than targeted senolytic because they originate from natural sources such as foods and other natural compounds. They have promising clinical applications in treatment and developing more potent and specific senolytic drugs (Li, Qin, et al., 2019).

Nutritional status in aging

Nutritional status is associated with age-related changes. Because oxidative stress and free radicals have been recognized to have roles in the aging process, therefore dietary interventions with antioxidants have gained a lot of attention for their potential anti-aging effects. Calorie restriction is another dietary intervention that can modulate oxidative stress and ultimately slow the aging process (Meydani, 2001). Dietary supplements are generally accepted as important and safe forms of preventative medicine, but they can also be utilized to slow down or sometimes reverse age-associated physiological and biochemical changes resulting from aging. Examples of potential supplements for anti-aging are vitamin C, vitamin E, coenzyme Q10, alpha lipoic acid, chromium, L-carnitine, and quercetin (Janson, 2006). The intake of supplements and antioxidant vitamins has also been found to reduce the risk of age-associated diseases, like some cancers, cataracts, cognitive impairment, and cardiovascular disease. The cognitive decline observed in aging is closely related to oxidative stress, so dietary antioxidants

may have a preventive effect on vascular dementia, stroke, and Alzheimer's disease (Roth et al., 2007). Specifically, the curcumin analog EF24 was found to be a potent and broad-spectrum senolytic agent (Li, He, et al., 2019).

Curcumin and its anti-aging effects

Curcumin is a bioactive compound that is acquired from the *Curcuma longa* (Tumeric) rhizomes, the main stem of the plant. It belongs to the *Zingiberaceae* family and is mainly cultivated in Southeast Asia and India (Amalraj et al., 2017; Nebrisi, 2021). Curcumin has a bright yellow color that has resulted in its broad use as a food additive, dietary spice, and herbal remedy across Asia. Several studies have shown that curcumin is a potent nutraceutical product due to its possession of biochemical and biological activities such as anti-inflammatory, antioxidant, anti-diabetic, and anti-aging activities.

Aging is characterized by chronic low-grade inflammation (Calder et al., 2017). This implies that polyphenol-rich foods, which possess anti-inflammatory and antioxidant functions, can alleviate pathophysiological traits of aging. Several studies have indicated that curcumin has an anti-aging role due to its anti-inflammatory and antioxidant properties (Li, He, et al., 2019; Salvioli et al., 2007; Sandur et al., 2007; E Sikora et al., 2010; Ewa Sikora et al., 2010). Recently, a vast number of clinical trials enumerated the therapeutic function of curcumin against age-related chronic disorders such as metabolic, neurological, cardiovascular, and pulmonary diseases (Bielak-Zmijewska et al., 2019; Hatcher et al., 2008; Kotha & Luthria, 2019; Rahmani et al., 2018). Epidemiological studies in India revealed that long-term curcumin supplementation substantially lowered the incidence rate of neurodegenerative disease cases.

Another epidemiological study that curcumin consumption markedly reduced (4.4 times) the prevalence of Alzheimer's disease (AD) in India compared to the United States (Ganguli et al., 2000; Ng et al., 2006). Curcumin is a good candidate for a senolytic agent because it is readily available, easy to consume, cost-effective, and safe.

Along with these results, some data suggest that the anti-aging function of curcumin is the result of its ability to regulate cellular senescence. However, it is still unknown whether curcumin has an effect to postpone cellular senescence in the aged mouse liver.

Age-associated metabolic disease: Type 2 diabetes (T2D)

T2D is an increasing worldwide problem and represents over 90 percent of diabetes diagnoses (Kalra & Sharma, 2018). T2D can lead to the development of many complications. This prevalent disorder is caused by an insulin deficiency in individuals resulting from β-cell dysfunction and insulin resistance in specific organs including the liver (Chatterjee et al., 2017; Tokarz et al., 2018). The core pathophysiology of T2D is still not completely understood, but it is thought to be the result of increased concentration of fatty acid metabolite concentration, which can cause serine kinase cascades. Ultimately, this leads to insulin signaling deficiencies downstream of the insulin receptor (Liu et al., 2009; Saini, 2010). Like Type 1 Diabetes, T2D is affected by genetic changes in specific environments, such as obesogenic environments (Araki & Ito, 2009; Chatterjee et al., 2017; Shepherd et al., 2009).

T2D is different from Type 1 Diabetes in that it occurs at an increased rate in older individuals. Currently, 18-30% of the United States elderly population has T2D (Kalra & Sharma, 2018; Kirkman et al., 2012). This is believed to have been caused by increased insulin

resistance resulting from increased adiposity, decreased lean muscle mass, and reduced physical activity. These factors are all associated with aging (Araki & Ito, 2009; Kalyani et al., 2020). Other studies have attempted to identify a relationship between age and insulin resistance, plasma glucose clearance, and beta cell proliferation yet they have been inconclusive (Bouwens & Rooman, 2005; Kahn et al., 2006). Currently, it is known that there is a reduction in β-cell proliferation related to aging caused by reduced cell cycle activators and increased cell cycle inhibitors (Bouwens & Rooman, 2005; Gunasekaran & Gannon, 2011).

CHAPTER III

METHODS

Animals and treatment

All animal procedures were performed in an American Association for Accreditation of Laboratory Animal Care accredited facility and the procedures were approved by the Animal Care and Use Committee of the NIA Intramural Program and Oklahoma State University Institutional Animal Care and Use Committee (IACUC). Aged male C57BL/6 mice (18-20 months old) were obtained from the National Institute on Aging (NIA) Aged Rodent Colony housed at Charles River Laboratories (Frederick, MD). For 15 weeks treated mice, it was transferred into the NIA intramural housing facility (Baltimore, MD) and animals were acclimated to the facility for 1 week with standard NIH chow (Teklad Global Rodent Diet, Envigo, Indianapolis, IN). For 8 weeks treated mice, it was transferred to the Oklahoma State University Animal Resources (Stillwater, OK) and animals were acclimated to the facility for 3 weeks. Animals were subject to baseline assessments such as body weight and body composition, then blood samples were collected for analyses of plasma markers. After baseline assessment, they were randomized into four groups: a normal chow diet (NCD; n = 9), a normal chow diet containing 0.4% (w/w) of curcumin (NCD+CUR; n = 9) a high fat/high sugar diet (HFHSD; n = 8) or an HFHSD

Table 2. Formulation of Diet

Ingredients	NCD (per kg)	NCD+CUR (per kg)	HFHSD (per kg)	HFHSD+CUR (per kg)
Curcumin (CUR)	0	4	0	4
Carbohydrates				
Cornstarch (g)	398.562	394.562	310.462	306.562
Dyetrose (g)	132	132	50	50
Sucrose (g)	90	90	90	90
Total (g)	620.562	616.562	450.462	446.562
Protein				
High Nitrogen Casein (g)	200	200	200	200
Total (g)	200	200	200	200
Fat				
Whole Butter (18% Water)	0	0	181	181
Soybean Oil	70	70	70	70
Lard	0	0	20	20
Total (g)	70	70	271	271
Fiber				
Cellulose	50	50	50	50
Total (g)	50	50	50	50
Vitamin mix (g)	10	10	10	10
Mineral mix (g)	35	35	35	35
Supplement (g)	10	10	10	10
Choline Chloride (g)	1.4	1.4	1.4	1.4
L-Cystine (g)	3	3	3	3
Ethoxyquin (g)	0.024	0.024	0.024	0.024
Red Dye (g)	0	0	0.1	0

containing 0.4% (w/w) of curcumin (HFHSD+CUR; n = 7) for 15 weeks. Curcumin for diets was purchased from Sigma-Aldrich (St. Louis, MO) and all the diets were formulated by Dyets Inc. (Bethlehem, PA) **(Table 2)**. The dose of curcumin in this study was determined based on our previous study using middle-aged mice (Kim et al., 2019). This amount of curcumin is equivalent to 2 g/day for a 60 kg adult calculated with an equivalent surface area dosage conversion method (Freireich et al., 1966). Mice were allowed *ad libitum* access to food and water throughout the study. The average body weight and food consumption were calculated weekly for 15 weeks.

Insulin tolerance test and Glucose tolerance test

For the insulin tolerance test (ITT), blood glucose levels were measured from tail veins of 6 hours-fasted mice at 0, 15, 30, 60, 90, and 120 minutes following intraperitoneal administration of recombinant human insulin from Novo Nordisk (1 U insulin/kg body weight). For the glucose tolerance test (GTT), blood glucose levels were measured from the tail veins of 16 hours-fasted mice at 0, 15, 30, 60, 90, and 120 minutes following intraperitoneal administration of 50% (w/w) glucose solution (Alpha Teknova, Hollister, CA). Blood glucose levels were measured using a hand-held glucometer (Contour, Bayer, model 7160-P). Blood plasma was used to measure insulin levels using an Ultra-Sensitive Mouse Insulin ELISA kit (Crystal Chemical Inc., Downers Grove, IL).

Table 3. Primer Sequence List for qRT-PCR

Target gene	Gene ID	5' – 3'	Sequence
Cxcl10	15945	Forward Reverse	5'-CGC TGC AAC TGC ATC CAT ATC G -3' 5'-CCG GAT TCA GAC ATC TCT GCT C -3'
IL-6	16193	Forward Reverse	5'-TGA GAA AAG AGT TGT GCA ATG G-3' 5'-GGT ACT CCA GAA GAC CAG AGG -3'
Cxcl2	20310	Forward Reverse	5'-CCC AGA CAG AAG TCA TAG CCA C-3' 5'-TGG TTC TTC CGT TGA GGG AC-3'
FoxO3	56484	Forward Reverse	5'-CTCCATCCGGCACAACCT-3' 5'-TTGCCCGTCCCTTCATTC-3'
18s Ribosomal RNA	19791	Forward Reverse	5'-AGT CCC TGC CCT TTG TAC ACA -3' 5'-CGA TCC GAG GGC CTC ACT A -3'

RT-PCR, reverse transcription polymerase chain reaction; Cxcl, chemokine (C-X-C motif) ligand; IL, interleukin; FoxO3, forkhead box O3.

Real-time reverse-transcription polymerase chain reaction (RT-PCR)

Total RNA was extracted from individual frozen tissue samples using TRIzol Rea-gent (Invitrogen; Thermo Fisher Scientific) and quantified using a NanoDrop OneC Microvolume UV-Vis Spectrophotometer (Thermo Fisher Scientific). The normalized RNA was reverse transcribed with an iScript™ Reverse Transcription Supermix for RT-qPCR (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Gene expression was assessed using Power SYBR® Green Master Mix (Applied Biosystems; Thermo Fisher Scientific) on a CFX Opus 384 Real-Time PCR System (Bio-Rad Laboratories, Inc.) with the following thermal cycling conditions: 95 °C for 10 min, followed by 39 cycles of 95 °C for 15 sec and 60°C for 1 min. The fluorescence cycle threshold value (Ct) data were normalized to 18S. The primer sequences are displayed in **Table 3**.

Immunoblotting analysis

Mouse liver tissue samples were homogenized in tissue lysis buffer (25 mM Tris (pH 7.4), 2 mM Na_3VO_4, 10 mM NaF, 10 mM $Na_4P_2O_7$, 1 mM EGTA, 1 mM EDTA, and 1% NP-40) containing phosphatase and protease inhibitor cocktails in the OMNI BeadRuptor 24 (Omni-Inc, Kennesaw, GA). Protein was quantified using a BCA Assay (ThermoFisher Scientific, Rockford, IL) and then protein loading samples were resolved in SDS-PAGE under reducing conditions and transferred to a polyvinylidene fluoride (PVDF) membrane. Membranes were blocked in blocking reagent (LI-COR, Lincoln, NE) assay system for 1 hour at room temperature and incubated with primary antibodies overnight at 4°C as follows: p-extracellular signal-regulated kinase (ERK) 1/2 (1:1000), ERK1/2 (1:1000), p-p38 (1:1000), p38 (1:1000), p-MAP kinase-activated protein kinase (MK) 2 (1:1000), MK2 (1:1000), p-nuclear factor kappa B (NF-kB) (1:1000), NF-kB (1:1000),

cyclophilin B (1:1000), and β-actin (1:3000) from Cell Signaling Technology (Danvers, MA). Membranes were washed with tris-based saline-tween 20 (TBS-T) and the appropriate secondary antibody (1:20000), ThermoScientific, Rockford, IL) was added in blocking reagent for 1 hour at room temperature. Membranes were washed three times with TBS-T and developed using a chemiluminescence assay system. Bands on the membrane were visualized on autoradiography film. Western blot images were scanned, saved as Tiff files, inverted, and integrated density was analyzed using ImageJ software (NIH). Phosphorylated protein levels were normalized to the respective total protein levels.

Assessment of body composition using NMR spectroscopy

Mouse body composition was measured at weeks 0, 8, and 14. Mice were placed on a Bruker Minispec LF90 NMR (Bruker, Billerica, MA, USA). Readouts were lean, fat, and fluid mass. Data are presented as percent body fat and lean masses.

RNA and mRNA library preparation

Total RNA was extracted from the left lateral lobe of the liver. RNA purity was determined by using a NanoDrop8000 spectrophotometer. RNA integrity was assessed using an Agilent Technologies 2100 Bioanalyzer with an RNA Integrity Number (RIN) value. The mRNA sequencing libraries were prepared from 1 μg of RNA, according to the manufacturer's instructions (Illumina Truseq stranded mRNA library prep kit). The quality of the amplified libraries was verified by capillary electrophoresis (Bioanalyzer, Agilent). The index-tagged libraries were

pooled on equimolar quantities, post to the quantitative real-time PCR using SYBR Green PCR Master Mix (Applied Biosystems). The sequencing was performed on Novaseq 6000 sequencing system (Illumina) with a 2 x 100bp read length. The rate at which the required readings for each sample are mapped to the reference genome is presented in Supplementary Table 1.

RNA sequencing analysis

The sequencing reads for each sample were mapped to the reference mm10 genome of *Mus musculus* (C57BL/6J strain) by Tophat (v2.0.13). The aligned results were added to Cuffdiff, a program of Cufflinks (v2.2.0) to identify differentially expressed genes (DEGs) using the UCSC genome annotations and default parameters. For library normalization and dispersion estimation, pooled methods were applied. Raw and processed data have been deposited to NCBI with GEO accession numbers (GSE186971). Genes exhibiting p value < 0.05 were considered significant for False Discovery Rate (FDR)/multiple comparison corrections. The sequencing and initial data analysis were performed by the DNA LINK sequencing lab (Los Angeles, CA).

Gene Ontology and pathway analysis

The differentially expressed genes (DEGs) were classified into Gene Ontology terms (GO-Term) through enrichment using DAVID (Database for Annotation, Visualization, and Integrated Discovery) online tool (https://david.ncifcrf.gov/home.jsp). The terms were categorized into GO-Molecular Function (MF), GO-Biological Process (BP), and GO-Cellular Component (CC). Functional pathway analysis was performed using Ingenuity Pathway Analysis (IPA, QIAGEN

Inc., https://www.qiagenbioinformatics.com/products/ingenuity-pathway-analysis). The right-tailed Fisher's exact test p-values were used to identify significant GO-Terms and pathways. The gene list associated with specific pathways was obtained from the KEGG database (https://www.genome.jp/kegg/pathway.html) and used to construct the heatmaps shown.

Data analysis and visualization

Data analysis was done using JMP-SAS, MS-Excel, GraphPad prism, and R Studio 1.4.1106 (http://www.R-project.org). Volcano plots were generated using the EnhancedVolcano package of R. To better visualize and explore the enriched GO-Terms, the Gene Ontology obtained from the DAVID database and the expression data were combined and represented as GOCircle plots using the Goplot R package. The R package Dplyr was used for data processing and the ggplot package was used to construct graphs. Heatmaps were constructed using the pheatmap library. The gene list for signaling pathways was obtained from the IPA and KEGG pathway databases. The figures were assembled in Adobe Illustrator and Adobe Photoshop.

Statistical analysis

All data were analyzed by GraphPad Prism (Prism 9; GraphPad Inc.). Ordinary one-way ANOVA was held for fasting plasma insulin, the protein expression level of phosphorylated ERK/ERK in the liver. Two-way ANOVA repeated measure was held for body weight, food intake, body composition, ITT, and GTT followed by Tukey's multiple comparisons tests; NMR followed by Šídák's multiple comparisons test. Student t-tests were held for NF-κB, phospho-p38/p38, and phosphor-MK2/MK2 protein expression levels in the liver sample. Quantitative data are represented as the mean ± SEM. Quantification analysis for AUC, western blot band density,

and imaging pixels was conducted using one-way ANOVA followed by Tukey's multiple comparisons after the outlier test (α=0.05). * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, and **** $p \leq 0.0001$ were considered statistically significant.

CHAPTER IV

RESULTS

Curcumin supplementation suppresses body weight gain and fat accumulation in diet-induced obese (DIO) aged mice

For this study, 18-20 months old male C57BL/6 mice (n = 7-9 in each group) were administered a dietary intervention for 15 weeks **(Figure 2A)**. Two dietary regimes were used in four groups of mice: a normal chow diet (NCD) with or without curcumin (CUR) supplementation and a high-fat high-sugar diet (HFHSD) with or without CUR **(Figure 2A)**. HFHSD+CUR-fed mice had markedly lower body weights (39.57 ± 1.42 g vs. 43.67 ± 2.01 g) and less body weight gain (4.72 ± 1.78 g vs. 7.83 ± 1.64 g) compared to HFHSD fed mice **(Figure 2B and 2C)**, without any change in food intake **(Figure 2D)**. On the other hand, NCD and NCD+CUR-fed mice maintained similar body weights and food intake **(Figure 2B and 2D)**.

Because of the weight loss due to CUR supplementation, body composition was analyzed using NMR. As expected, HFHSD significantly increased fat/body percentage (%) compared to NCD after 14 weeks ($p = 0.0003$). CUR-fed mice had lower total body fat mass in both dietary regimes from week 8 onwards and decreased body fat accumulation

in the HFHSD group such that their fat mass was similar to NCD mice, despite no alteration in their food intake (16.88 ± 1.92% vs 24.02 ± 1.00%, **Figure 3A**). As a gradual decline of lean body mass is known to occur with aging in mice (Kalyani et al., 2020), lean body mass was also monitored. A significant loss of lean body mass was observed in the HFHSD group compared to the NCD group by week 14 ($p = 0.0056$). Along with this result, a protective effect of CUR on the loss of lean body mass was also observed in both dietary regimes compared to the respective controls by week 8 (mouse age: 20-22 months old). However, this effect was not maintained by the end of the study **(Figure 3B)**. In addition, overall fat, lean body mass (%), and fluids (%) were analyzed at week 14 and it was found that only fat mass was reduced by CUR **(Figure 3C)**.

Dietary curcumin alters gene expression associated with senescence pathways and insulin signaling pathways in the aged mice

To characterize any CUR-driven transcriptional changes in the liver of aged mice, whole transcriptome RNA sequencing (RNAseq) analysis was performed. The analysis generated 40,785,830 to 71,480,323 reads, which represented a 90-98% mapping rate to the reference genome **(Supplementary Table S1)**. To investigate the biological significance of the differentially regulated genes, GO (Gene Ontology) terms and canonical pathways associated with DEGs were identified. The most significant enrichments, especially in the HFHSD+CUR group, were for (a) glucose metabolism- and homeostasis-related pathways, (b) aging and aging-related pathways, and (c) pathways that control inflammation such as Th1, Th2, inflammasome, PD-1/PD-L1 pathways **(Figure 4A and 4B)**. The notable canonical pathways shared between both CUR groups in the different dietary regimens included insulin secretion and insulin receptor signaling pathways,

senescence pathway, IGF-1, mTOR, Hif1α signaling, and Type 2 diabetes mellitus signaling **(Figure 4C)**. Heatmap analysis for genes in the senescence pathway revealed that CUR in both dietary regimes led to the downregulation of genes that attenuate senescence pathway transduction **(Figure 4E)**. This response was more evident in HFHSD groups. Analysis of upstream transcription regulators (TRs) associated with DEGs in HFHSD+CUR compared to HFHSD groups identified 187 significant TRs of which 15 had positive Z-scores associated with activation and 16 with inhibition **(Supplementary Figure 1)**. The genes regulated by hepatic nuclear factors, *Hnf1A* (n = 112 target molecules in the dataset) and *Hnf4A* (n = 527) TRs, whose mutations are known to cause maturity-onset diabetes of the young (MODY) and insulin-independent diabetes mellitus (Shepherd et al., 2009; Yamagata et al., 1996), were predicted to be inhibited by CUR. The activation Z-scores for Hnf1A and Hnf4A were -4.749 and -3.314, respectively. Thus, the RNAseq data suggests that CUR prevents reduction in insulin sensitivity in the liver in HFHSD-fed aged mice through transcriptional regulation of the senescence pathway.

Dietary curcumin treatment downregulates p38-MAPK pathway in DIO-aged mice

Considering that CUR supplementation altered hepatic genes in the senescence pathway, this pathway was explored more closely. After exposure to senescence-induced stimuli, MAPK pathways – mainly p38 and ERK1/2 – lead to the senescence phenotype development (Anerillas et al., 2020) **(Figure 5A)**. To identify the mechanism(s) of curcumin in hepatic senescence, immunoblotting was conducted, and analyzed two major regulators, the ERK1/2 and p38 protein expression levels in the liver samples from all four groups **(Figure 5B and 5C).** A ratio of phosphorylated p38 to p38 protein expression levels was downregulated in CUR-treated obese

aged mice ($p = 0.0505$, **Figure 5C**). However, no differences were shown in phosphorylated ERK to ERK in all four groups **(Figure 5B)**.

Dietary curcumin regulates inflammation pathways associated with senescence in the aged mice

In addition to the heatmap analysis for genes in the senescence pathway, we sorted inflammation-related pathway genes for heatmap analysis since most SASPs disrupt liver functions by inducing chronic inflammation (Coppé et al., 2010). This revealed that CUR in HFHSD dietary regimes led to the downregulation of genes in inflammation-related pathways **(Figure 6A)**. *Stab1, Fdxr, Ppp3r1*, and *Pde8a* were the most significant fold changes associated with inflammation pathways based on Ingenuity Pathway Analysis (IPA) **(Figure 6B)**. Among SASPs, mRNA expression levels of interleukin 6 (*Il-6*) were suppressed in the same group compared to its control group ($p = 0.0664$, **Figure 7A**). Additionally, HFHSD+CUR mice had significantly downregulated gene expression levels of chemokine ligand 10 (*Cxcl10*), *Cxcl2*, and forkhead box O3 (*FoxO3*) ($p < 0.05$, **Figure 7A)**. To confirm the effects of dietary CUR on the regulation of inflammatory genes, a pivotal component of the senescence effector, NF-κB (p65) (Chen et al., 2003; Freund et al., 2010) protein expression levels were evaluated. Of importance, CUR supplementation decreased hepatic NF-κB expression levels compared to control mice in both NCD and HFHSD groups ($p = 0.0562$; NCD, $p = 0.0723$; HFHSD, **Figure 7B and 7C**).

The senolytic effect of curcumin protects against impaired insulin homeostasis in the aged mice

Previous studies revealed that hepatic senescence is involved in impaired glucose homeostasis and insulin signaling (Bonnet et al., 2022; Cheng et al., 2021; Michael et al., 2000). Therefore, we evaluated glucose and insulin homeostasis in our mouse model setting. Fasting plasma insulin (FPI) was maintained over time in NCD+CUR group while the NCD-fed group showed around a 50% increase at Week 15 compared to the baseline (1.10 ± 0.53 vs. 1.52 ± 0.38 ng/mL: $p = 0.058$). As expected, HFHSD feeding for 15 weeks caused a markedly increased FPI compared to Week 0. Also, FPI in HFHSD-fed mice was significantly higher than levels in NCD-fed mice at Week 15 ($p < 0.01$). Of note, HFHSD+CUR group showed lower FPI at Week 15 than HFHSD group (1.08 ± 0.49 vs. 1.53 ± 0.61 ng/mL; $p = 0.14$). HFHSD+CUR-fed mice maintained FPI over time and it was similar to that of NCD-fed mice **(Figure 8A)**. To check whether the change in FPI affected glucose tolerance level, we conducted GTT (2g/kg body weight) at Week 14 but showed no differences between CUR treatments **(Figure 8B)**. However, during an ITT (1 U insulin/kg body weight) at Week 14, the HFHSD+CUR group had similar insulin sensitivity to NCD and NCD+CUR fed groups, whereas the HFHSD fed group, as expected, had reduced insulin sensitivity **(Figure 8C)**. To confirm the results of GTT at Week 14, another set of mice – 8 weeks of treatment – conducted GTT. 8 weeks of CUR treatment was decided since Week 8 was the timepoint that showed a significant difference in body weight comparing HFHSD and HFHSD+CUR. Along with GTT results at Week 14, there were no differences between the CUR-treated mice and the control mice in both dietary regimens **(Figure 9A)**. However, as shown at Week 14, the ITT result at Week 8 showed increased insulin sensitivity in both dietary regimens when CUR was supplemented **(Figure 9B)**. These *in vivo* results lend credence to the finding that CUR positively modulates insulin sensitivity.

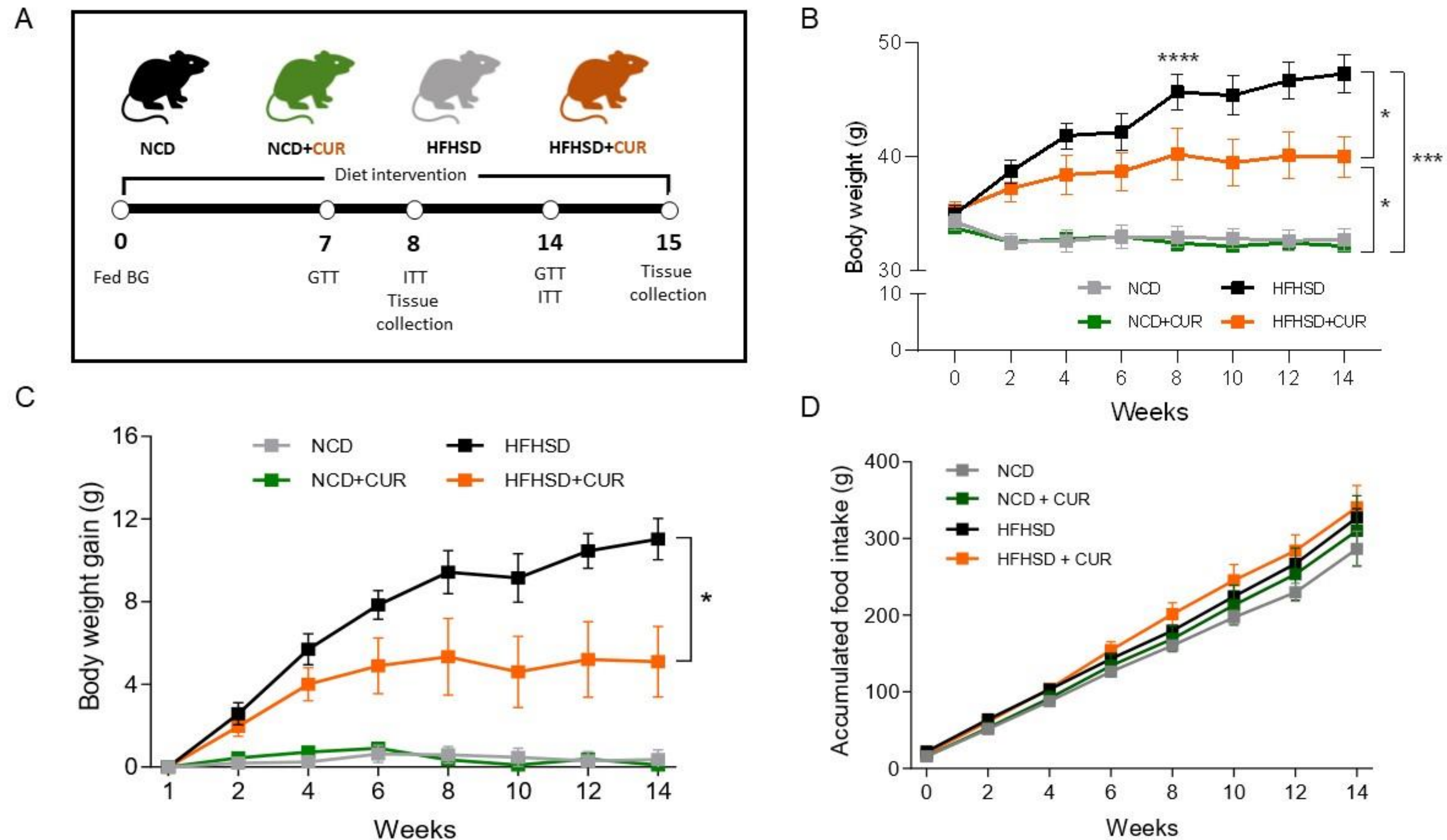

Figure 2. Animal study design, body weight and food intake (A) Scheme of animal study design **(B)** body weight (g), **(C)** body weight gain (g), **(D)** accumulated food intake (g) (n = 7-9). $*p \leq 0.05$ and $***p \leq 0.001$.

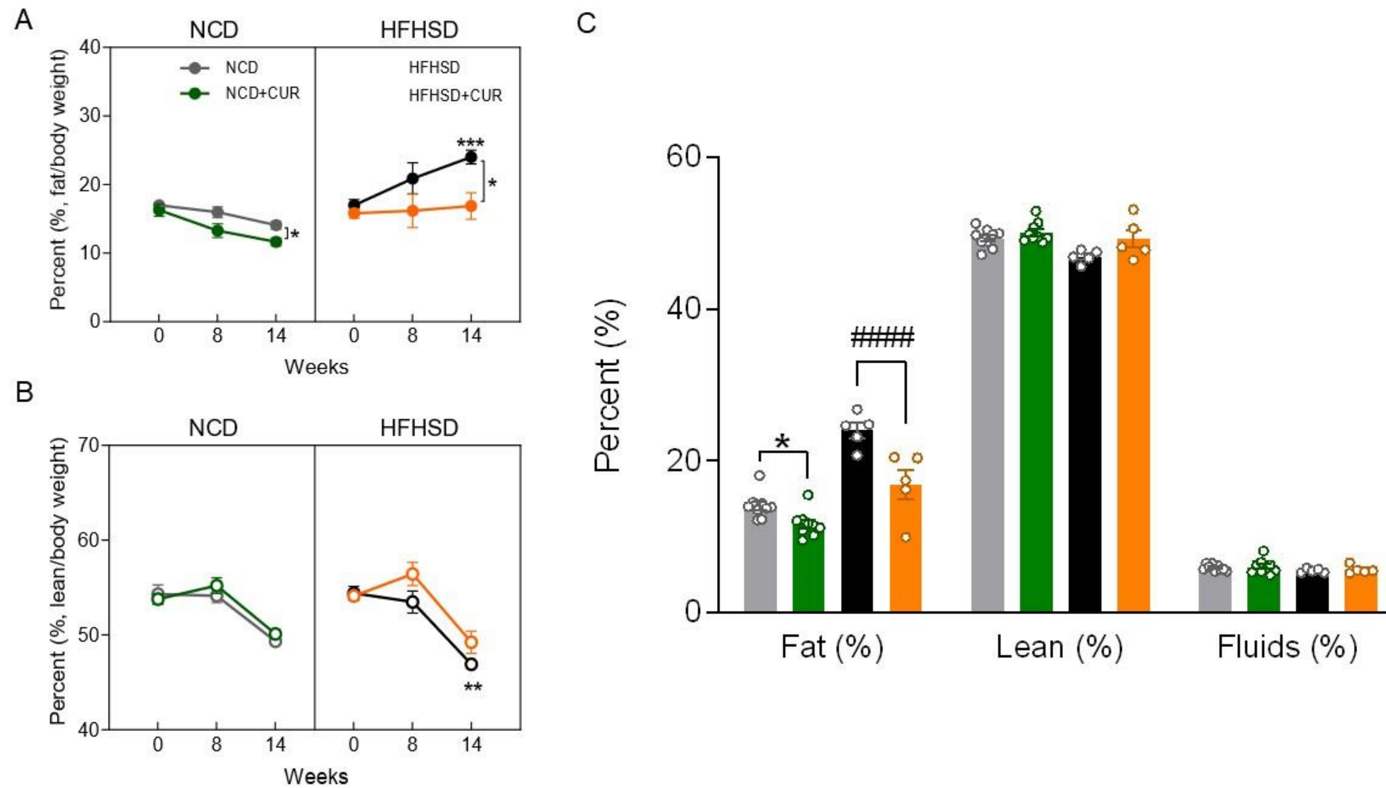

Figure 3. NMR spectrometry results. NMR spectrometry was conducted with a normal chow diet (NCD), curcumin-supplemented normal control diet (NCD+CUR), high-fat high-sugar diet (HFHSD), and curcumin-supplemented high-fat high-sugar diet (HFHSD+CUR) fed mice at baseline, 8 weeks, and 14 weeks of diet (n = 5-9). **(A)** fat/body weight percentage (%) **(B)** lean/body weight percentage (%) **(C)** overall comparison of fat/body weight percentage (%), lean/body weight percentage (%), and fluid/body weight percentage (%) at week 14. $*p \leq 0.05$, $**p \leq 0.01$, $***p \leq 0.001$ and $^{####}p \leq 0.0001$.

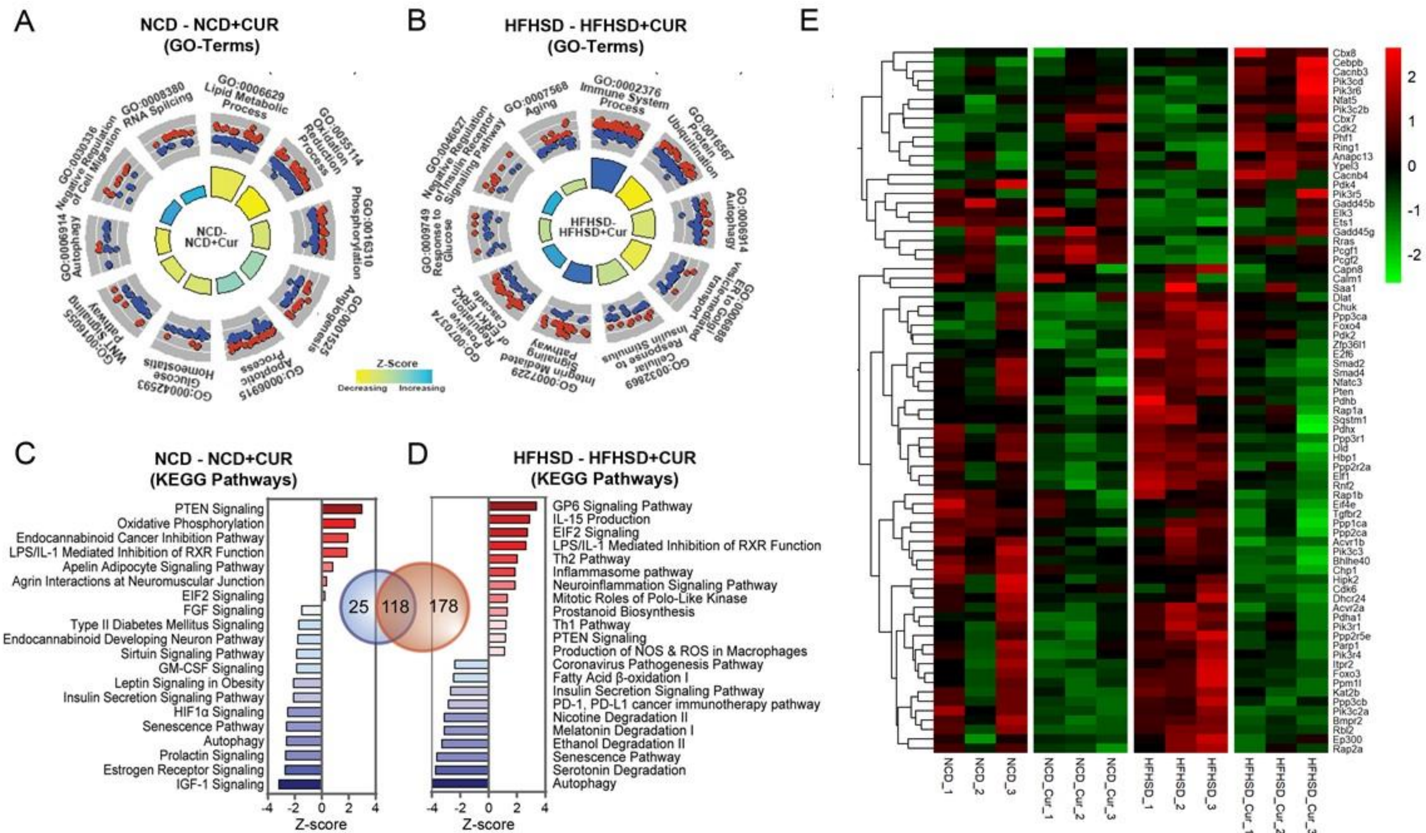

Figure 4. RNA Sequencing results related with senescence pathway (A and B) Significant Gene Ontology (GO)-Biological Processes enriched in Differentially Expressed Genes (DEGs) in aged mice liver receiving curcumin-supplemented normal control diet (NCD+CUR) or high-fat, high-sugar diet (HFHSD+CUR) compared with their respective non-supplemented control groups (NCD or HFHSD) (n = 3 per group). The relevant top 10 GO terms are presented in the GO circle plots. Within each GO-Term, the upregulated and downregulated genes are represented in red and blue circles. The breadth of inner rectangles represents the strength of p-value significance. Yellow color represents GO-Terms with negative Z-scores and blue, positive Z-scores. **(C and D)** Bar charts of top significantly affected canonical pathways based on IPA presented based on the Z-scores. The red color indicates activation, and the blue color indicates suppression. **(E)** Heatmap analysis of Senescence pathway: Heatmap of RNA expression is measured by FPKM ($p < 0.05$ in HFHSD comparison; FPKM > 1.5) from the senescence pathway. Red indicates a positive Z-Score and green, a negative Z-score.

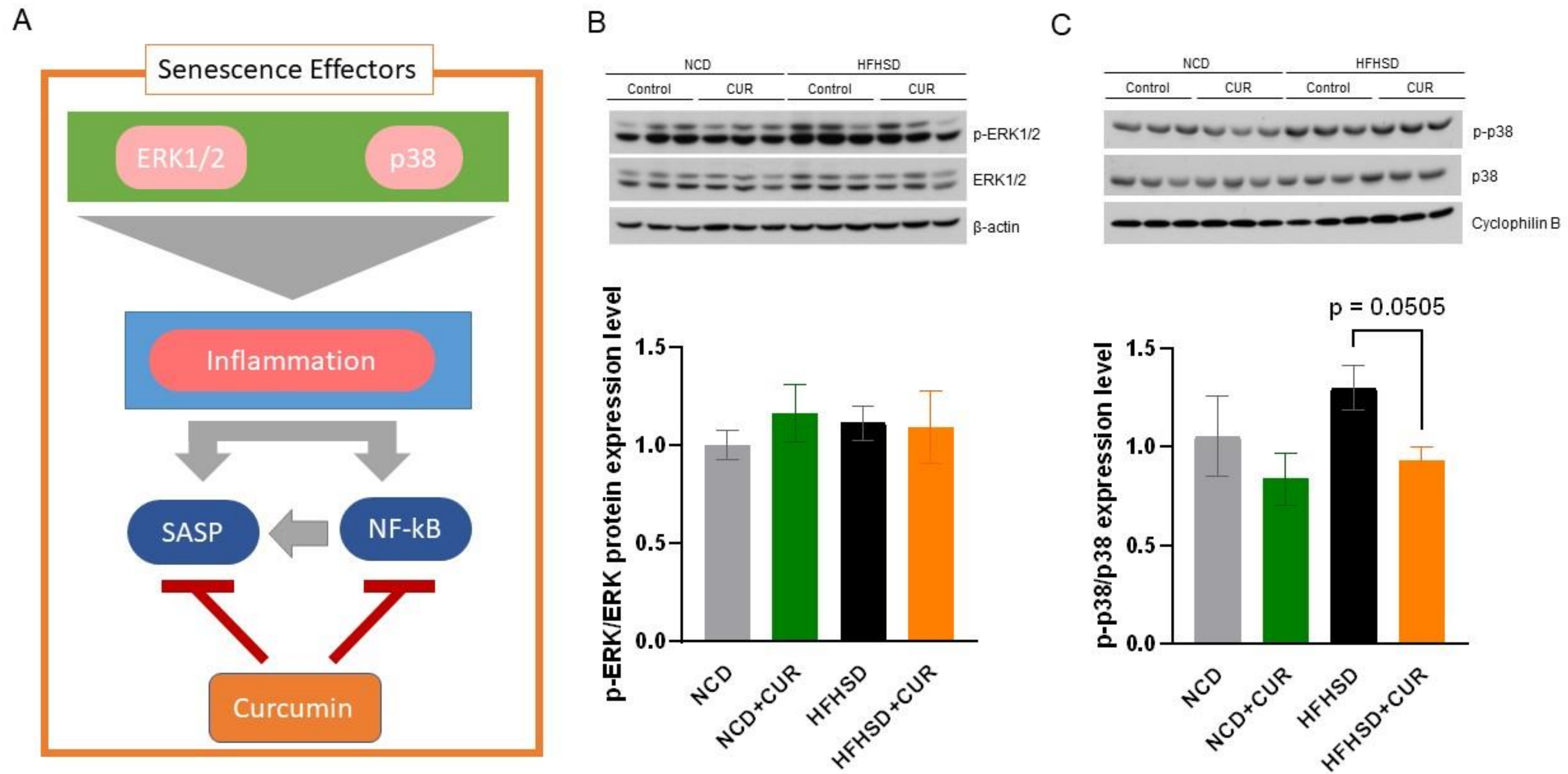

Figure 5. Expression level of main senescence effectors (A) Scheme of senescence effectors in cellular senescence**. (B-C)** Immunoblots and quantification of protein expression level in liver tissue lysate after the diet treatment (n = 3); **(B)** p-ERK/ERK in the liver from mice with dietary intervention for15 weeks, **(C)** p-p38/p38 in the liver from mice with dietary intervention for 8 weeks.

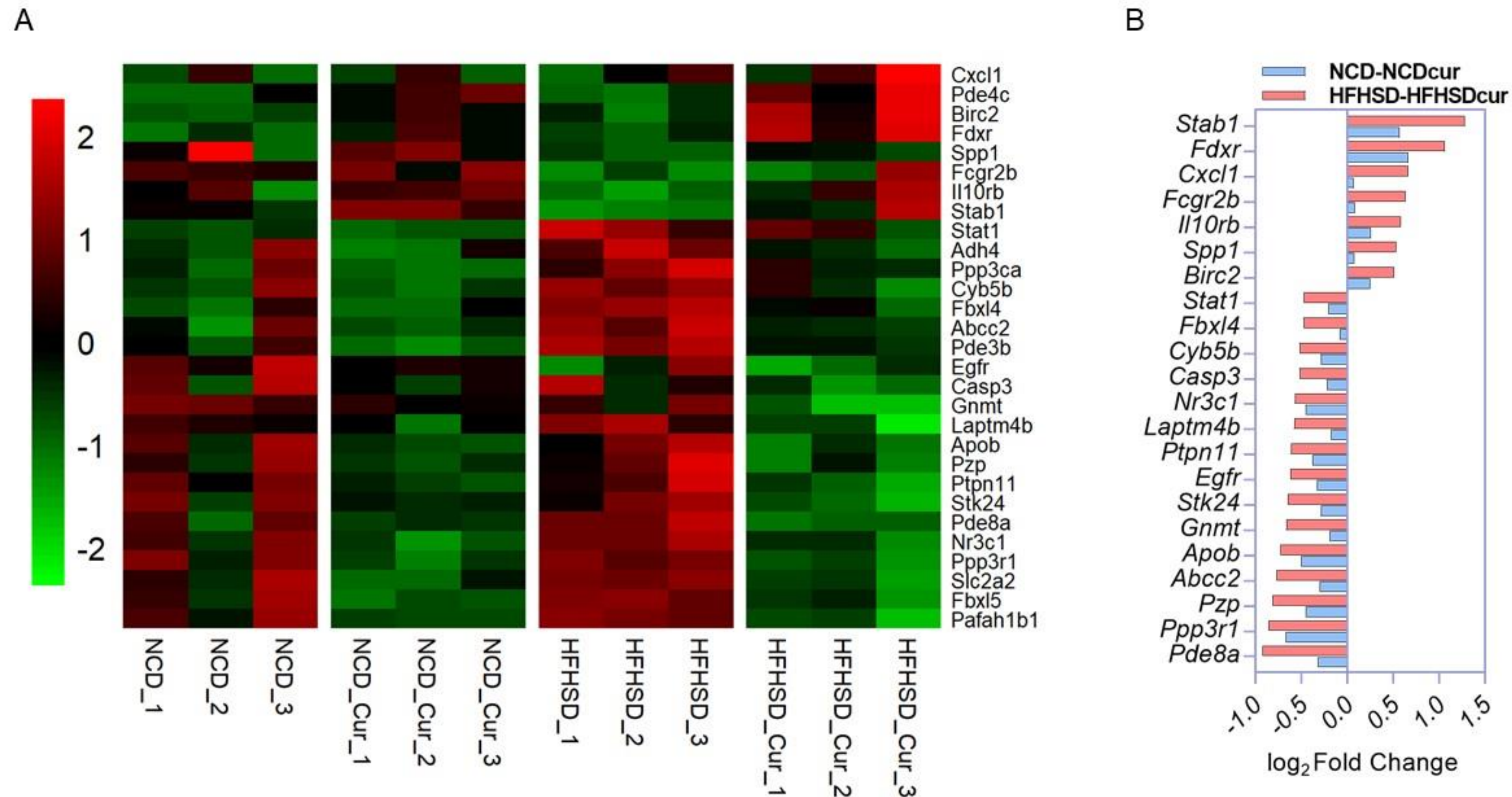

Figure 6. RNA Sequencing results related with inflammation pathway (A) Heatmap analysis of inflammation-related pathway: Heatmap of RNA expression is measured by FPKM ($p < 0.05$ in HFHSD comparison; FPKM > 1.5) from Senescence Pathway based on GO term and KEGG analysis. Red indicates a positive Z-Score and green, a negative Z-score. **(B)** Bar charts of genes with the most significant fold change associated with inflammation pathways based on IPA presented based on the Z-scores. The red color indicates activation, and the blue color indicates suppression.

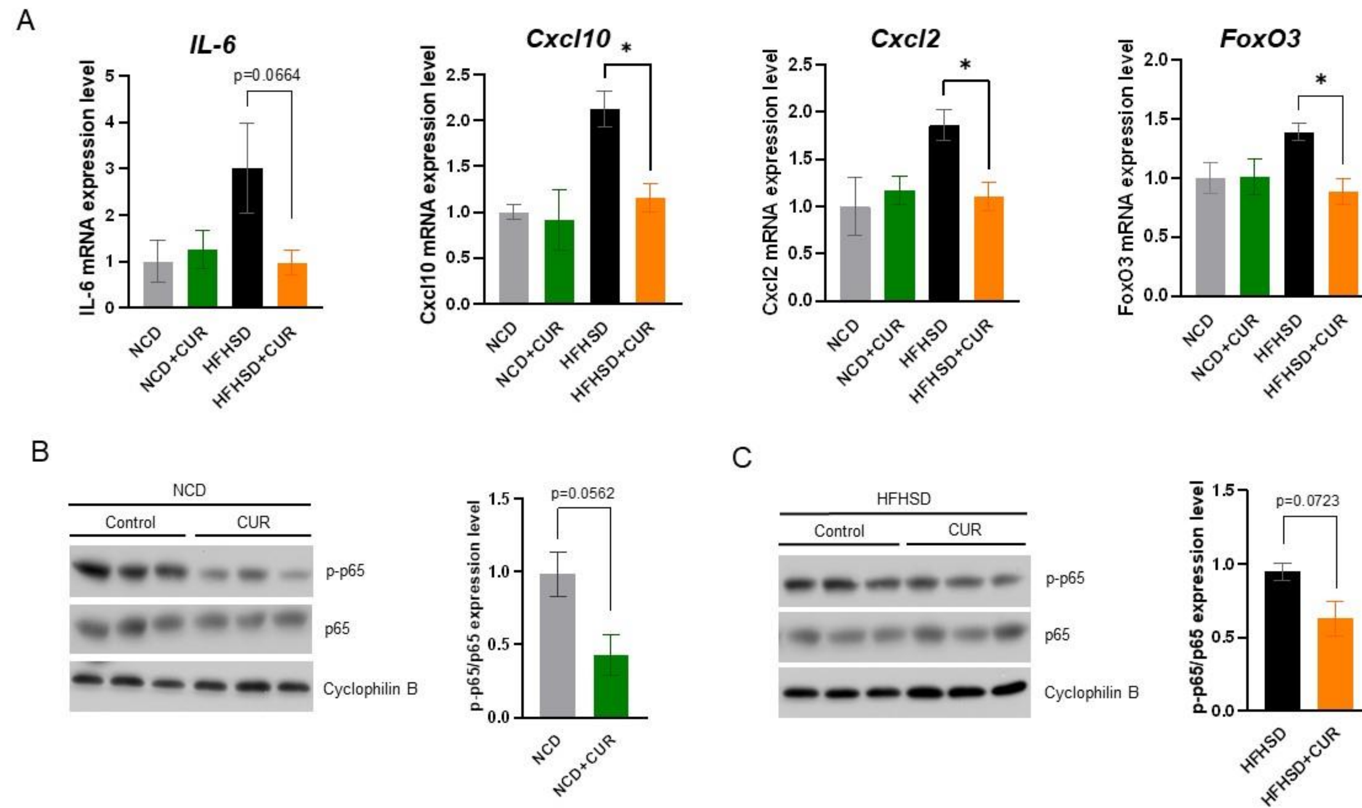

Figure 7. Relative mRNA abundance in SASP. Quantification of the mRNA expression level of aged mouse liver for 8 weeks treatment; **(A)** *IL-6*, *Cxcl10, Cxcl2,* and *Foxo3*. Proteins were extracted from livers and then immunoblotted. Quantification of p-p65/p65 protein expression levels; **(B)** Normal chow diet (n=3) **(C)** High-fat high-sugar diet groups (n = 3). p-values were from a two-tailed unpaired t-test. *$p \leq 0.05$.

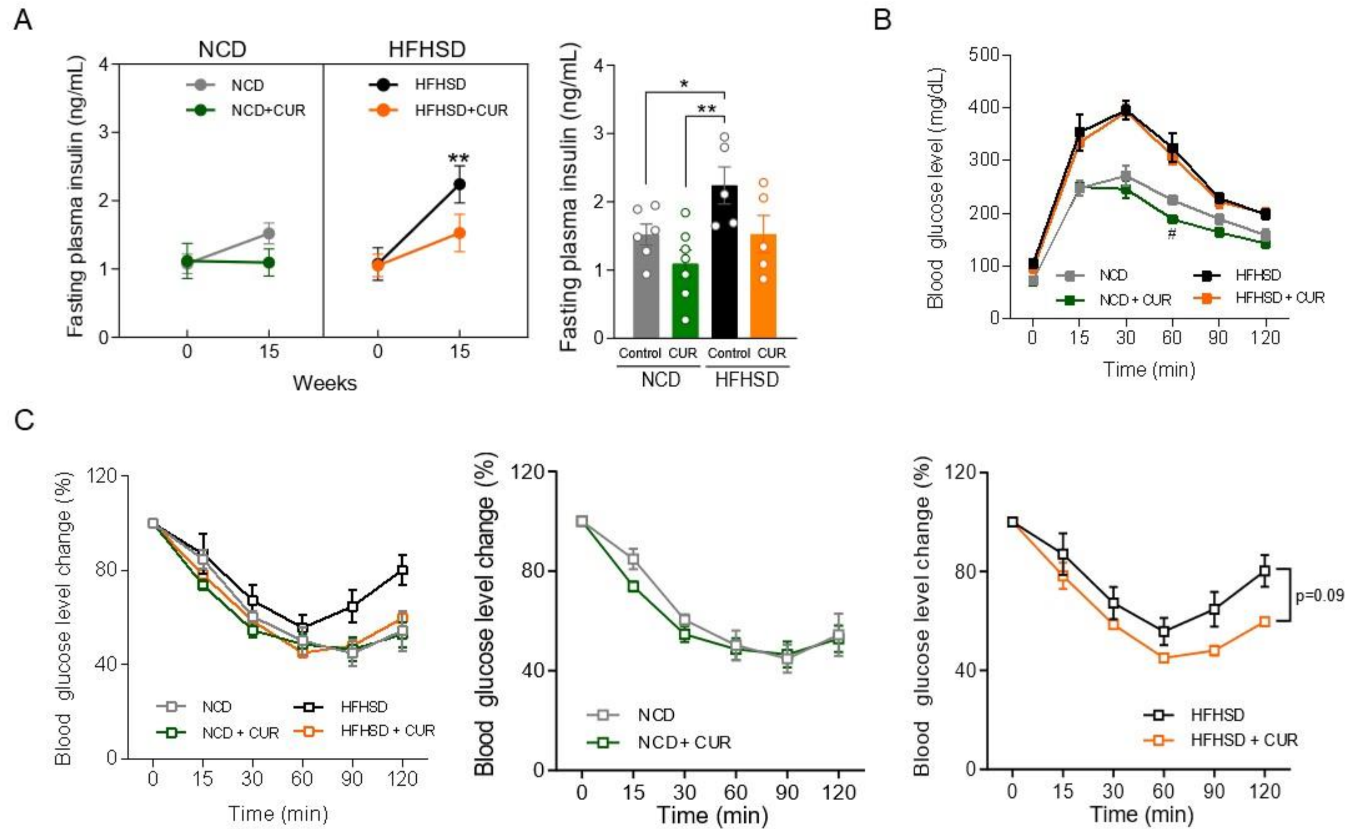

Figure 8. GTT and ITT results with 15 weeks treated mice (A) 6-hour fasting insulin levels (ng/mL) at week 15 (n = 5-7). **(B)** Glucose Tolerance Test (GTT) was performed at Week 14 after the diet treatment **(C)** Insulin Tolerance Test (ITT) was performed at Week 14 after the diet treatment (n = 4-8). #p ≤ 0.05, *p ≤ 0.05 and **p ≤ 0.01.

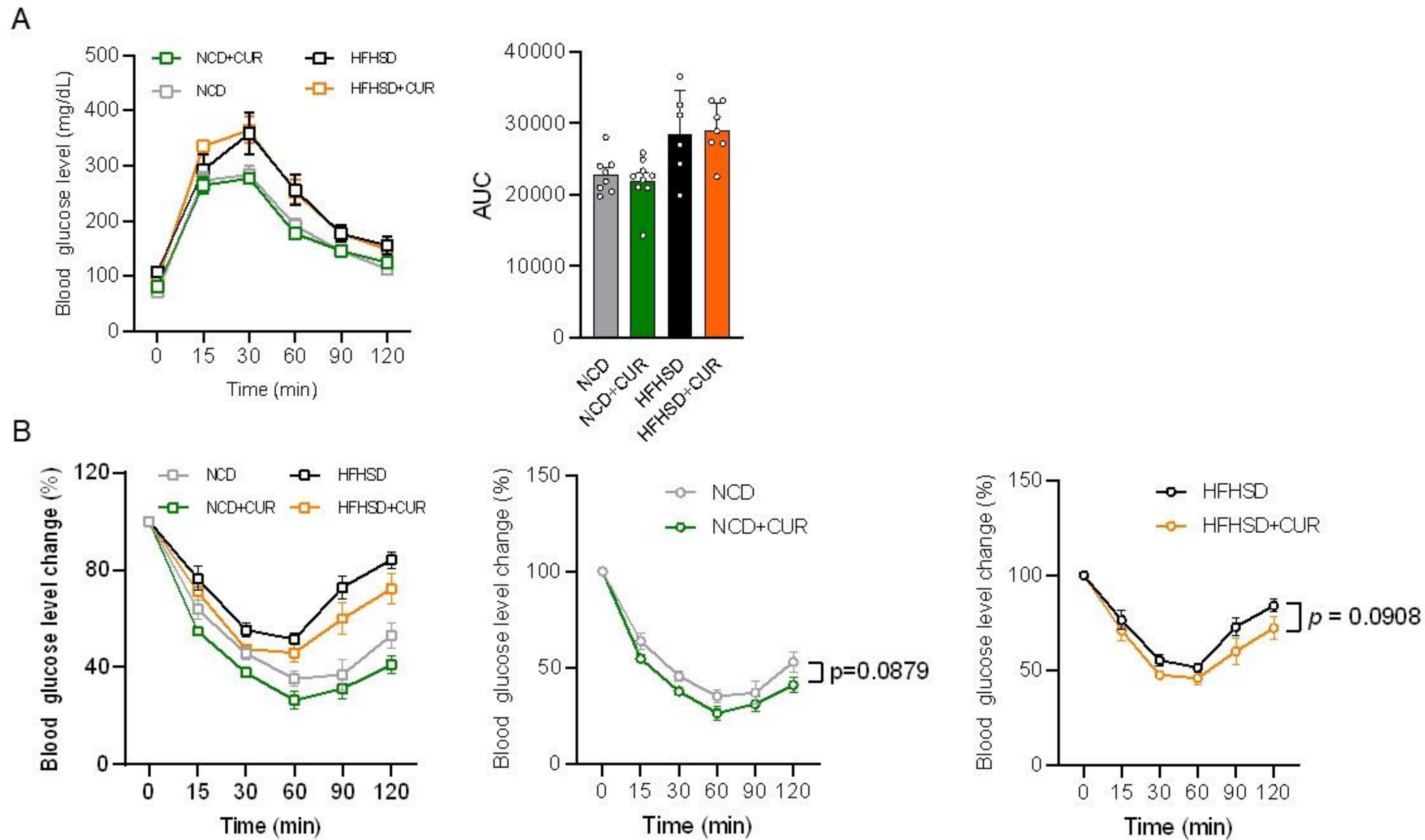

Figure 9. GTT and ITT results with 7 weeks treated mice (A) GTT was performed at Week 7 after the diet treatment, and the area under the curve (AUC) analysis **(B)** ITT was performed at Week 8 after the diet treatment (left), percentage of blood glucose level change in NCD (middle) and HFHSD (right). P-values were from a two-way ANOVA test. $^{\#}p \leq 0.05$, $^{*}p \leq 0.05$ and $^{**}p \leq 0.01$.

CHAPTER V

DISCUSSION

Cellular senescence, irreversible cell-cycle arrest coupled with SASP, is a characteristic of aging. Therefore, a promising approach to delay or prevent age- and cellular senescence-associated pathologies is to eliminate or reduce the senescence effectors. Curcumin is renowned for its beneficial effects on factors that contribute to age-related declines in function because of its anti-inflammatory, anti-viral, anti-carcinogenic, and antioxidant effects when it is consumed (Kotha & Luthria, 2019; Lee et al., 2022; Nebrisi, 2021; Sundar Dhilip Kumar et al., 2018); however, before this study, the effect of curcumin in hepatic senescence of naturally aged mice has not been well described.

Recent studies have demonstrated the regulation of senescent traits by MAPK cascades (Martínez-Zamudio et al., 2017; Xu et al., 2014). MAPK pathways can change the cellular environments and allow adaptive responses by stimulating senescence traits. These traits are mediated by growth suppression, resistance to apoptosis, and regulation of SASPs (Anerillas et al., 2020; Kumari & Jat, 2021). Thus, eliminating the exploitation of MAPKs is the strategy to suppress the growth of senescent cells (Anerillas et al., 2020). Our results with the ratio of phosphorylated p38 to p38 protein expression levels indicated that curcumin supplementation suppressed p38 activation more effectively in the aged obese mouse model. This discrepancy was

derived from the addition of the obesity factor since obesity aggravates inflammation severely in aged individuals (Luo et al., 2017; Tang et al., 2019). However, curcumin did not regulate ERK activation in all four regimes since phosphorylated ERK to ERK protein expression level had none of the difference. With canonical MAPK pathways, ERK1/2 and p38 have a different chain of cascade and their downstream nuclear target (Anerillas et al., 2020). However, growing evidence arouse the notion of alternative MAPK signaling: MAPKs cross-talk each other that ultimately activating transcription factor that involves with inflammation (Pimienta & Pascual, 2007; Plotnikov et al., 2011). Interestingly, major SASP gene expression levels indicated that CUR downregulated inflammation-related markers. These findings indicate that curcumin supplementation has the potential to be a senolytic agent that would ameliorate metabolic dysfunction. There is support that a feature of age-related metabolic diseases is occurred by accumulated senescent cells in metabolic organs (Bonnet et al., 2022; Bonomini et al., 2015; Palmer et al., 2019).

The global liver transcriptomic analyses confirm the beneficial effects of dietary curcumin supplementation on senescence pathway-related gene expressions in aged mouse livers. Although the curcumin-induced transcriptional response was different between aged mice on a normal chow diet and nutritionally challenged mice, curcumin supplementation upregulated and downregulated genes (550 and 787 genes, respectively) in both groups. Additionally, the senescence pathway was among the top 10 most affected pathways and notably suppressed (Z-score = -3.679, HFHSD+CUR vs HFHSD), indicating the potential benefits of curcumin supplementation as an anti-aging agent. These data lend to hepatic senescence in aged mice. Moreover, the GO enrichment analysis identified insulin receptor binding (GO:0005158; $p = 0.019$) and the KEGG

pathway analysis did the insulin resistance pathway as the most significant pathway associated with the commonly regulated genes (p = 7. 31$E^{-0.4}$; fold enrichment = 2.497).

Of note, the autophagy pathway was regulated by curcumin treatment of both NCD and HFHSD based on GO-term and KEGG pathway enrichment analyses. Autophagy shares overlapped canonical pathways with immune metabolism and affects both innate and adaptive immune cells and immune responses (Deretic, 2021). Also, recent studies have demonstrated that upregulating autophagy genes lead to the amelioration of the aging phenotype since it improves immune responses (Kitada & Koya, 2021; Rajendran et al., 2019; Zhang et al., 2016). However, whether autophagy regulates senescence positively or negatively is still controversial regarding the cellular stress responses (Kang & Elledge, 2016; Patel et al., 2021; Young et al., 2021).

CHAPTER VI

CONCLUSION

In summary, this study illustrates that curcumin supplementation in aged individuals is likely to play a role in mitigating reduced hepatic senescence pathway due to aging *per se* and its combination with dietary challenges. It seems convincing that curcumin suppresses hepatic senescence through the downregulation of p38-MAPK pathway. This is indicated by the reduced activation of the pro-inflammatory pathways. Thus, curcumin is a potent, natural therapeutic agent which acts in a multifaceted manner to protect against aging-associated metabolic disorders. Taken together, the results presented in this study suggest that curcumin could function as a novel senolytic by suppressing hepatic cellular senescence.

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APPENDICES

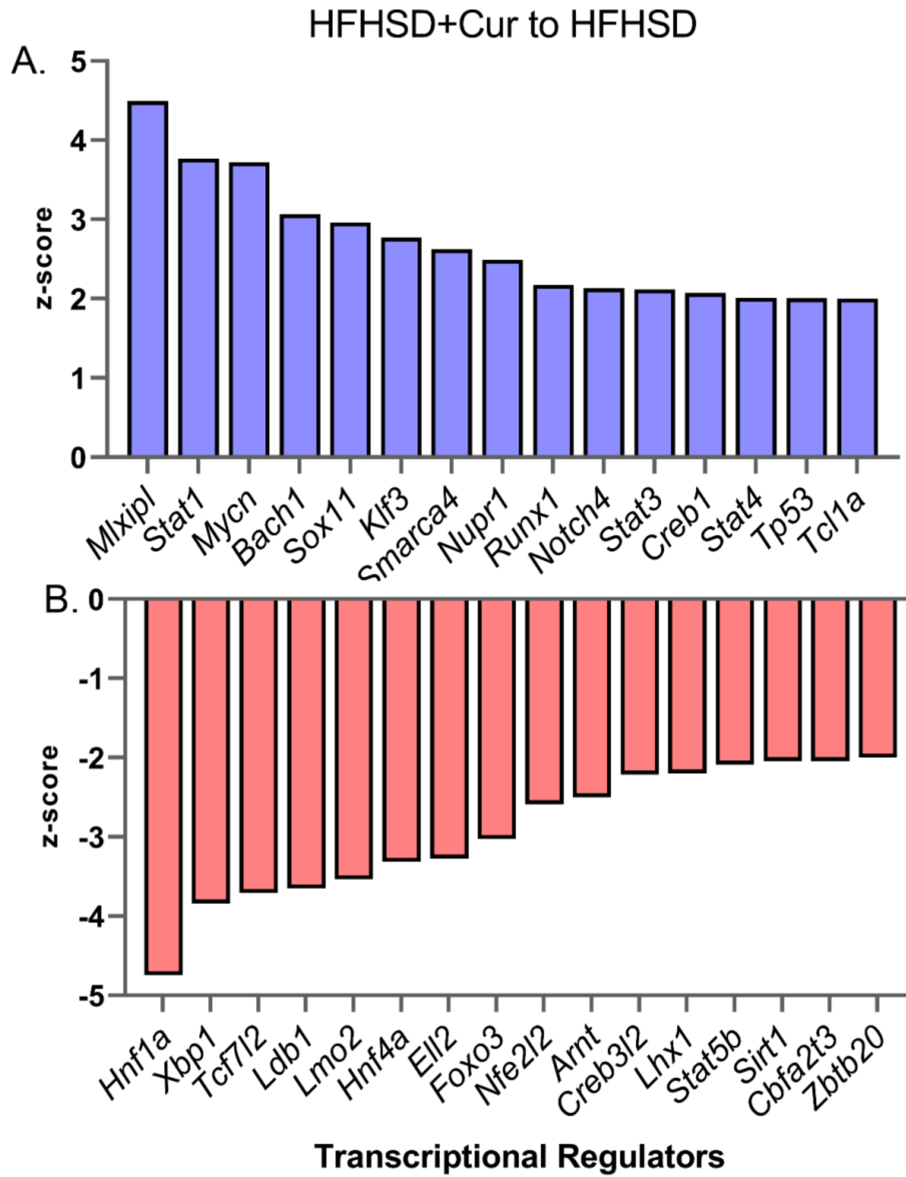

Figure 10. Transcriptional Regulators associated with differentially expressed genes.

The z-scores as predicted by Ingenuity Pathway Analysis for upstream transcriptional regulators (TRs) associated with differentially expressed genes are shown for those predicted to be associated with activation (A) or inhibition (B) of downstream genes. The comparison is provided for aging mice fed with HFHSD+CUR and the non-supplemented HFHSD control group.

Table 4. Alignment Summary

Sample	Reads	Mapped_Reads	Mapping_Rate
HFHSD_1	44,712,286	42,287,128	94.5761
HFHSD_2	55,922,916	53,161,527	95.0622
HFHSD_3	72,968,900	71,480,323	97.9600
HFHSD_Cur_1	56,314,214	51,156,887	90.8419
HFHSD_Cur_2	47,353,264	45,271,212	95.6031
HFHSD_Cur_3	53,605,040	51,339,781	95.7742
NCD_1	46,635,284	43,886,507	94.1058
NCD_2	49,468,774	46,352,953	93.7014
NCD_3	72,347,684	70,571,108	97.5444
NCD_Cur_1	48,303,724	45,757,951	94.7297
NCD_Cur_2	43,365,404	40,785,830	94.0515
NCD_Cur_3	45,002,994	42,087,349	93.5212
Average	53,000,040	50,344,880	94.7900

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