VNIVERSITAT DE VALÈNCIA

Doctoral Program in Physiology
Department of Physiology
Faculty of Medicine and Odontology

ROLE OF RESVERATROL AND OTHER NATURAL COMPONENTS ON LIPID CATABOLISM, MITOCHONDRIAL FUNCTION, AND LONGEVITY

Doctoral Thesis presented by

Kristine Bakklund Strømsnes

Supervised by:

Professor Juan Gambini Buchón

Professor José Viña Ribes

Professor Lucia Gimeno Mallench

April 2024

D/Dña Juan Gambini Buchón, Titular del Dpto. de Fisiología, Universitat de València

D/Dña José Viña Ribes, Catedrático del Dpto. de Fisiología, Universitat de València

D/Dña Lucia Gimeno Mallench, Profesora Colaboradora Doctora, Universidad CEU Cardenal Herrera

CERTIFICA/N:

Que la presente memoria, titulada "**Role of Resveratrol and Other Natural Components on Lipid Catabolism, Mitochondrial Function, and Longevity**", corresponde al trabajo realizado bajo su dirección por D/Dña. **Kristine Bakklund Stromsnes**, para su presentación como Tesis Doctoral en el Programa de Doctorado en Fisiología de la Universitat de València.

Y para que conste firma/n el presente certificado en Valencia, a 18 de abril 2024.

JUAN| GAMBINI| BUCHON
Firmado digitalmente por JUAN|GAMBINI| BUCHON
Fecha: 2024.04.25 09:55:27 +02'00'

JOSE VIÑA RIBES - NIF:19454510Y
Firmado digitalmente por JOSE VIÑA RIBES - NIF:19454510Y
Fecha: 2024.04.24 22:20:38 +02'00'

LUCIA| GIMENO| MALLENCH
Firmado digitalmente por LUCIA| GIMENO| MALLENCH
Fecha: 2024.04.25 06:57:34 +02'00'

Juan Gambini Buchón José Viña Ribes Lucia Gimeno Mallench

ACKNOWLEDGEMENTS

First and foremost, I want to give my deepest gratitude to my directors.

Juan, mi tutor y director. Muchas gracias por todo lo que me has enseñado durante estos años. Gracias por siempre tener la puerta abierta y el móvil encendido para cualquier duda. No me podría imaginar otro jefe que contestaría el móvil estando en la playa para ayudarme (a manejar una máquina del siglo tres), sin molestarse e incluso animarme en el proceso. Gracias por tu infinita paciencia, por ayudarme siempre, por escucharme y por confiar en mí. Gracias por dejarme explorar mis propias ideas, por discutir las cosas con profesionalidad y por hacerme sentir como una compañera en vez de estudiante. Gracias por ayudarme a ser la investigadora que soy.

A mi director Pepe, el jefe del jefe. Muchas gracias por compartir tus conocimientos con nosotros en las reuniones semanales. Gracias por cuestionar cada paso que daba para ayudarme a reflexionar sobre la relevancia de mis experimentos. Gracias por enseñarme a pensar de forma crítica y por hacerme mejor investigadora.

A mi directora, Lucia. Gracias por todo lo que me has enseñado, por ayudarme y guiarme siempre.

Secondly, I want to give my thanks to the personnel at the laboratory.

Quiero agradecer especialmente a todo el equipo Ejercicio/Nutrición. Gracias por estar siempre dispuestos a ayudar ante cualquier duda. Gracias por hacer que cada día en el laboratorio fuese más ameno.

A mis chicas, Alicia, Abril, Ángela, Artemis, Blanca, Maria y Silvana. Estos años hubiesen sido insoportables sin vosotras. Gracias por celebrar los días felices conmigo y por hacer que los días malos se mejoraran. Gracias por los ánimos, los consejos, el apoyo, las risas y las cervezas.

Silvana. I feel like we connected so deeply in so little time. Every time I see you in the lab, my day gets brighter. Thank you for always being there, for listening and guiding me. I am so thankful that you started working with us and I hope we will have the opportunity to work together for a long time and that our friendship can continue to grow.

Maria, no sabes cuánto agradezco que te incorporaras al grupo. Los meses que hemos pasado juntas en el laboratorio han sido de los mejores. Gracias a ti, hasta el mes más estresante de toda la tesis se convirtió en uno de los más divertidos. Por más estresada y agobiada que estuviera, tenía ganas de ir al laboratorio para hacer más experimentos contigo. Eres una gran científica y

maravillosa persona y espero poder trabajar a tu lado durante muchos años.

A los técnicos, Vicky, Mar, María y Marta. Sin vosotras esto no hubiese sido posible. Gracias por vuestra paciencia infinita, por guiándonos y ayudándonos. También quiero agradecer a Sergio del UCIM, por la toda la ayuda, por siempre dejarnos hueco en el incubador cuando el nuestro fallaba y por todo el material que nos dejaste.

Thirdly, I would like to give my thanks to my loved ones.

To my fiancée, Rafa. Thank you for supporting me every step of the way, for cheering for me when things went well, and motivating me when I was ready to give up. Thank you for making me feel capable. For making me feel valued and loved. I feel so thankful to have had the opportunity to embark on this journey with you by my side. I am so proud of you for everything you have accomplished. I cannot wait to go on new adventures with you. You are the light of my life, my love, my home.

Til mamma og pappa. Uten dere hadde jeg aldri kommet så langt. Tusen takk for all støtten dere ga meg gjennom alle disse årene. Fra bachelorgrad i Korea til doktorgrad i Valencia. Takk for at dere aldri ga opp håpet når alt så mørkt ut. Takk for at dere alltid viste meg veien framover. Takk for de forbildene dere satt for oss og arbeidsmoralen dere ga. Takk for den ubetingede

kjærligheten dere viser og for at dere alltid fikk meg til å føle at dere var stolte av meg og at jeg kunne klare alt.

Martine. Takk for at du alltid har vært der og heiet på meg, for at du alltid har kommet på besøk og vist interesse for det jeg holdt på med, uansett hvor langt unna vi var. Du er en av de mest optimistiske personene jeg kjenner, du er som en sol som sprer glede til alle rundt deg. Takk for at du har holdt ut med meg i alle disse etappene i livet.

Martine. My chosen sister. Har ikke ord for hvor mye jeg setter pris på deg. Jeg tørr nesten ikke å tenke på hvordan livet mitt hadde sett ut om jeg ikke hadde møtt deg. Jeg kunne skrevet en hel bok om hvor mye du betyr for meg, men opponentene forventer en doktoroppgave, så jeg holder det kort: Takk for at du er du.

To Mackenzie, for forcing me to take monthly breaks to do something completely different. Thank you for the good talks, the delicious food, for taking care of our kitty, and the beautiful picture you made of him, the embarrassing painting class you took me to, and the great book recommendations. Thank you for cheering me along and celebrating every objective I checked off the list of experiments I had to finish. I´m so happy we met and I feel truly thankful to have you as my friend.

INDEX

FIGURE INDEX

INTRODUCTION

MATERIAL AND METHODS

RESULTS

TABLE INDEX

MATERIAL AND METHODS

RESULTS

ABBREVIATIONS

AMPK: AMP-activated protein kinase

ACC1: Acetyl-CoA carboxylase

acyl-CoA: acyl coenzyme A

ACSL: Acyl-CoA Synthase

ATP: Adenosine triphosphate

ATCC: American type culture collection

BSA: Bovine serum albumin

CACT: Carnitine acylcarnitine translocase

cAMKK: Calcium/calmodulin dependent protein kinase kinase

CAT: Catalase

cDNA: complementary deoxyribonucleic acid

CPT: Carnitine palmitoyl transferase

Cu-SOD: Copper-dependent superoxide dismutase

CO_2: Carbon dioxide

CT: Cycle threshold

DNA: Deoxyribonucleic acid

dNTP: Deoxynucleotide triphosphate

DMEM: Dulbecco's Modified Eagle Medium

DMSO: Dimethyl sulfoxide

ECH: Enoyl-CoA hydratase

EGTA: ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid)

ETC: Electron transport chain

$FADH_2$: Flavin adenine dinucleotide

FBS: Fetal bovine serum

FCCP: Carbonyl cyanide-p-trifluoromethoxyphenylhydrazone

GAPDH: Glyceraldehyde 3-phosphate dehydrogenase

GPX: Glutathion peroxidase

H_2O_2: Hydrogen peroxide

HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HS: Horse serum

HSP: Heat shock protein

KCl: Potassium chloride

KOH: Potassium hydroxide

KH_2PO_4: Monopotassium phosphate

LCFA: Long-chain fatty acids

METC: Mitochondrial electron transport chain

MnSOD: Manganese-dependent superoxide dismutase

MDA: Malondialdehyde

$MgCl_2$: Magnesium chloride

mRNA: Messenger ribonucleic acid

mtDNA: Mitochondrial deoxyribonucleic acid

MTPP: 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine

NADH: Nicotinamide adenine dinucleotide

NRF: Nuclear respiratory factor

O2•-: Superoxide anion

OCR: Oxygen consumption rate

OH•: Hydroxyl radical

PBS: Phosphate buffer solution

PGC-1α: Peroxisome proliferator-activated receptor-gamma coactivator 1 alpha

R-: Alkyl radical

RO-: Alkoxyl radical

ROO-: Peroxyl radical

ROS: Reactive oxygen species

RNA: Ribonucleic acid

RNase: Ribonucleases

rRNA: Ribosomal ribonucleic acid

RT-qPCR: Real-time quantitative polymerase chain reaction

SDS: Sodium dodecyl sulfate

SOD: Superoxide dismutase

tDNA: Transfer deoxyribonucleic acid

TFAM: Mitochondrial transcription factor A

TRIS: 2-amino-2-hidroximetil-1,3-propanodiol

tRNA: Transfer ribonucleic acid

UCP: Uncoupling protein

UPLC: Ultraperformance liquid chromatography

UV: Ultraviolet

Zn-SOD: Zinc-dependent superoxide dismutase

ABSTRACT

INTRODUCTION

Metabolism is defined as the complete set of all chemical reactions in the organism. These constitute the pathways that extract energy from nutrients, which is used for work and storage of excess energy to be used later. Lipids are involved in the formation of cell membranes and protein modification and act as signaling molecules, and their oxidation is one of the main sources of energy. Changes in nutritional status trigger changes in lipid metabolism. The mitochondrial oxidation of long-chain fatty acids plays an important role in energy homeostasis, as it constitutes the main source of energy in the tissues specialized in carrying out fatty acid synthesis - the muscle, liver, and adipose tissue. Mitochondria are known to play an important role in the regulation of cell survival and apoptosis as well as being the metabolic powerhouse for the fatty acid pathway, tricarboxylic acid and urea cycle, biosynthesis of amino acids, purines, and steroids, among others. The mitochondrial content and activity of the respiratory chain is susceptible to regulation depending on cellular conditions, different physiological changes produced by hormones, changes in growth factors, or changes in physiological activity. Activation of the mitochondria could induce oxidative stress if not accompanied by an upregulation of endogenous antioxidant defenses. Mitochondrial biogenesis is defined as the growth and division of mitochondria.

Mitochondria are descendants of an α-protobacteria endosymbiont and therefore have their own genome and have the ability to autoreplicate. Mitochondrial biogenesis requires a coordinated synthesis and import of approximately 1000 proteins encoded by the nuclear genome which is synthesized on cytosolic ribosomes. It can be affected by environmental factors such as oxidative stress, diet, exercise, temperature changes, cell division, renewal, and differentiation.

Oxidative stress is defined as an alteration of the balance between prooxidant and antioxidant species, in favor of the former. Despite the physiological role played by some activated oxygen species, they can also give rise to unwanted oxidation reactions, against which organisms have had to develop antioxidant defenses. The formation of a certain amount of free radicals is a normal and inevitable process, since they are the product of an infinite number of chemical reactions that are essential for cellular life. These reactive species do not cause oxidative damage under normal conditions because the cell is provided with a large number of antioxidant mechanisms. However, when the antioxidant substances are lower than the prooxidants, oxidative damage is produced which can affect various molecules and can be reflected in their physiological functions. Thus, oxidative stress can be caused by an excess of prooxidant

substances, a deficiency of antioxidant agents, or a combination of both.

Resveratrol (3,5,4'-trihydroxystilbene) is a natural polyphenol with a stilbene structure formed by two phenolic rings bound by a double styrene bond. Resveratrol is present in numerous plants and fruits, such as blueberries and blackberries, nuts, grapes, and red wine, which is the largest contribution to resveratrol intake in the Mediterranean diet. Resveratrol has received great interest in the scientific community due to its many biological properties including anti-inflammatory, antioxidant, antiplatelet, anticarcinogenic, neuroprotective, and lipolysis regulatory effects. It is one of the most studied red wine molecules and is considered responsible for what is known as the "French-paradox", which describes the low risk of cardiovascular disease despite a diet rich in saturated fats and cholesterol. This suggests that resveratrol can provide an essential role in lipidic metabolism. Indeed, resveratrol has been found to have multiple metabolic effects in relation to insulin resistance, especially in improving energy metabolism in diabetes-induced mice. Additionally, resveratrol has been proven to increase lipidic oxidation in healthy, aged mice by inhibiting fatty acid synthesis and activating fatty acid degradation and mobilization. Piceid, or polydatin, is a natural

glycosylated derivative of resveratrol found in red wine, cocoa-containing products, grapes, lentils, and peanuts. Previous studies have shown that piceid exhibits various beneficial effects, mainly due to its antioxidant and anti-inflammatory activity. According to previous studies, the absorption of some phenols in diet is enhanced by conjugation with glucose. If glucosides facilitate absorption, piceid could be more efficiently absorbed than resveratrol in its aglycone form. There are many studies published on the beneficial effects of piceid, including protection against myocardial ischemia, endometriosis, small intestine injury during hemorrhagic shock, mitochondrial- dependent apoptosis, liver inflammation, diabetic renal fibrosis, and spinal cord injury.

Hydroxytyrosol is a polyphenol with a phenylethyl-alcohol structure found in olive oil and olive leaves. It is an amphipathic compound that is both liposoluble and highly soluble in water, it is stable in the free form and penetrates readily into tissues. Hydroxytyrosol is usually absorbed in the intestine through bi-directional passive diffusion with an efficiency between 75% to 100%. The absorption depends on the composition of the food matrix through which hydroxytyrosol is administered, as its absorption is greater in its natural form present in extra virgin

olive oil as opposed to when added in refined olive oil. Hydroxytyrosol has been found to have anti-inflammatory and antioxidant effects through multiple signaling pathways. Is has also been shown to exert protective effects against a number of conditions. Some of them include endothelial dysfunction, ulcerative colitis, coronary heart disease, steatosis and hypertriglyceridemia, and Covid-19.

Harmol is a β-carboline alkaloid present in coffee and alcoholic beverages. It has been found to exert a variety of bioactivities, including antifungal, antiviral, and antioxidant, properties. Additionally, it has been reported to improve object recognition in mice. Furthermore, harmol inhibits human monoamine oxidase, which has been linked to depression. In a recent study, harmol was described as a mitohormetic compound, which are defined as mild mitochondrial stressors that trigger compensatory responses that ultimately results in an improvement of mitochondrial function.

The fly *Drosophila melanogaster* has been used for longevity studies for decades and has also become widely accepted for the analysis of developmental genetics in the recent years. The main advantages of *Drosophila melanogaster* as a model organism are centered on its easy handling due to its small size, its abundant offspring, and its relatively short lifespan.

In recent decades, life expectancy has significantly increased in industrialized countries. This is primarily attributed to new pharmacological therapies and the adoption of a healthy lifestyle by the population. Among these factors, nutritional habits have played a particularly relevant role. Consequently, there has been a shift in the population pyramid. It is estimated that by 2030, 1 in 6 people will be over 60 years of age and the number of people aged 80 years or older is expected to triple by 2050, reaching 425 million people worldwide.

This shift has posed a new challenge for society as it has led to an increase in the percentage of individuals facing physical and mental limitations caused by age-related diseases. Among these, frailty, associated with age, is a geriatric syndrome that describes vulnerable individuals with increasing disability, institutionalization, and even death, as first described by Linda Fried et al. in 2001.

MATERIALS AND METHODS

For our *in vitro* studies, C2C12 cells were cultured and differentiated before treatment with 10 nM resveratrol or piceid alone or in combination with 1 μM androgen receptor inhibitor bicalutamide. Another group of cells were treated with 10 nM,

100 nM, and 10 μM hydroxytyrosol. The cells were harvested after 24-hour treatment.

To determine the effects of these polyphenols on catabolism and mitochondrial biogenesis RT-qPCR were performed. Redox status was analyzed using RT-qPCR as well as UPLC to evaluate lipid peroxidation levels, western blot to determine protein oxidation, and fluorimeter to measure hydrogen peroxide levels. To determine the effects of piceid and harmol on longevity and frailty parameters, Oregon R *Drosophila melanogaster* flies were separated into frail and robust groups and treated with 10 nM harmol and piceid separately. A longevity curve was preformed, and frailty parameters adapted by those proposed by Linda Fried were performed at 90%, 50%, and 10% survival. Flies were also collected for gene expression analysis at these same time points to measure genes related to the immune system and antioxidant enzymes.

RESULTS

The first thing we set out to do was study if resveratrol exerts effects on lipid catabolism at nutritionally relevant concentrations *in vitro*, specifically in murine skeletal muscle cells, C2C12. To determine the optimal concentration and treatment duration, we performed a concentration curve and

time course. The cells were treated with resveratrol at 1 nM, 10 nM, 50 nM, and 100 nM for 6, 12, 24, and 48 hours. We then measured genes related to lipid transport and fatty acid β-oxidation. The cells treated with 10 nM resveratrol for 24 hours showed a significant upregulation in *Ampk, Acsl, Cpt1, Cact*, and *Ech1*. Additionally, *Acc1* was downregulated in the cells treated with 1 nM, 10 nM, and 50 nM. Furthermore, treatment with 100 nM increased *Ech1* expression after 24 hours.

To study the effects of resveratrol and its potential link to androgen receptor on fatty acid transport to the mitochondria, we treated C2C12 cells with resveratrol alone and in combination with bicalutamide. We found that *Ampk* and p-AMPK increase in the cells treated with resveratrol, but not in the cells treated with a combination of resveratrol and bicalutamide. Similarly, gene expression of *Cpt1* and *Ech1* increased in the cells treated with resveratrol, but not in those treated with the combination of resveratrol and bicalutamide. These results indicate that resveratrol activates lipid catabolism through androgen receptor.

Secondly, we set out to study the effects of resveratrol and the implication of bicalutamide in the oxygen consumption of the cells in real-time. that maximal respiration, a parameter to measure the ability of the cells to meet a metabolic challenge, was

increased when treated with resveratrol. Interestingly, it was also increased in the cells treated with the combination of resveratrol and bicalutamide, but not with bicalutamide alone. Next, we measured the spare capacity of the cell, which is used as a parameter to analyze the fitness or flexibility of a cell. As with the maximal respiration, we also found that the spare capacity was increased in the cells treated with resveratrol alone and in combination with bicalutamide, but when those treated with bicalutamide alone. Lastly, we measured proton leak, which is defined as the remaining basal respiration not coupled to ATP production. An increased proton leak can be a sign of mitochondrial damage. We found a decrease in proton leak only in the cell treated with resveratrol alone.

To study if bicalutamide affects the antioxidant properties of resveratrol, we analyzed mRNA expression of the antioxidant genes catalase, glutathione peroxidase (*Gpx*), and Manganese-dependent superoxide dismutase (*MnSOD*) in comparison to the housekeeping gene *Gapdh.* We found that neither resveratrol nor bicalutamide had any effects on the expression of *MnSOD* or *Cat*. However, the expression of *Gpx* was found to be significantly increased in the cells treated with resveratrol, but not in those treated with resveratrol in combination with bicalutamide. To analyze if bicalutamide influences the endogenous redox state of the cells, we measured hydrogen peroxide levels. We found that

the cells treated with resveratrol showed significantly lower levels of H_2O_2 in comparison to the control and resveratrol with bicalutamide treated cells. To analyze the effects of resveratrol and bicalutamide on lipid and protein oxidation, we measured carbonylated proteins and MDA as a marker for lipid peroxidation. We found that neither resveratrol alone or in combination with bicalutamide affected protein carbonylation nor MDA levels. These results all indicate that resveratrol acts through androgen receptor.

To study if resveratrol binds to membrane or nuclear receptor, we treated the cells with piceid alone and in combination with bicalutamide. As piceid is a glycosylated derivative of resveratrol, it will not be able to readily enter the cell membrane, and we can therefore assess the location of the receptor by comparing the effects of piceid to those of resveratrol. Treatment with piceid significantly increased expression of *Ampkα-1* but did not affect the expression of *Acc1* or the mitochondrial enzymes *Cpt1*, *Cact*, or *Ech1.* Furthermore, treatment of piceid did not affect the antioxidant enzymes *MnSOD*, *Gpx*, or *catalase*, nor the levels of MDA as a marker for lipid peroxidation. That piceid did not exert the same effects as resveratrol indicates that resveratrol acts through intracellular androgen receptor.

The polyphenol hydroxytyrosol has been found to exert multiple protective effects. However, most of the studies published are performed with concentrations only available as supplements. Therefore, we set out to study if hydroxytyrosol at nutritionally relevant concentrations could exert the same effects as previously reported at high concentrations. To study the effects of hydroxytyrosol on mitochondrial biogenesis, we analyzed the gene expression of *Pgc-1α*, *Nrf1*, and *Tfam*. We found no differences in the expression of *Nrf1* and *Tfam* in any of the experimental groups. However, treatment with 100 nM and 10 μM increased expression of *Pgc-1α*. Additionally, hydroxytyrosol at 10 nM significantly increased expression of *Ampkα-1* and treatment with 10 and 100 nM increased *Cpt1* expression. In regard to mitochondrial respiration, we found that maximal respiration, spare capacity, and ATP production increased significantly after treatment with 10 nM, 100 nM, and 10 μM hydroxytyrosol. Regarding redox status, improvements were only found in the cells treated with 10 μM.

To analyze the possible effects of piceid and harmol treatment on longevity, we performed a survival curve with *Drosophila Melanogaster*. Piceid did not affect longevity in the flies, but harmol significantly improved longevity in the frail individuals.

Neither geotaxis not phototaxis performance as parameters of frailty was improved in any of the treatment groups. However, as the flies aged, we observed an improvement in gene expression. At 50% of lifespan, we found an upregulation of *Attacin C* and *Diptericin* in the fragile treated group in comparison to the fragile controls. We also found an upregulation of *Cecropin* in the robust flies administered piceid in comparison to the control robust group. At 10% longevity we found significantly higher levels of all the genes measured in the piceid-treated fragile flies in comparison to the fragile control group. Additionally, *Diptericin* was upregulated in the robust flies treated with piceid in comparison to the robust controls. Regarding redox status, no differences were found in the antioxidant enzymes *cat* or *MnSOD* in any age group. However, p53 was upregulated at 50% survival both in the frail and robust groups treated with piceid. Surprisingly, we found a decrease in *cecropin c* in the harmol-treated robust group compared to the robust control group. Similarly, at 50% survival, we found a decrease in the levels of *attacin C* in the robust harmol-treated flies in comparison to the robust control group. Harmol treatment did however succeed in activating *diptericin* and *16s rRNA* in the fragile flies. Although interestingly, *16s rRNA* was downregulated in the robust harmol group in comparison to the fragile flies administered harmol treatment. No differences were

found in the expression of any of the genes measured at 10% survival following treatment with harmol.

DISCUSSION

As previously mentioned, there are numerous studies on the beneficial effects of resveratrol. However, the majority of these studies are performed utilizing doses only obtainable through supplements and studies showing the effects of resveratrol at dosis achievable through diet are scarce.

In a study published by our laboratory show that resveratrol at 2.3 μg/kg/day, equating approximately 100 nM, induces lipid catabolism in old mice. Therefore, we were interested in understanding the mechanism behind this change and to see if the same effects were replicated at even lower concentrations.

Our experiments *in vitro* show that the lowest effective concentration to activate genes related to lipid catabolism was 10 nM with a 24-hour incubation. We therefore set out to study the effects of 10 nM resveratrol treatment on mitochondrial respiration and the antioxidant defense system.

We found that resveratrol at 10 nM increased maximal respiration and spare capacity of our cells, while also decreasing proton leak. This activation of the mitochondria was

accompanied by an activation of the oxidant defense system through an upregulation of *Gpx* and a reduction in H_2O_2 levels.

Although resveratrol significantly increased maximal respiration and spare capacity, it did not affect ATP production. This could be do to a dissipation of the proton gradient. The transfer of electrons from substrates to oxygen in the respiratory chain is coupled to translocation of protons across the membrane. Mitochondrial uncoupling occurs when uncoupling proteins (UCP) causes an increase in the permeability of protons to the inner mitochondrial membrane. This causes protons to return to the mitochondrial matrix without passing through ATP synthase, thereby causing an increase in temperature instead of ATP production. In fact, previous studies have shown resveratrol increases protein expression of UCP2, thereby increasing energy dissipation, causing a decrease in body-fat in rats. Further studies on the action mechanism to determine whether UCP could have caused the lack of ATP production found in our experiments.

The concentration of resveratrol used in our studies could be achieved through diet by consuming foods rich in resveratrol. However, the bioavailability of polyphenols remains a challenge in the confirmation of their effectiveness. In this context, it is

worth highlighting studies on the absorption and bioavailability of resveratrol.

A study published in 2004 shows that more than 70% of resveratrol is absorbed. In this study, six healthy volunteers were administered 25 mg resveratrol before plasma levels were measured. They reported peak plasma levels of 491 ng/mL of resveratrol and its metabolites and 5 ng/mL of unchanged resveratrol. These doses equal approximately 2 μM and 22 nM respectively. To obtain 10 nM unchanged resveratrol based on this uptake rate, we would need to consume around 11 mg resveratrol. This could be achieved by consuming 300 mL of red wine, 200 grams of lingonberries, and 100 grams of peanuts. However, if you take into consideration the concentrations of resveratrol with its metabolites found in plasma, one would only need to consume 0.11 mg of resveratrol.

Another study performed on men with well-controlled type 2 diabetes found a prolonged intake of resveratrol to be much more bioavailable. The participants were administered 150 mg resveratrol per day for 30 days and found plasma levels of unchanged resveratrol as high as 378 ng/mL, which equates to 1.66 μM. To obtain the 10 nM used in our experiments, based on these plasma concentrations, we would only need to consume 0.9 mg resveratrol. This would equate to less than 100 mL of red

wine, 30 grams of lingonberries, or 80 grams of peanuts a day. We do have to take into account that the uptake of resveratrol was measured after 30 days of daily intake. As resveratrol is a liposoluble molecule, a repeated intake of resveratrol allows it to be stored in the fat, thereby generating which results in an enhanced bioavailability. Therefore, it could be possible to achieve these concentrations by following a diet rich in these resveratrol-containing foods.

Once the doses and treatment time were established, we set out to study the pharmacodynamics of resveratrol. Seeing as resveratrol was able to exert effects at concentrations as low as 10 nM, we speculated that this effect could be mediated by a receptor, and specifically a hormone receptor. We therefore set out to study whether resveratrol is mediated by an androgen receptor, as we know they are abundant in skeletal muscle cells.

Resveratrol is thought to activate mitochondrial fatty acid β-oxidation through the activation of AMPK, although the exact signaling pathway is still unknown. Resveratrol additionally stimulates Ca2+/Calmodulin-dependent Protein Kinase Kinase 2 (CaMKK2), which phosphorylates and thereby activates AMPK in response to cytoplasmic Ca^{2+} increase. Interestingly, it has been proven that CaMKK2 is transcriptionally regulated by androgen receptor. Testosterone deficiency has been found to promote

insulin resistance in skeletal muscle, at least partially, of both males and females via an androgen receptor-dependent mechanism involving a decrease in PGC1α-mediated oxidative and insulin-sensitive muscle fibers. Whether resveratrol is mediated by androgen receptors and acts through the same pathway as cAMKK2 remains to be found.

There are many studies published on the effects of high concentrations of resveratrol on androgen receptor-dependent transcriptional activity in cancer cells. A study published in 2010, reported that 10 μM and 50 μM resveratrol decreased prostate-specific antigen expression, whereas 1 μM was suggested to increase androgen receptor transcriptional activity in androgen-dependent LNCaP cells, but not in its androgen-independent sub-clone, C4-2 cells. These results are confirmed by a similar study on the effects of resveratrol on prostate cancer. They reported that the activation of androgen receptor-driven gene expression following low-dose resveratrol treatment was dependent on the presence of androgen. Therefore, in prostate cells containing high levels of endogenous androgen, 1 - 10 μM resveratrol was found to stimulate, whereas 100 μM was found to inhibit luciferase activity. Interestingly, concentrations of 0.1 μM or lower were reported to have no effect on luciferase activity or androgen receptor protein levels.

Despite these findings, there is a lack of studies on the effects of resveratrol and its correlation with androgen receptors in other cell types and at lower concentrations. Our experiments show that resveratrol at nutritionally relevant concentrations activates lipid catabolism and improves certain parameters of oxidative stress, and most importantly that these effects are not produced when the cells are treated with resveratrol in combination with the androgen receptor inhibitor bicalutamide.

Thus, in this doctoral thesis, we show for the first time that resveratrol acts similarly to a hormone by activating lipid catabolism and reducing oxidative stress through androgen receptors at nutritionally relevant concentrations in skeletal muscle cells.

It is well known that various androgen receptors exist. We therefore set out to determine the location of the receptor through which resveratrol acts. By studying the effects of piceid and comparing them to those of resveratrol, we could analyze whether the receptor is intracellular or on the cell membrane. As piceid is glycosylated it cannot readily enter the cell without the presence of a specific transporter. Our analysis of the effects of piceid on genes related to lipid catabolism and antioxidant enzymes, as well as MDA as a marker for lipid peroxidation, showed that piceid does not exert the same effects as resveratrol.

This indicates that resveratrol acts through intracellular androgen receptors.

After determining the effects of resveratrol and androgen receptor on lipid catabolism, we were interested in analyzing whether resveratrol could increase mitochondrial respiration at nutritionally relevant concentrations and if this increase could be produced by activating androgen receptors. We found that resveratrol at 10 nM increased maximal respiration and spare capacity while decreasing proton leak. However, maximal respiration and spare capacity were also increased in the cells treated with resveratrol in combination with bicalutamide, although not when treated with bicalutamide alone. These results indicate that resveratrol improves mitochondrial respiration through an androgen-independent pathway. However, it is important to take into account that this experiment was run using an acute treatment of 30 minutes and not 24 hours as with the other parameters analyzed.

Hydroxytyrosol is a polyphenol abundant in olive oil, which is widely consumed in the Mediterranean Diet. Hydroxytyrosol has been reported to exert protective effects against endothelial dysfunction in human aortic endothelial cells. This study reported that both free hydroxytyrosol and its metabolites were

effective in the reduction of the endothelial dysfunction biomarkers at physiological doses. We were therefore interested in researching the potential effects of this polyphenol on mitochondrial function at low doses in skeletal muscle cells.

In regard to mitochondrial biogenesis and fatty acid transport hydroxytyrosol upregulated *pgc-1α*, *ampk-1α*, and *cpt-1* at nutritionally relevant concentrations. Similar results have been found in both 3T3-L1 adipocytes and hepatocytes. We also found that mitochondrial respiration was significantly increased in the cells treated with hydroxytyrosol of all the doses studied, suggesting 10 nM is sufficient to effectively increase mitochondrial respiration and ATP production. To our knowledge, ours is the first study analyzing the effects of hydroxytyrosol on mitochondrial respiration in real time.

As previously explained, the mitochondria are one of the largest sources of endogenous oxidants in the body. Therefore, it is important that the activation of the mitochondria is accompanied by an activation of the antioxidant system of the body. Unfortunately, the redox status of our cells did not seem to improve in the majority of the parameters measured, and the few defense systems found were only present at 10 μM. Hydroxytyrisol has been found to exert antioxidant effects in previous studies both *in vitro* and *in vivo*. However, these studies

were performed with considerably higher doses than the ones used in our studies. This suggests that hydroxytyrosol might not be able to combat oxidant stressors following an increase in mitochondrial respiration at nutritionally relevant concentrations in skeletal muscle cells.

In this doctoral thesis, we show that hydroxytyrosol at doses obtainable through diet can exert beneficial effects on mitochondrial function. However, as with all nutrients, and especially polyphenols, we have to take into consideration its absorption and bioavailability.

A recent study on the bioavailability of hydroxytyrosol found plasma concentrations up to 3.79 ng/mL after a 5 mg intake, which would equate 24.6 nM. This study also showed how the bioavailability of hydroxytyrosol depended on the food matrix, showing how oily matrices favor absorption. This is important to take into consideration when planning our diet to obtain the maximum benefits of this phenolic compound.

Based on these findings, the concentrations of 10 nM, 100 nM, and 10 μM in this study could be achieved by consuming 2 mg, 20 mg, and 2000 mg respectively. Studies have found concentrations of hydroxytyrosol in olives as high as 413 mg/100 g, 3.47 mg/100 mL olive oil, and 0.96 mg/100 mL red wine. This indicates we could achieve both 10 nM and 100 nM

hydroxytyrosol easily following a diet rich in these compounds. To achieve 10 nM of hydroxytyrosol, one could consume 200 mL of red wine or less than 60 mL of olive oil, and for 100 nM one could consume 4.84 grams of black olives.

Another study administrating volunteers with 25 mL extra-virgin olive oil reported plasma concentrations of hydroxytyrosol of 4.40 ng/mL. This concentration equates approximately 28 nM, and one could therefore achieve 10 nM with one tablespoon, and 100 mM with 90 mL extra-virgin olive oil. Interestingly, only trace amounts were found in the subjects consuming ordinary olive oil, indicating that the quality of the oil is highly correlated with the bioavailability of hydroxytyrosol.

However, a study from 2013 comparing the difference in bioavailability of hydroxytyrosol in men and women reported greater plasma levels of hydroxytyrosol in men than in women after 9.7 mg supplementation. The mean peak concentration was reported to be 28 ng/mL in women, with values ranging from 9-94 ng/mL. Whereas in men, the mean peak plasma concentrations were 117 ng/mL, with values ranging between 42-325 ng/mL. This would indicate that women would need to consume 534 mg of hydroxytyrosol, equating to around 130 grams of olives to achieve plasma concentrations of 10 μM. In comparison, men would need to consume 30 grams of olive to

achieve this same concentration. Although, based on these findings, both sexes would be able to achieve both 10 nM and 100 nM by regularly including red wine, olive oil, or olives in their diet.

Interestingly, the difference in bioavailability between sexes does not seem to translate to rats. In a study published in 2015, plasma concentrations of hydroxytyrosol were found to be higher in female rats in comparison to males. Further studies on bioavailability in all animal models should be performed to confirm the differences in sexes in this regard.

In a study previously performed in our laboratory have shown that moderate red wine consumption can increase longevity in *Drosophila melanogaster.* We were interested in studying if this effect could be partly due to the presence of piceid. To this end, the first thing we did was perform a longevity curve and evaluate parameters associated with frailty, adapted from those published by Linda Fried.

Under optimal conditions of life, it is in the last stage of life that the probability of death increases. It is important to keep in mind that aging is not a disease and must be separated from the causes of death due to old age. Aging refers to post-maturational processes that lead to a decrease in homeostasis and an increase

in the vulnerability of the organism, which leads to a greater predisposition to suffer from different pathologies that end with the death of the individual.

Frailty however is considered a geriatric syndrome occurring in vulnerable individuals with increased disabilities and institutionalization. The Medical Research Council Manual Muscle Testing is widely used in patients to evaluate weakness and can be effective in differentiating true weakness from imbalance or poor endurance. In this way, overcoming gravity to perform a movement, with or without resistance, will determine muscular strength of the patient. Similarly, the fly will have less ability to ascend the test tube in frail individuals where muscle weakness is present. We correlated low physical activity with phototaxis, as the fly is naturally attracted to light and would instinctively fly towards sources of light. Their reluctance or inability to do so could therefore indicate low levels of activity. Similarly, measuring sarcopenia or weight loss in the fly would be complicated. We therefore opted for a measurement of food consumption of the fly to evaluate this parameter. Although piceid did not improve any of the frailty parameters, we did find a significant increase in both longevity and phototaxis in the robust groups in comparison to the frail flies, indicating that the methods used to evaluate frailty are effective.

To this day, various theories of aging exist, among them the immunological theory of aging. The immunological theory of aging states that the immune system progressively deteriorates over time as antibodies lose their effectiveness. This increases vulnerability to infectious disease, which causes cellular stress and eventually death. The immune system has proven to play a critical role in various age-related disease and in the year 2000 the term "inflamm-aging" was coined. This phenomenon refers to a global reduction in the capacity to cope with stressors during aging, leading to a concomitant progressive increase in pro-inflammatory status.

In light of this, we set out to study the effects of nutritionally relevant doses of piceid on immune system related genes in *Drosophila melanogaster.* At 80% survival we found no differences in the expression of the genes of interest. This was expected, as the flies at this time point are still quite young. At 50% survival we did find an increased expression in *attacin C* and *diptericin* in the fragile group supplemented with piceid in comparison to the control. Additionally, *cecropin C* was overexpressed in the robust piceid group in comparison to both the robust controls and the fragile group treated with piceid. At 10% of survival piceid activated all of the genes of interest in the fragile individuals as well as *diptericin* in the robust flies. This upregulation in the fragile individuals suggests that piceid could exert a protective

effect against infections in aged flies. The lack of differences in the control and piceid treated robust individuals was also expected, as the robust individuals have been shown to have greater longevity and would therefore already have a more active immune system.

Another well described theory of the decreased health status occurring as we age is the free radical theory of aging. This term was coined by Denham in 1956 where he proposed that the effects of free radicals on cell constituents cause the damage and degenerative diseases associated with aging. It has therefore been proposed that the administration of antioxidant could prevent this damage caused by these radicals and therefore prevent age-related degenerative diseases. In later years this theory has been proposed to be a theory causing frailty rather than aging as it increases the risk of disability. Therefore, we were interested in studying the possible effects of piceid on antioxidant enzymes. Unfortunately, no changes were found in any of the treatment groups.

Piceid at 10 nM induced expression of immune system genes in aged flies and could therefore protect flies from infections as they age. However, at the doses used in this project, piceid was not able to reduce oxidative stress or improve frailty parameters nor longevity of the flies. As higher doses of piceid have been found

to reduce oxidative stress in other model, further studies should be performed with piceid at other concentrations to better determine its function on frailty parameters and longevity in *Drosophila melanogaster.*

Harmol is a scantly studied alkaloid, with only two studies published on its effects in *Drosophila melanogaster.* Out of these two studies, one analyzed its effects on longevity. This study by Costa-Machado, Luis Filipe et al. found that harmol at 25 μg/ml increased longevity in *Caenorhabditis Elegans* and *Drosophila melanogaster.* We were therefore interested in studying if low doses of harmol could replicate these effects, as well as analyze its effect on frailty parameters in aged flies.

Although longevity was improved by a significant increase in median survival in fragile flies treated with harmol, no significant differences were found in the parameters we proposed to measure frailty. However, a clear tendency is shown in the phototaxis assay at 10% survival rate, suggesting harmol might exert some protective effects against frailty at the later stages of life.

Interestingly, at 50% survival, *16s rRNA* was improved in the frail group treated with harmol. This ribosomal mitochondrial transcript has been reported to correlate with the shape of the

longevity curve in male flies, preceding the decrease in survival. This supports our findings of improvement of median survival rate, but not life span as this gene is not upregulated at 10% survival. However, some of the genes related to the immune system are downregulated in the robust flies treated with harmol. Taking this together with the fact that no improvements were found in the expression antioxidant enzymes or on the frailty parameters indicates that although harmol positively affects longevity, this increase is not accompanied by an improved health status of the aged flies.

It is important to take into consideration that these studied were performed exclusively in male *Drosophila melanogaster* populations. Further studies should be performed to determine the effects of these compounds on female flies.

CONCLUSIONS

Based on the results presented in this doctoral thesis, we can draw the following conclusions:

1. Resveratrol activates lipid catabolism and exerts protective effects against oxidative stress at nutritionally relevant concentrations in murine skeletal muscle cells

2. Resveratrol exerts its beneficial effects on mitochondrial function and redox status through intracellular androgen receptors
3. Hydroxytyrosol activates lipid catabolism and augments mitochondrial respiration, but does not activate antioxidative systems at nutritionally relevant concentrations
4. Piceid activates genes related to the immune system but does not improve longevity or frailty or redox status of aged male *Drosophila melanogaster* at nutritionally relevant concentrations
5. Harmol improves longevity, but not frailty parameters or health status of male *Drosophila melanogaster*

RESUMEN

INTRODUCCIÓN

El metabolismo se define como el conjunto completo de todas las reacciones químicas del organismo. Constituyen las vías que extraen energía de los nutrientes, que se utiliza para el trabajo y el almacenamiento del exceso de energía. Los lípidos intervienen en la formación de las membranas celulares y en la modificación de las proteínas y actúan como moléculas señalizadoras, y su oxidación es una de las principales fuentes de energía. Los cambios en el estado nutricional desencadenan cambios en el metabolismo de los lípidos. La oxidación mitocondrial de los ácidos grasos de cadena larga desempeña un papel importante en la homeostasis energética, ya que constituye la principal fuente de energía en los tejidos especializados en llevar a cabo la síntesis de ácidos grasos: el músculo, el hígado y el tejido adiposo. Se sabe que las mitocondrias desempeñan un papel importante en la regulación de la supervivencia celular y la apoptosis, además de ser la central metabólica de la vía de los ácidos grasos, el ciclo del ácido tricarboxílico y la urea, la biosíntesis de aminoácidos, purinas y esteroides, entre otros. El contenido mitocondrial y la actividad de la cadena respiratoria son susceptibles de regulación en función de las condiciones celulares, diferentes cambios fisiológicos producidos por hormonas, cambios en los factores de crecimiento o cambios en la actividad fisiológica. Cabe destacar que la activación de las

mitocondrias podría inducir estrés oxidativo si no va acompañada de un aumento de las defensas antioxidantes endógenas. La biogénesis mitocondrial se define como el crecimiento y la división de las mitocondrias. Las mitocondrias son descendientes de una α-protobacteria endosimbionte y, por lo tanto, tienen su propio genoma y la capacidad de autoreplicarse. La biogénesis mitocondrial requiere una síntesis e importación coordinadas de aproximadamente 1000 proteínas codificadas por el genoma nuclear que se sintetiza en ribosomas citosólicos. Puede verse afectada por factores ambientales como el estrés oxidativo, la dieta, el ejercicio, los cambios de temperatura, la división celular, la renovación y la diferenciación.

El estrés oxidativo se define como una alteración del equilibrio entre especies prooxidantes y antioxidantes, a favor de las primeras. A pesar del papel fisiológico que desempeñan algunas especies de oxígeno activadas, también pueden dar lugar a reacciones de oxidación no deseadas, contra las que los organismos han tenido que desarrollar defensas antioxidantes. La formación de una cierta cantidad de radicales libres es un proceso normal e inevitable, ya que son el producto de infinitas reacciones químicas esenciales para la vida celular. Estas especies reactivas no causan daños oxidativos en condiciones normales porque la célula tiene un gran número de mecanismos antioxidantes. Sin embargo, cuando las sustancias antioxidantes

son inferiores a las prooxidantes, se produce un daño oxidativo que puede afectar a diversas moléculas que pueden reflejarse en sus funciones fisiológicas. Así pues, el estrés oxidativo puede deberse a un exceso de sustancias prooxidantes, a una deficiencia de agentes antioxidantes o a una combinación de ambos.

El resveratrol (3,5,4'-trihidroxiestilbeno) es un polifenol natural con una estructura estilbénica formada por dos anillos fenólicos unidos por un doble enlace estireno. El resveratrol está presente en numerosas plantas y frutas, como los arándanos y las moras, los frutos secos, las uvas y el vino tinto, que constituye la mayor contribución a la ingesta de resveratrol en la dieta mediterránea. El resveratrol ha llamado un gran interés en la comunidad científica debido a sus numerosas propiedades biológicas, entre las que se incluyen efectos antiinflamatorios, antioxidantes, antiagregantes plaquetarios, anticancerígenos, neuroprotectores y reguladores de la lipólisis. Es una de las moléculas del vino tinto más estudiadas y se considera responsable de lo que se conoce como la "paradoja francesa", que describe el bajo riesgo de enfermedades cardiovasculares a pesar de una dieta rica en grasas saturadas y colesterol. Esto sugiere que el resveratrol puede desempeñar un papel esencial en el metabolismo lipídico. De hecho, se ha descubierto que el resveratrol tiene múltiples

efectos metabólicos en relación con la resistencia a la insulina, especialmente en la mejora del metabolismo energético en ratones inducidos por la diabetes. Además, se ha demostrado que el resveratrol aumenta la oxidación lipídica en ratones sanos y envejecidos, inhibiendo la síntesis de ácidos grasos y activando su degradación y movilización. El piceid, o polidatina, es un derivado natural glucosilado del resveratrol que se encuentra en el vino tinto, el cacao, las uvas, las lentejas y los cacahuetes. Estudios anteriores han demostrado que el piceid ejerce diversos efectos beneficiosos, principalmente debido a su actividad antioxidante y antiinflamatoria. Según estudios anteriores, la absorción de algunos fenoles en la dieta se ve favorecida por la conjugación con glucosa. Si los glucósidos facilitan la absorción, el piceid podría absorberse más eficazmente que el resveratrol en su forma aglicona. Se han publicado numerosos estudios sobre los efectos beneficiosos del piceid, como la protección contra la isquemia miocárdica, la endometriosis, la lesión del intestino delgado durante el choque hemorrágico, la apoptosis dependiente de las mitocondrias, la inflamación hepática, la fibrosis renal diabética y la lesión de la médula espinal.

El hidroxitirosol es un polifenol con estructura feniletilalcohólica que se encuentra en el aceite de oliva y las hojas de oliva. Es un compuesto anfipático liposoluble y soluble en agua, estable en forma libre y que penetra fácilmente en los tejidos. El

hidroxitirosol suele absorberse en el intestino mediante difusión pasiva bidireccional con una eficacia de entre el 75% y el 100%. La absorción depende de la composición de la matriz alimentaria a través de la cual se administra el hidroxitirosol, ya que su absorción es mayor en su forma natural presente en el aceite de oliva virgen extra que cuando se añade en el aceite de oliva refinado. Se ha descubierto que el hidroxitirosol tiene efectos antiinflamatorios y antioxidantes a través de múltiples vías de señalización. También se ha demostrado que ejerce efectos protectores contra una serie de afecciones. Algunas de ellas siendo la disfunción endotelial, la colitis ulcerosa, la cardiopatía coronaria, la esteatosis y la hipertrigliceridemia, y la Covid-19.

El harmol es un alcaloide β-carbolina presente en el café y algunas bebidas alcohólicas. Se ha descubierto que ejerce efectos beneficiosos, como propiedades antifúngicas, antivirales y antioxidantes. Además, se ha observado que mejora el reconocimiento de objetos en ratones. Por otra parte, el harmol inhibe la monoaminooxidasa humana, que se ha relacionado con la depresión. En un estudio reciente, el harmol se describió como un compuesto mitohormetico, y se define como un estresor mitocondrial leve que desencadena respuestas compensatorias que, en última instancia, se traducen en una mejora de la función mitocondrial.

La mosca *Drosophila melanogaster* se ha utilizado durante décadas para estudios de longevidad y en los últimos años también se ha aceptado ampliamente para el análisis de la genética del desarrollo. Las principales ventajas de *Drosophila melanogaster* como organismo modelo se centran en su fácil manejo debido a su pequeño tamaño, su abundante descendencia y su relativamente corta promedia de vida.

En las últimas décadas, la esperanza de vida ha aumentado considerablemente en los países industrializados. Esto se atribuye principalmente a las nuevas terapias farmacológicas y a la adopción de un estilo de vida saludable por parte de la población. Entre estos factores, los hábitos nutricionales han desempeñado un papel especialmente relevante. En consecuencia, se ha producido un cambio en la pirámide de población. Se estima que en 2030, 1 de cada 6 personas tendrá más de 60 años y que el número de personas de 80 años o más se triplicará hasta 2050, alcanzando 425 millones de personas mundialmente.

Este cambio ha supuesto un nuevo reto para la sociedad, ya que ha provocado un aumento del porcentaje de individuos que se enfrentan a limitaciones físicas y mentales causadas por enfermedades relacionadas con la edad. Entre ellas, la fragilidad, asociada a la edad, es un síndrome geriátrico que describe a

individuos vulnerables con discapacidades crecientes, institucionalización e incluso la muerte, como describieron por primera vez Linda Fried et al. en 2001.

MATERIAL Y METODOS

Para nuestros estudios *in vitro*, se cultivaron y diferenciaron células C2C12 antes del tratamiento con 10 nM de resveratrol o piceid solos o en combinación con 1 μM de bicalutamida, inhibidor del receptor androgénico. Otro grupo de células se trató con 10 nM, 100 nM y 10 μM de hidroxitirosol. Las células se aislaron tras 24 horas de tratamiento para su posterior analisis.

Para determinar los efectos de estos polifenoles sobre el catabolismo y la biogénesis mitocondrial se realizaron RT-qPCR. El estado redox se analizó mediante RT-qPCR, así como mediante UPLC para evaluar los niveles de peroxidación lipídica, western blot para determinar la oxidación proteica y fluorímetro para medir los niveles de peróxido de hidrógeno.

Para determinar los efectos de piceid y harmol en los parámetros de longevidad y fragilidad, se separaron moscas *Drosophila melanogaster* Oregon R en grupos frágiles y robustos y se trataron con 10 nM de harmol o piceid. Se realizó una curva de longevidad y se midieron parámetros de fragilidad adaptados

por los propuestos por Linda Fried al 90%, 50% y 10% de supervivencia. Además, se recogieron moscas para realizar análisis de expresión génica en estos mismos puntos de vida con el fin de medir los genes relacionados con la longevidad, el sistema inmunitario y las enzimas antioxidantes.

RESULTADOS

Lo primero que nos propusimos fue estudiar si el resveratrol ejerce efectos sobre el catabolismo lipídico a concentraciones nutricionalmente relevantes in vitro, concretamente en células musculares esqueléticas murinas, C2C12. Para determinar la concentración óptima y la duración del tratamiento, realizamos una curva de concentración y un curso temporal. Las células fueron tratadas con resveratrol a 1 nM, 10 nM, 50 nM y 100 nM durante 6, 12, 24 y 48 horas. A continuación, se midieron los genes relacionados con el transporte de lípidos y la β-oxidación de ácidos grasos. Las células tratadas con 10 nM de resveratrol durante 24 horas mostraron un aumento significativo de *Ampk*, *Acsl*, *Cpt1*, *Cact* y *Ech1*. Además, *Acc1* se redujo en las células tratadas con 1 nM, 10 nM y 50 nM. Además, el tratamiento con 100 nM aumentó la expresión de *Ech1* después de 24 horas.

Para estudiar los efectos del resveratrol y su posible relación con el receptor androgénico en el transporte de ácidos grasos a la mitocondria, se trataron las células C2C12 con resveratrol solo y en combinación con bicalutamida. Encontramos que *Ampk* y p-AMPK aumentaron en las células tratadas con resveratrol, pero no en las células tratadas con la combinación de resveratrol y bicalutamida. Del mismo modo, la expresión génica de *Cpt1* y *Ech1* aumentó en las células tratadas con resveratrol, pero no en las tratadas con la combinación de resveratrol y bicalutamida. Estos resultados indican que el resveratrol activa el catabolismo lipídico a través del receptor de andrógenos.

En segundo lugar, nos propusimos estudiar los efectos del resveratrol y la implicación de la bicalutamida en el consumo de oxígeno de las células en tiempo real. La respiración máxima, un parámetro para medir la capacidad de las células para hacer frente a un desafío metabólico, se incrementó cuando se trataron las células con resveratrol. Curiosamente, también aumentó en las células tratadas con la combinación de resveratrol y bicalutamida, pero no con la bicalutamida sola. A continuación, medimos la capacidad de reserva de la célula, que se utiliza como parámetro para analizar la aptitud o flexibilidad de una célula. Al igual que con la respiración máxima, también observamos que la capacidad de reserva aumentaba en las células tratadas con resveratrol y en combinación con bicalutamida, pero no en las

tratadas únicamente con bicalutamida. Por último, medimos la fuga de protones, que se define como la respiración basal en reposo no acoplada a la producción de ATP. Un aumento de la fuga de protones puede ser un signo de daño mitocondrial. Encontramos una disminución en la fuga de protones únicamente en las células tratadas solo con resveratrol.

Para estudiar si la bicalutamida afecta a las propiedades antioxidantes del resveratrol, analizamos la expresión de ARNm de los genes antioxidantes catalasa, glutatión peroxidasa (Gpx) y superóxido dismutasa dependiente de manganeso (MnSOD) en comparación con el gen control *Gapdh*. Encontramos que ni el resveratrol ni la bicalutamida tenían ningún efecto sobre la expresión de *MnSOD* o *Cat*. Sin embargo, la expresión de *Gpx* aumentó significativamente en las células tratadas con resveratrol, pero no en las tratadas con resveratrol en combinación con bicalutamida. Para analizar si la bicalutamida influye en el estado redox endógeno de las células, medimos los niveles de H_2O_2. Descubrimos que las células tratadas con resveratrol mostraban niveles significativamente más bajos de H_2O_2 en comparación con las células control y las tratadas con resveratrol en combinación con bicalutamida. Para analizar los efectos del resveratrol y la bicalutamida en la oxidación de lípidos y proteínas, se midieron las proteínas carboniladas y MDA como un marcador de la peroxidación lipídica.

Encontramos que ni el resveratrol solo o en combinación con bicalutamida afectó a la carbonilación de proteínas ni los niveles de MDA. Estos resultados indicaban que el resveratrol actúa a través del receptor de andrógenos.

Para estudiar si el resveratrol se una a la membrana o a un receptor nuclear, tratamos las células con piceid y con la combinación de piceid con bicalutamida. Como el piceid es un derivado glicosilado del resveratrol, no puede penetrar fácilmente en la membrana celular, por lo que podemos evaluar la localización del receptor comparando los efectos del piceid con los del resveratrol. El tratamiento con piceid aumentó significativamente la expresión de *Ampkα-1*, pero no afectó a la expresión de *Acc1* o de las enzimas mitocondriales *Cpt1*, *Cact* o *Ech1*. Además, el tratamiento con piceid no afectó a las enzimas antioxidantes *MnSOD, Gpx* o *cat*, ni a los niveles de MDA como marcador de la peroxidación lipídica. El hecho de que el piceid no ejerciera los mismos efectos que el resveratrol indica que el resveratrol actúa a través del receptor de andrógenos intracelular.

Se ha descubierto que el polifenol hidroxitirosol ejerce múltiples efectos protectores. Sin embargo, la mayoría de los estudios publicados se realizan con concentraciones sólo disponibles como suplementos. Por lo tanto, nos propusimos investigar si el

hidroxitirosol en concentraciones nutricionalmente relevantes podría ejercer los mismos efectos que los descritos anteriormente en concentraciones elevadas. Para estudiar los efectos del hidroxitirosol en la biogénesis mitocondrial, analizamos la expresión génica de *Pgc-1α*, *Nrf1* y *Tfam*. No encontramos diferencias en la expresión de *Nrf1* y *Tfam* en ninguno de los grupos experimentales. Sin embargo, el tratamiento con 100 nM y 10 μM aumentó la expresión de *Pgc-1α*. Además, el hidroxitirosol a 10 nM aumentó significativamente la expresión de *Ampkα-1*, mientras que el tratamiento con 10 y 100 nM aumentó la expresión de *Cpt1*. Con respecto a la respiración mitocondrial, encontramos que la respiración máxima, la capacidad de reserva y la producción de ATP aumentaron significativamente tras el tratamiento con 10 nM, 100 nM y 10 μM de hidroxitirosol. En cuanto al estado redox, sólo se encontraron mejoras en las células tratadas con 10 μM.

Para analizar los posibles efectos del tratamiento con piceid y harmol sobre la longevidad, realizamos una curva de supervivencia con Drosophila Melanogaster. El piceid no afectó a la longevidad de las moscas, pero el harmol sí mejoró significativamente la longevidad de los individuos frágiles. Ni la geotaxia ni la fototaxia como parámetros de fragilidad mejoraron

en ninguno de los grupos de tratamiento. Sin embargo, a medida que las moscas envejecían, observamos una mejora en la expresión génica. Al 50% de la vida útil, encontramos una sobreexpresión de la *Attacina C* y la *Diptericina* en el grupo frágil tratado en comparación con los controles frágiles. También encontramos una sobreexpresión de la Cecropina en las moscas robustas a las que se les administró piceid en comparación con el grupo control robusto. Al 10% de longevidad, encontramos niveles significativamente más altos de todos los genes medidos en las moscas frágiles tratadas con piceid en comparación con el grupo de control frágil. Además, la diptericina se sobreexpresó en las moscas robustas tratadas con piceid en comparación con los controles robustos. En cuanto al estado redox, no se encontraron diferencias en las enzimas antioxidantes *cat* o *MnSOD* en ningún grupo de edad. Sin embargo, *p53* se elevó al 50% de supervivencia tanto en los grupos frágiles como en los robustos tratados con piceid. Sorprendentemente, encontramos una disminución de la *cecropina c* en el grupo robusto tratado con harmol en comparación con el grupo robusto de control. Del mismo modo, al 50% de supervivencia, encontramos una disminución de los niveles de *attacina C* en las moscas robustas tratadas con harmol en comparación con el grupo control robusto. Sin embargo, el tratamiento con harmol consiguió activar la diptericina y el *ARNr 16s* en las moscas frágiles.

Aunque, curiosamente, la expresión del *ARNr 16s* disminuyó en el grupo de harmol robusto en comparación con las moscas frágiles a las que se administró tratamiento con harmol. No se encontraron diferencias en la expresión de ninguno de los genes medidos al 10% de supervivencia tras el tratamiento con harmol.

DISCUSSION

Como ya se ha mencionado, existen numerosos estudios sobre los efectos beneficiosos del resveratrol. Sin embargo, la mayoría de estos estudios se realizan utilizando dosis que sólo pueden obtenerse a través de suplementos y por tanto los estudios que muestran los efectos del resveratrol en dosis alcanzables a través de la dieta son escasos.

En un estudio publicado por nuestro laboratorio se muestra que el resveratrol a 2,3 μg/kg/día, lo que equivale aproximadamente a 100 nM, induce el catabolismo lipídico en ratones viejos. Por lo tanto, estábamos interesados en comprender el mecanismo detrás de este cambio y para ver si los mismos efectos se replicaron en concentraciones aún más bajas.

Nuestros experimentos *in vitro* muestran que la concentración efectiva más baja para activar genes relacionados con el catabolismo lipídico fue de 10 nM y que el tiempo de incubación

mas eficiente era de 24 horas. Por lo tanto, nos propusimos estudiar los efectos del tratamiento con resveratrol a 10 nM sobre la respiración mitocondrial y el sistema de defensa antioxidante.

Descubrimos que el resveratrol a 10 nM aumentaba la respiración máxima y la capacidad de reserva de nuestras células, a la vez que disminuía la fuga de protones. Esta activación de las mitocondrias fue acompañada por una activación del sistema de defensa oxidante a través de una sobreexpresión de *Gpx* y una reducción de los niveles de H_2O_2.

Aunque el resveratrol aumentó significativamente la respiración máxima y la capacidad de reserva, no produjo efectos en la producción de ATP. Esto podría deberse a una disipación del gradiente de protones. La transferencia de electrones de los sustratos al oxígeno en la cadena respiratoria está acoplada a la translocación de protones a través de la membrana. El desacoplamiento mitocondrial se produce cuando las proteínas de desacoplamiento (UCP) provocan un aumento de la permeabilidad de los protones a la membrana mitocondrial interna. Esto hace que los protones vuelvan a la matriz mitocondrial sin pasar por la ATP sintasa, provocando así un aumento de la temperatura en lugar de la producción de ATP. De hecho, estudios anteriores han demostrado que el resveratrol aumenta la expresión proteica de la UCP2, incrementando así la

disipación de energía, lo que provoca una disminución de la grasa corporal en ratas. Más estudios sobre el mecanismo de acción son necesarios para determinar si UCP podría haber causado la falta de producción de ATP encontrado en nuestros experimentos.

La concentración de resveratrol utilizada en nuestros estudios podría conseguirse a través de la dieta consumiendo alimentos ricos en resveratrol. Sin embargo, la biodisponibilidad de los polifenoles sigue siendo un reto para la confirmación de su eficacia. En este contexto, cabe destacar los estudios sobre la absorción y la biodisponibilidad del resveratrol.

Un estudio publicado en 2004 muestra que más del 70% del resveratrol se absorbe. En este estudio, administraron 25 mg de resveratrol a seis voluntarios sanos antes de medir sus niveles plasmáticos. Registraron niveles plasmáticos máximos de 491 ng/mL de resveratrol y sus metabolitos y 5 ng/mL de resveratrol inalterado. Estas dosis equivalen aproximadamente a 2 μM y 22 nM respectivamente. Para obtener 10 nM de resveratrol inalterado basándonos en esta tasa de absorción, tendríamos que consumir unos 11 mg de resveratrol. Esto podría conseguirse consumiendo 300 mL de vino tinto, 200 gramos de arándanos rojos y 100 gramos de cacahuetes. Sin embargo, si se tiene en cuenta las concentraciones de resveratrol con sus metabolitos

que se encuentran en el plasma, sólo sería necesario consumir 0,11 mg de resveratrol.

Otro estudio realizado en hombres con diabetes tipo 2 controlada descubrió que tras ingesta prolongada de resveratrol la biodisponibilidad era mucho mayor. Se administró a los participantes 150 mg de resveratrol al día durante 30 días y se observaron niveles plasmáticos de resveratrol inalterado de hasta 378 ng/mL, lo que equivale a 1,66 μM. Para obtener los 10 nM utilizados en nuestros experimentos, basándonos en estas concentraciones plasmáticas, sólo necesitaríamos consumir 0,9 mg de resveratrol. Esto equivaldría a menos de 30 gramos de arándanos rojos, 80 gramos de cacahuetes o 100 mL de vino tinto, al día. Como el resveratrol es una molécula liposoluble, una ingesta repetida de resveratrol permite que se almacene en la grasa, generando así una reserva, lo que resulta en una mayor biodisponibilidad. Por lo tanto, sería posible alcanzar estas concentraciones siguiendo una dieta rica en estos alimentos que contienen resveratrol.

Una vez establecidas las dosis y el tiempo de tratamiento, nos propusimos a estudiar la farmacodinámica del resveratrol. Al ver que el resveratrol era capaz de ejercer efectos a concentraciones tan bajas como 10 nM, especulamos que este efecto podría estar mediado por un receptor, y en concreto por un receptor

hormonal. Por lo tanto, nos propusimos estudiar si el resveratrol está mediado por un receptor andrógeno, ya que sabemos que son abundantes en las células de músculo esquelética.

Se sabe que el resveratrol activa la β-oxidación mitocondrial de los ácidos grasos a través de la activación de la AMPK, aunque aún se desconoce la vía de señalización exacta. Además, el resveratrol estimula la proteína quinasa 2 dependiente de Ca2+/calmodulina (CaMKK2), que fosforila y, por tanto, activa la AMPK en respuesta al aumento del Ca2+ citoplasmático. Curiosamente, se ha demostrado que la CaMKK2 está regulada transcripcionalmente por un receptor andrógeno. Se ha descubierto que la deficiencia de testosterona promueve la resistencia a la insulina en el músculo esquelético, al menos parcialmente, tanto en hombres como en mujeres a través de un mecanismo dependiente del receptor andrógeno, lo que implica una disminución de las fibras musculares oxidativas y sensibles a la insulina mediadas por PGC1α. Queda por determinar si el resveratrol está mediado por receptores andrógenos y si actúa a través de la misma vía que la cAMKK2.

Hay muchos estudios publicados sobre los efectos de altas concentraciones de resveratrol en la actividad transcripcional dependiente de receptores andrógenos en las células cancerígenas. Un estudio publicado en 2010 señaló que 10 μM y

50 μM de resveratrol disminuían la expresión del antígeno prostático específico, mientras que 1 μM aumentaba la actividad transcripcional del receptor andrógeno en las células LNCaP que son androgeno-dependientes, pero no en su subclón andrógeno-independiente, las células C4-2. Estos resultados se ven confirmados por un estudio similar sobre los efectos del resveratrol en el cáncer de próstata. Éstos informaron de que la activación de la expresión génica impulsada por el receptor de andrógenos tras el tratamiento con dosis bajas de resveratrol dependía de la presencia de andrógenos. Por lo tanto, en las células de próstata que contienen altos niveles de andrógenos endógenos, 1 - 10 μM resveratrol se estimuló, mientras que a 100 μM se inhibió la actividad de luciferasa. Curiosamente, se observó que las concentraciones de 0,1 μM o inferiores no afectaban a la actividad de la luciferasa ni a los niveles de proteína del receptor androgéno.

A pesar de estos hallazgos, faltan estudios sobre los efectos del resveratrol y su correlación con los receptores de andrógenos en otros tipos celulares y a bajas concentraciones. Nuestros experimentos demuestran que el resveratrol a concentraciones nutricionalmente relevantes activa el catabolismo lipídico y mejora ciertos parámetros de estrés oxidativo, y más importante, que estos efectos no se producen cuando las células son tratadas

con resveratrol en combinación con el inhibidor del receptor andrógeno, bicalutamida.

En esta tesis doctoral, demostramos por primera vez que el resveratrol actúa de forma similar a una hormona activando el catabolismo lipídico y reduciendo el estrés oxidativo a través de los receptores androgénicos a concentraciones nutricionalmente relevantes en células de músculo esquelético.

Se sabe que que existen varios receptores andrógenos. Por ello, nos propusimos determinar la localización del receptor a través del cual actúa el resveratrol. Estudiando los efectos del piceid y comparándolos con los del resveratrol, pudimos analizar si el receptor es intracelular o de membrana. Al estar glucosilado, el piceid no puede entrar fácilmente en la célula sin la presencia de un transportador específico. Nuestro análisis de los efectos del piceid sobre los genes relacionados con el catabolismo lipídico y las enzimas antioxidantes, así como sobre el MDA como marcador de la peroxidación lipídica, mostró que el piceid no ejerce los mismos efectos que el resveratrol. Esto indica que el resveratrol actúa a través de los receptores andrógenos intracelulares.

Después de determinar los efectos del resveratrol y de los receptores andrógenos en el catabolismo lipídico, estábamos interesados en analizar si el resveratrol podría aumentar la

respiración mitocondrial en concentraciones nutricionalmente relevantes y si este aumento podría ser producido por la activación de los receptores andrógenos. Encontramos que el resveratrol a 10 nM aumentaba la respiración máxima y la capacidad de reserva mientras disminuía la fuga de protones. Sin embargo, la respiración máxima y la capacidad de reserva también aumentaron en las células tratadas con resveratrol en combinación con bicalutamida, aunque no cuando se trataron con bicalutamida sola. Estos resultados indican que el resveratrol mejora la respiración mitocondrial a través de una vía independiente de los andrógenos. Sin embargo, es importante tener en cuenta que este experimento se realizó con un tratamiento agudo de 30 minutos y no de 24 horas como con los otros parámetros analizados.

El hidroxitirosol es un polifenol abundante en el aceite de oliva, abuntante en la Dieta Mediterránea. Se ha descrito que el hidroxitirosol ejerce efectos protectores contra la disfunción endotelial en células endoteliales de aorta humana. Este estudio determinó que tanto el hidroxitirosol libre como sus metabolitos eran eficaces en la reducción de los biomarcadores de disfunción endotelial a dosis fisiológicas. Por lo tanto, nos propusimos

investigar los efectos de este polifenol sobre la función mitocondrial a dosis bajas en células musculares esqueléticas.

Con respecto a la biogénesis mitocondrial y el transporte de ácidos grasos, el hidroxitirosol aumentó la *pgc-1α*, *ampk-1α* y *cpt-1* en concentraciones nutricionalmente relevantes. Se han descrito anteriormente resultados similares tanto en adipocitos 3T3-L1 como en hepatocitos. También encontramos que la respiración mitocondrial se incrementó significativamente en las células tratadas con hidroxitirosol de todas las dosis estudiadas, lo que sugiere que 10 nM es suficiente para aumentar la respiración mitocondrial y la producción de ATP de manera eficiente. A nuestro saber, el nuestro es el primer estudio que analiza los efectos del hidroxitirosol sobre la respiración mitocondrial en tiempo real.

Como se ha explicado anteriormente, las mitocondrias son una de las mayores fuentes de oxidantes endógenos del organismo. Por lo tanto, es importante que la activación de las mitocondrias vaya acompañada de una activación del sistema antioxidante del organismo. Desafortunadamente, el estado redox de nuestras células no pareció mejorar en la mayoría de los parámetros medidos, y las pocas mejoras de los sistemas de defensa encontradas sólo estaban presentes en las células tratadas con 10 μM. En estudios anteriores se ha observado que el hidroxitirisol

ejerce efectos antioxidantes tanto *in vitro* como *in vivo*. Sin embargo, estos estudios se realizaron con dosis considerablemente más altas que las utilizadas en nuestros estudios. Esto sugiere que el hidroxitirosol podría no ser capaz de combatir estresores oxidantes tras un aumento de la respiración mitocondrial a concentraciones nutricionalmente relevantes en células de músculo esquelético.

En esta tesis doctoral, demostramos que el hidroxitirosol en dosis obtenibles a través de la dieta puede ejercer efectos beneficiosos sobre la función mitocondrial. Sin embargo, como ocurre con todos los nutrientes, y especialmente con los polifenoles, hay que tener en cuenta su absorción y biodisponibilidad.

Un estudio reciente sobre la biodisponibilidad del hidroxitirosol encontró concentraciones plasmáticas de hasta 3,79 ng/mL tras una ingesta de 5 mg, lo que equivaldría a 24,6 nM. Este estudio también mostró cómo la biodisponibilidad del hidroxitirosol dependía de la matriz alimentaria, mostrando cómo las matrices aceitosas favorecen su absorción. Es importante tener esto en cuenta a la hora de planificar nuestra dieta para obtener los máximos beneficios de este compuesto fenólico.

Basándonos en estos hallazgos, las concentraciones de 10 nM, 100 nM y 10 μM en este estudio podrían alcanzarse consumiendo 2 mg, 20 mg y 2000 mg respectivamente. Los estudios han

encontrado concentraciones de hidroxitirosol en aceitunas de hasta 413 mg/100 g, 3,47 mg/100 mL de aceite de oliva y 0,96 mg/100 mL de vino tinto. Esto indica que podríamos alcanzar fácilmente tanto 10 nM como 100 nM de hidroxitirosol siguiendo una dieta rica en estos compuestos. Para alcanzar 10 nM de hidroxitirosol, se podrían consumir 200 mL de vino tinto o menos de 60 mL de aceite de oliva, se podría alcanzar los 100 nM consumiendo solo 4,84 gramos de aceitunas negras.

Otro estudio en el que se administró a voluntarios 25 mL de aceite de oliva virgen extra registró concentraciones plasmáticas de hidroxitirosol de 4,40 ng/mL. Esta concentración equivale aproximadamente a 28 nM, por lo que se podrían alcanzar 10 nM con una cucharada y 100 mM con 90 mL de aceite de oliva virgen extra. Curiosamente, solo se encontraron trazas en los sujetos que consumían aceite de oliva corriente, lo que indica que la calidad del aceite está muy correlacionada con la biodisponibilidad del hidroxitirosol.

Sin embargo, un estudio de 2013 en el que se comparaba la diferencia en la biodisponibilidad del hidroxitirosol en hombres y mujeres informó de mayores niveles plasmáticos de hidroxitirosol en hombres que en mujeres tras una suplementación de 9,7 mg. La concentración pico media fue de 28 ng/mL en mujeres, con valores que oscilaban entre 9-94 ng/mL.

Mientras que en los hombres, las concentraciones plasmáticas máximas medias fueron de 117 ng/mL, con valores que oscilaron entre 42-325 ng/mL. Esto indicaría que las mujeres necesitarían consumir 534 mg de media de hidroxitirosol, lo que equivale a unos 130 gramos de aceitunas, para alcanzar concentraciones plasmáticas de 10 μM. En comparación, los hombres necesitarían consumir 30 gramos de aceitunas para alcanzar esta misma concentración. Aunque, según estos resultados, ambos sexos podrían alcanzar tanto 10 nM como 100 nM incluyendo regularmente aceite de oliva, vino tinto o aceitunas en su dieta.

Curiosamente, la diferencia de biodisponibilidad entre sexos no parece trasladarse a las ratas. En un estudio publicado en 2015, se observó que las concentraciones plasmáticas de hidroxitirosol eran mayores en las ratas hembras en comparación con los machos. Deberían realizarse más estudios sobre la biodisponibilidad en todos los modelos animales para confirmar las diferencias entre sexos a este respecto.

En un estudio realizado previamente en nuestro laboratorio se ha demostrado que el consumo moderado de vino tinto puede aumentar la longevidad en *Drosophila melanogaster*. Nos interesaba estudiar si este efecto podía deberse en parte a la presencia de piceid. Para ello, lo primero que hicimos fue realizar

una curva de longevidad y evaluar parámetros asociados a la fragilidad, adaptados de los publicados por Linda Fried.

En condiciones óptimas de vida, es en la última etapa de la vida cuando aumenta la probabilidad de muerte. Es importante tener en cuenta que el envejecimiento no es una enfermedad y debe separarse de las causas de muerte por vejez. El envejecimiento se refiere a procesos post-madurativos que conducen a una disminución de la homeostasis y a un aumento de la vulnerabilidad del organismo, lo que conlleva una mayor predisposición a padecer diferentes patologías que terminan con la muerte del individuo.

Sin embargo, la fragilidad se considera un síndrome geriátrico que se presenta en individuos vulnerables con un aumento de las discapacidades y de la institucionalización. Las Pruebas Musculares Manuales del Consejo de Investigación Médica se utilizan ampliamente en pacientes para evaluar la debilidad y pueden ser eficaces para diferenciar la verdadera debilidad del desequilibrio o de la escasa resistencia. De este modo, la superación de la gravedad para realizar un movimiento, con o sin resistencia, determinará la fuerza muscular del paciente. Del mismo modo, la mosca tendrá menos capacidad para ascender por el tubo de ensayo en individuos frágiles en los que tenga debilidad muscular. Correlacionamos la baja actividad física con

la fototaxia, ya que la mosca se siente naturalmente atraída por la luz e instintivamente volaría hacia las fuentes de luz. Su renuencia o incapacidad para hacerlo podría indicar, por tanto, bajos niveles de actividad. Del mismo modo, medir la sarcopenia o pérdida de peso en la mosca sería complicado. Por tanto, optamos por una medición del consumo de alimento de la mosca para evaluar este parámetro. Aunque el piceid no mejoró ninguno de los parámetros de fragilidad, sí encontramos un aumento significativo tanto de la longevidad como de la fototaxia en los grupos robustos en comparación con las moscas frágiles, lo que indica que los métodos utilizados para evaluar la fragilidad son eficaces.

A día de hoy, existen varias teorías del envejecimiento, entre ellas la teoría inmunológica del envejecimiento. La teoría inmunológica del envejecimiento afirma que el sistema inmunitario se deteriora progresivamente con el tiempo a medida que los anticuerpos pierden su eficacia. Esto aumenta la vulnerabilidad a las enfermedades infecciosas, que provocan estrés celular y, finalmente, la muerte. Se ha demostrado que el sistema inmunitario desempeña un papel fundamental en diversas enfermedades relacionadas con la edad y en el año 2000 se acuñó el término "*inflamm-aging*". Este fenómeno hace referencia a una reducción global de la capacidad para hacer frente a los factores estresantes durante el envejecimiento, lo que

conlleva un aumento progresivo concomitante del estado proinflamatorio.

En vista de ello, nos propusimos estudiar los efectos de dosis nutricionalmente relevantes de piceid en genes relacionados con el sistema inmunitario en *Drosophila melanogaster*. Al 80% de supervivencia no encontramos diferencias en la expresión de los genes de interés. Esto era de esperar, ya que las moscas en este momento son todavía bastante jóvenes. Al 50% de supervivencia, encontramos una mayor expresión de *attacina C* y *diptericina* en el grupo frágil suplementado con piceid en comparación con el control. Además, la *cecropina C* se sobreexpresó en el grupo robusto tratado con piceid en comparación con los controles robustos y el grupo frágil tratado con piceid. Al 10% de supervivencia, el piceid activó todos los genes de interés en los individuos frágiles, así como *la diptericina* en las moscas robustas. Esta sobreexpresión en los individuos frágiles sugiere que el piceid podría ejercer un efecto protector contra las infecciones en las moscas envejecidas. También era de esperar que no hubiera diferencias entre los individuos robustos tratados con piceid y los de control, ya que se ha demostrado que los individuos robustos tienen una mayor longevidad y, por tanto, ya tendrían un sistema inmunitario más activo.

Otra teoría bien descrita de la disminución del estado de salud que se produce a medida que envejecemos es la teoría de los radicales libres del envejecimiento. Este término fue acuñado por Denham en 1956, quien propuso que los efectos de los radicales libres en los componentes celulares causan los daños y las enfermedades degenerativas asociadas al envejecimiento. Por lo tanto, se propuso que la administración de antioxidantes podría prevenir este daño causado por estos radicales y, por lo tanto, prevenir las enfermedades degenerativas relacionadas con la edad. En años posteriores se ha propuesto que esta teoría es la causante de la fragilidad y no del envejecimiento, ya que aumenta el riesgo de discapacidad. Por lo tanto, estábamos interesados en estudiar los posibles efectos del piceid sobre las enzimas antioxidantes. Desafortunadamente, no se encontraron cambios en ninguno de los grupos de tratamiento.

Como se ha mencionado anteriormente, el piceid a 10 nM indujo la expresión de genes del sistema inmunitario en moscas envejecidas y, por tanto, podría proteger a las moscas de las infecciones a medida que envejecen. Sin embargo, a las dosis utilizadas en este proyecto, piceid no fue capaz de reducir el estrés oxidativo ni de mejorar los parámetros de fragilidad ni la longevidad de las moscas. Dado que se ha observado que dosis más elevadas de piceid reducen el estrés oxidativo en otros modelos, deberían realizarse más estudios con piceid a otras

concentraciones para determinar mejor su función sobre los parámetros de fragilidad y longevidad en *Drosophila melanogaster*.

El harmol es un alcaloide escasamente estudiado, con sólo dos estudios publicados sobre sus efectos en *Drosophila melanogaster*. De estos dos estudios, uno analizó sus efectos sobre la longevidad. Este estudio de Costa-Machado, Luis Filipe et al. encontró que el harmol a 25 μg/ml aumentaba la longevidad en *Caenorhabditis Elegans* y *Drosophila melanogaster*. Por ello, nos interesaba estudiar si dosis bajas de harmol podían replicar estos efectos, así como analizar su efecto sobre los parámetros de fragilidad en moscas envejecidas.

Aunque la longevidad mejoró con un aumento significativo de la supervivencia media en las moscas frágiles tratadas con harmol, no se encontraron diferencias significativas en los parámetros que propusimos para medir la fragilidad. Sin embargo, se muestra una clara tendencia en el ensayo de fototaxia a una tasa de supervivencia del 10%, lo que sugiere que harmol podría ejercer algunos efectos protectores contra la fragilidad en las últimas etapas de la vida.

Curiosamente, al 50% de supervivencia, el *16s rRNA* mejoró en el grupo frágil tratado con harmol. Se ha descrito que este transcrito mitocondrial ribosómico se correlaciona con la forma

de la curva de longevidad en moscas macho, precediendo a la disminución de la supervivencia. Esto apoya nuestros hallazgos de mejora de la tasa media de supervivencia, pero no de la duración de la vida, ya que este gen no se sobreexpresa al 10% de supervivencia. Sin embargo, algunos de los genes relacionados con el sistema inmunitario se expresa menos en las moscas robustas tratadas con harmol. Esto, unido al hecho de que no se encontraron mejoras en la expresión de enzimas antioxidantes ni en los parámetros de fragilidad, indica que, aunque el harmol afecta positivamente a la longevidad, este aumento no va acompañado de una mejora del estado de salud de las moscas envejecidas.

Es importante tener en cuenta que estos estudios se realizaron exclusivamente en poblaciones de machos de *Drosophila melanogaster*. Deberían realizarse más estudios para determinar los efectos de estos compuestos en las moscas hembra.

CONCLUSIONES

A partir de los resultados presentados en esta tesis doctoral, podemos extraer las siguientes conclusiones:

1. El resveratrol activa el catabolismo lipídico y ejerce efectos protectores frente al estrés oxidativo a

concentraciones nutricionalmente relevantes en células murinas de músculo esquelético.

2. El resveratrol ejerce sus efectos beneficiosos sobre la función mitocondrial y el estado redox a través de los receptores andrógenos intracelulares.
3. El hidroxitirosol activa el catabolismo lipídico y aumenta la respiración mitocondrial, pero no activa los sistemas antioxidantes a concentraciones nutricionalmente relevantes.
4. El piceid activa los genes relacionados con el sistema inmunitario, pero no mejora la longevidad, la fragilidad ni el estado redox de los machos envejecidos de *Drosophila melanogaster* a concentraciones nutricionalmente relevantes.
5. Harmol mejora la longevidad, pero no los parámetros de fragilidad o el estado de salud de los machos envejecidos de *Drosophila melanogaster*.

INTRODUCTION

INTRODUCTION

1.1 Metabolism

Metabolism is defined as the complete set of all chemical reactions in the organism. These constitute the pathways that extract energy from nutrients, which are used for work and storage of excess energy to be used later. Anabolic pathways are those that synthesize large molecules from simpler ones, while catabolic pathways are those that break down large molecules. Some of the energy generated by the metabolism is transformed into heat, which is used to maintain proper body temperature. The rest of the energy is transformed into work, or to be stored as high-energy molecules to be released in various processes when needed (1,2).

1.1.1 Lipid metabolism

Lipids are involved in the formation of cell membranes, protein modification, they act as signaling molecules, and their oxidation is one of the main sources of energy. The mitochondrial oxidation of long-chain fatty acids (LCFA) plays an important role in energy homeostasis, as it constitutes the main source of energy in the tissues specialized in carrying out fatty acid synthesis - the muscle, liver, and adipose tissue (3).

When the extracellular concentration of LCFAs is high (of micromolar magnitudes), they are able to freely pass the plasma membranes through passive diffusion, a mechanism identical to that of the entry of short- and medium-chain fatty acids. On the other hand, when the plasma concentration of LCFAs is low (of nanomolar magnitudes), their transport must be carried out by facilitated transport (4). Once taken up by the cell, the fatty acids are transported by fatty acid transport proteins to the mitochondria, peroxisomes, or the endoplasmic reticulum for their use or storage (5).

Once entered the cells, the fatty acids must be activated by long-chain acyl coenzyme A (acyl-CoA) synthetase in the cytosol, the only reaction of fatty acid catabolism that requires ATP (6,7). After they have been activated, these long-chain acyl-CoAs are introduced into the mitochondria for oxidation. This transport is carried out by carnitine palmitoyl transferase 1 (CPT-1), which catalyzes the reaction that produces acyl-carnitine, and carnitine-acylcarnitine translocase (CACT) transfers the fatty acid towards the mitochondrial matrix (8,9). Once inside the mitochondrial matrix, carnitine palmitoyl transferase 2 (CPT-2) catalyzes the exchange of the carnitine group for the CoA group in acyl, thereby obtaining acyl-CoA (10).

Acyl-CoA in the mitochondrial matrix is degraded by β-oxidation enzymes in a process of four steps that are repeated until the acyl-CoA molecule is completely broken down into acetyl-CoA molecules. The first step is dehydrogenation, where an enoyl-CoA molecule is obtained. Next, this molecule is transformed into hydroxyacyl-CoA in a process called hydration, by incorporating a water molecule by the action of enoyl-CoA hydratase. The hydroxyacyl-CoA is oxidized through dehydrogenation, and a ketoacyl-CoA molecule and $NADH_2$ is obtained. The final step is thiolic rupture, where the free CoA molecule generates an acetyl-CoA molecule and an acyl-CoA containing two carbons less in its chain (11) (Figure 1).

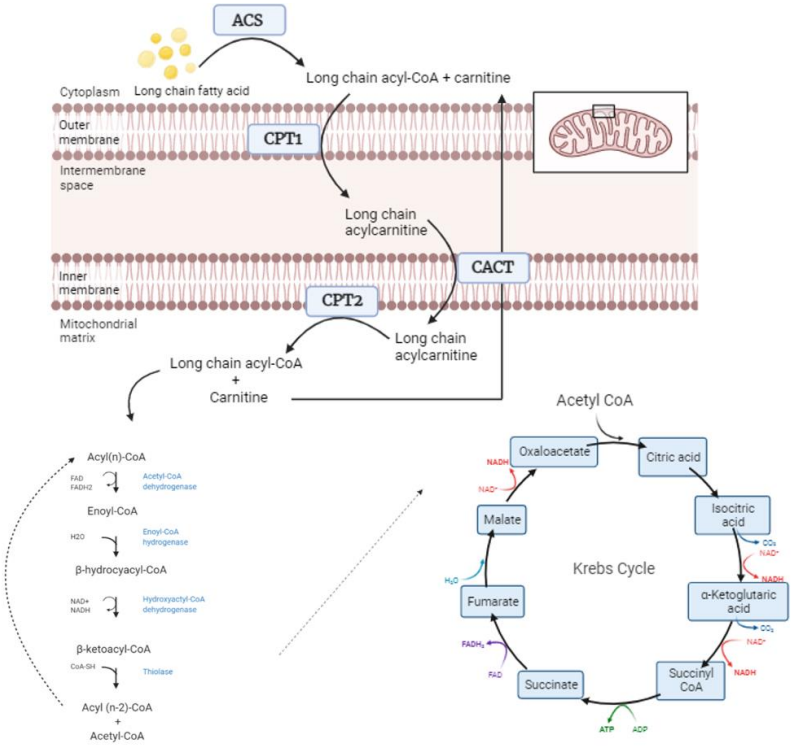

Figure 1. Transport and β-oxidation of long-chain fatty acids

Created with BioRender.com

Acetyl-CoA enters the Krebs cycle to generate reduced compounds to be used for oxidative phosphorylation. It can also be transported to the exterior of the mitochondria in the form of citrate and reconverted to acetyl-CoA in the cytosol. Due to the action of acetyl-CoA carboxylase (ACC), acetyl-CoA is then transformed into malonyl-CoA, an essential metabolite in

maintaining the balance between the synthesis and oxidation of fatty acids (12).

Another main regulator of fatty acid synthesis has been found to be AMP-activated protein kinase (AMPK). The activity of this enzyme is modulated by a wide variety of stimuli, including nutritional status, and once activated, it phosphorylates and inhibits ACC, thereby inhibiting the synthesis of malonyl-CoA (13,14). AMPK also regulates mitochondrial metabolism, mainly through peroxisome proliferator-activated receptor γ co-activator 1α (PGC1α). By directly phosphorylating and activating this transcription factor AMPK causes the transcription of genes involved in oxidative phosphorylation and mitochondrial biogenesis (15).

1.2 The mitochondria

The mitochondria are cellular organelles with an oval structure the size of a bacteria, approximately 2 μm and 0.5 μm diameter. The mitochondria contains an internal and external membrane and therefore make up two compartments: the intermembrane space situated between the external and internal membrane, and the matrix, which is enveloped by the inner membrane.

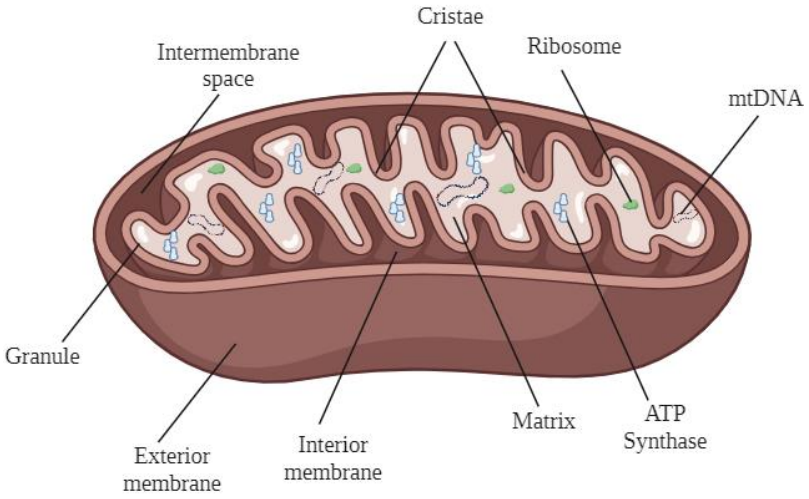

Figure 2. Simplified mitochondrial structure.

Created with BioRender.com

The matrix contains multiple copies of the circular mitochondrial genome, ribosomes, and numerous enzyme complexes necessary for both energetic functions and gene expression and replication. Specifically, the human mitochondrial genome contains 16,569 base pairs and encodes 13 respiratory chain proteins as well as the large and small ribosomal (ribonucleic acid) RNAs (rRNA) and enough transfer RNA (tRNA) to translate all codons. However, mitochondria also contain many proteins encoded by nuclear deoxyribonucleic acid (DNA). The intermembrane space contains enzymes, some of which have kinase activity, that mediate the transfer of substances between the mitochondrial matrix and the cytosol.

Similarly, their membranes show significant differences. The inner mitochondrial membrane is morphologically characterized by invaginations called cristae. They contain a large number of enzyme complexes, such as components of the electron transport and adenosine triphosphate (ATP) synthase and proteins that regulate the passage of metabolites, such as the adenine nucleotide translocator. The inner mitochondrial membrane is particularly impermeable to ions due to its high content of the phospholipid cardiolipin, an essential feature that allows it to maintain an electrochemical gradient necessary for the performance of the energetic function. On the other hand, the outer membrane lacks mitochondrial ridges and, under physiological conditions, is permeable to the passage of ions and metabolites with molecular weights below 6,000 Da due to the presence of the porin protein, now known as the voltage-dependent anion channel.

Despite the existence of these compartments, which at first sight seem watertight, the mitochondrial structure allows the regulated passage of proteins through processes controlled by transmembrane protein complexes -TIM (translocase of the inner membrane) in the inner membrane and TOM (translocase of the outer membrane) in the outer membrane. These are able to discriminate between the proteins that must go to the matrix and those that will go to the intermembrane space. Some members of

the heat shock protein (HSP) family, such as HSP 70 and HSP 60, are involved in this process (16). There are contact zones between mitochondrial membranes, termed dense zones, whose protein components are able to interact and lead to the formation of a channel, the MTPP (mitochondrial transient permeability pore), in which proteins from the cytoplasm and mitochondrial matrix participate.

1.2.1 Mitochondrial function

Mitochondria are known to play an important role in the regulation of cell survival and apoptosis as well as being the metabolic powerhouse for the fatty acid pathway, tricarboxylic acid and urea cycle, biosynthesis of amino acids, purines, and steroids, among others (17). Therefore, the mitochondrial content and activity of the respiratory chain are susceptible to regulation depending on cellular conditions. Thus, they should respond to the different physiological changes produced by hormones, changes in growth factors, or changes in physiological activity.

Among the functions carried out in the mitochondrion, oxidative phosphorylation is of great importance. This takes place in the electron transport chain, carried out by an enzyme complex that generates reducing energy formed by NADH and FADH2-,

which is used in the respiratory chain. Coupled with the transport of electrons through different redox levels, and thanks to the energy released, there is an outflow of three protons from the mitochondrial matrix into the intermembrane space. This creates an electrochemical gradient between the two compartments, resulting in an increase in free energy. This energy is used by ATP synthase (complex V), located in the inner membrane, to phosphorylate ADP molecules, thereby producing ATP, while protons return to the matrix down the electrochemical gradient (18) (Figure 3). The correct functioning of this enzyme complex is essential for cellular function, so any alteration in these enzymes can lead to degenerative diseases. There are many known inhibitors of the various components of the mitochondrial electron transport chain (METC), which are used widely in studies of mitochondrial function.

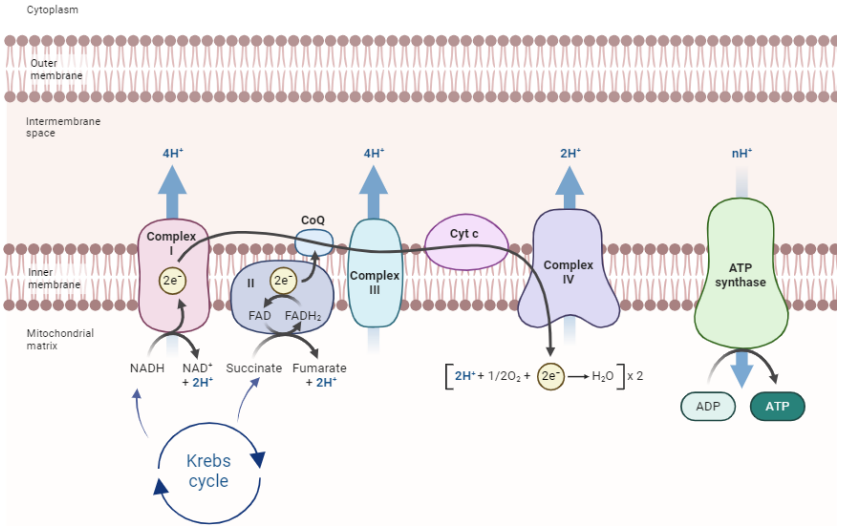

Figure 3. Mitochondrial electron transport chain.

Created with BioRender.com

1.2.2 Mitochondrial biogenesis

Mitochondrial biogenesis is defined as the growth and division of mitochondria. Mitochondria are descendants of an α-proteobacteria endosymbiont and therefore have their own genome and have the ability to autoreplicate. The mitochondrial DNA (mtDNA) is a double-stranded circular molecule of approximately 16.5 kb containing 37 genes that encode 13 subunits of the electron transport chain complexes. There are also 22 tRNA and two ribosomal RNAs (rRNA) necessary for the translation of the respiratory subunit messenger RNA (mRNA) within the mitochondrial matrix (19). Mitochondrial biogenesis

requires a coordinated synthesis and import of approximately 1000 proteins encoded by the nuclear genome which is synthesized on cytosolic ribosomes (20). It can be affected by environmental factors such as oxidative stress, diet, exercise, temperature changes, cell division, renewal, and differentiation.

AMPK is a major regulator of mitochondrial biogenesis, which regulates intracellular energy metabolism in response to AMP:ATP ratio. It is activated by metabolic stressors that inhibit ATP production, such as hypoxia, metabolic inhibitors, or glucose deprivation, and by stimulators of ATP consumption, such as motor proteins, ion channels, and pumps. AMPK is also modulated by cytokines that regulate energy balance, drugs used to treat diabetes type 2, and natural plant products (21). Once activated, AMPK switches on catabolic pathways, such as the uptake and metabolism of glucose and fatty acids, thereby generating ATP. Parallelly, it switches off ATP-consuming anabolic pathways, such as fatty acid, cholesterol, glycogen, or protein synthesis. AMPK achieves this through a rapid phosphorylation of transcription factors, metabolic enzymes, and co-activators that regulate gene expression (21).

Additionally, PPAR (peroxisome proliferator-activated receptor)-γ coactivator-1α (PGC-1α) is a co-transcriptional regulation factor regulated by AMPK that induces mitochondrial

biogenesis through the activation of different transcription factors, such as nuclear respiration factors 1 and 2 (NRF-1 and NRF-2). NRF-1 and NRF-2 in turn increase the transcription of key mitochondrial enzymes. They have also been shown to activate mitochondrial transcription factor A (TFAM), which drives transcription and replication of mtDNA, therebyinducing mitochondrial biogenesis (22).

1.3 Free radicals

Free radicals are chemical species that contain one or more unpaired electrons in their valence shell. The presence of this type of electron makes these species highly reactive. They are characterized by their high oxidizing power and because their half-life is usually very short. In nature, free radicals are mostly oxygen-derived compounds and are called reactive oxygen species (ROS).

Oxygen is naturally found mostly in diatomic form (O_2), containing 16 electrons correspondingly distributed in its various orbitals according to the theory of molecular orbitals (23). The most thermodynamically stable structure of the molecule forms a distribution of electrons that leaves two electrons in the valence shell unpaired, which enables it to react efficiently with

free radicals, although its reaction rate with radical species is usually low (24). There are a large number of oxygen-derived species that are very reactive or are capable of producing reactive species. Some of these species are true oxygen-derived free radicals, such as the hydroxyl radical ($OH^{\bullet}$) or superoxide anion radical ($O_2^{-\bullet}$). Others, such as hydrogen peroxide (H_2O_2), are not technically free radicals in the strict sense of the word as they do not lack electrons in their valence shell. However, they possess a strong oxidizing potential and are therefore known as non-radical oxidants (25).

1.3.1 Endogenous sources of free radicals

The body is constantly producing free radicals. Some of them occur through "chemical accidents", that is, unwanted secondary reactions between biomolecules, whereas other activated oxygen species are generated *in vivo* for a specific purpose, as in the case of activated phagocytes, which produce $O_2^{-\bullet}$ and H_2O_2 (26). It has been estimated that each cell of the body produces approximately 10^{10} superoxide anion molecules per day (27). However, 99% of these molecules go through dismutation to produce H_2O_2 (24).

$$2O_2^{-\bullet} + 2H \longrightarrow H_2O_2 + O_2$$

H_2O_2 is a relatively stable molecule in the absence of catalysts to promote its decomposition. Fenton discovered in 1894 that organic molecules could be oxidized by H_2O_2 and Fe^{2+} (Fenton's reagent) (28). Haber and Weiss later proved how Fe^{2+} reduces H_2O_2, which in turn decomposes into hydroxyl radical and hydroxyl ion, the most reactive of the species.

$$H_2O_2 + Fe^{2+} \longrightarrow Fe^{3+} + OH^- + OH^\bullet$$

H_2O_2 molecules can also be split into two hydroxyl radicals by UV rays. Hydrogen radicals can also be formed from superoxide anion and H_2O_2 catalyzed by metals such as copper and iron.

$$H_2O_2 + O_2^{-\bullet} \longrightarrow O_2 + OH^- + OH^\bullet$$

As previously mentioned, the electron transport chain produces energy in the form of ATP through oxidative phosphorylation. It is therefore one of the main sources of free radicals in the cell. Other endogenous sources of ROS include cellular organs that have a high oxygen consumption such as the endoplasmic reticulum and peroxisomes (29).

1.3.2 Exogenous sources of free radicals

Free radicals can also be produced by exogenous sources. These include environmental factors such as air and water pollution, ozone, pesticides, and industrial solvents. Lifestyle also has a big impact on the production of free radicals. Alcohol consumption and tobacco smoke, certain oils and smoked meat, exposure to high temperatures and ultraviolet light increase the risk of free radical production. Lastly, prolonged intake of drugs like paracetamol, anesthetics, and nonsteroidal anti-inflammatory drugs such as ibuprofen and naproxen are considered free radical sources (29,30).

1.3.3 Oxidative stress

Oxidative stress is defined as an alteration of the balance between prooxidant and antioxidant species, in favor of the former. Despite the physiological role played by some activated oxygen species, they can also give rise to unwanted oxidation reactions, against which organisms have had to develop antioxidant defenses (31). The formation of a certain amount of free radicals is a normal and inevitable process, since they are the product of an infinite number of chemical reactions that are essential for cellular life. These reactive species do not cause oxidative

damage under normal conditions because the cell is provided with a large number of antioxidant mechanisms (32). However, when the antioxidant substances are lower than the prooxidants, oxidative damage is produced which can affect various molecules and can be reflected in their physiological functions. Thus, oxidative stress can be caused by an excess of prooxidant substances, a deficiency of antioxidant agents, or a combination of both.

1.3.3.1 Lipid peroxidation

Of the main types of biomolecules, lipids, and especially polyunsaturated fatty acids, are the most susceptible to be attacked by free radicals with hydroxyl (HO-), peroxyl (ROO-), alkoxyl (RO-) and alkyl (R-) radicals being the main generators of oxidative damage to lipids (24).

The process of oxidative attack on lipids, known as lipid peroxidation, begins when a free radical attacks a carbon of the aliphatic chain of a fatty acid, releasing a hydrogen atom and forming an alkyl radical (33,34). This reaction occurs preferentially on the carbons adjacent to double bonds of polyunsaturated fatty acids since the radicals formed can be stabilized by resonance with the double bond. Peroxide radicals

can react with side chains of other polyunsaturated fatty acids, thereby propagating the radical chain reaction (33). Thus, a single attack by a free radical results in the formation of a large number of oxidation products, especially aldehydes such as MDA and 4-hydroxynonenal, and short-chain hydrocarbons such as ethane and pentane (24,26,35). Many of the aldehydes formed react rapidly with cellular components, causing mutations in DNA, and produce structural and functional damage when reacting with proteins (34). Lipid peroxidation is considered a very important factor in aging of aerobic cells (36). Oxidative damage to membrane lipids is most likely an important factor in the decrease of membrane fluidity (37).

1.3.3.2 Protein oxidation

All amino acids present in proteins have residues susceptible to being attacked by free radicals, particularly by the hydroxyl radical (38). Among the physiological amino acids, tyrosine, phenylalanine, tryptophan, histidine, methionine, and cysteine are those that suffer most from oxidative processes (39). This oxidation can lead to a conformational change of the protein and, therefore, to a loss or modification of its biological function.

Oxidative damage is usually irreversible and can lead to protein denaturation (40). Free radical-mediated enzyme oxidation has been proposed as a hallmark of protein turnover (38).

1.3.3.3 Oxidative damage in DNA

DNA is also susceptible to oxidative damage in all its components as oxygen can attack both bases and the sugar (41). Oxidative damage associated with proteins and DNA should not be considered independently. The accumulation of inactive forms of repair enzymes can increase the accumulation of oxidative DNA damage, so they can potentiate each other. When replication of damaged DNA takes place before repair or when damaged DNA is repaired incorrectly, a mutation occurs (42,43). The damaged DNA is repaired by enzymes that cut the affected part, which is then excreted in the urine. Given that the repair enzymes fail to eliminate all the lesions, they accumulate, so the number of mutations increases with age (44).

1.3.3.4 Carbohydrate oxidation

Carbohydrates react easily with the hydroxyl radical, although monosaccharides and disaccharides resist the action of oxygen-

free radicals. Glucose is a scavenger of the superoxide radical, retaining it and preventing its action on other molecules. Mannose and mannitol are hydroxyl radical scavengers. Therefore, it has been observed that various polysaccharides act as cellular protective agents (45). Oxidative damage to carbohydrates is of importance when dealing with polysaccharides of structural function since polysaccharides are depolymerized by free radicals leading to degenerative processes.

A particular example is that of hyaluronic acid, whose structural function is to maintain the viscosity of the synovial fluid. Exposure to oxidizing agents, especially the superoxide radical, causes its fragmentation, which leads to destabilization of the connective tissue and loss of synovial fluid viscosity (46).

1.3.4 Antioxidants

Since living beings have evolved in the presence of oxidizing substances, evolution has provided these organisms with systems capable of combating such reactive substances. Halliwell in 1995 defined an antioxidant as "any substance that, when present at low concentrations compared with that of an oxidizable substrate, significantly delays or inhibits oxidation of

that substrate" (47). Antioxidants can act in a number of ways, for example by preventing the formation of ROS or intercepting a ROS attack, sequestering reactive metabolites and converting them to less reactive molecules, amplifying the resistance of sensitive biological targets to ROS attack, facilitating the repair of damage caused by ROS or by maintaining a favorable environment for the action of other antioxidants.

From a cellular physiology point of view, they can be divided into primary, secondary, and tertiary antioxidants. Primary antioxidants prevent the formation of new free radical species. These antioxidants act by converting existing free radicals into less harmful molecules, or by preventing their formation from other molecules. This group includes superoxide dismutase (SOD), glutathione peroxidase (GPx), catalase (Cat), and metal binding proteins such as ferritin and ceruloplasmin that limit the availability of iron necessary for the formation of the OH- radical (48). Secondary antioxidants are non-enzymatic protectors or free radical scavengers that intervene when there is an overproduction of free radicals and enzyme systems are overwhelmed, thus preventing chain reactions. They include glutathione, vitamin E, vitamin C, uric acid, bilirubin, and albumin (49). Tertiary antioxidants repair biomolecules damaged by free radicals. These include intracellular proteolytic systems, which act by degrading oxidatively damaged proteins,

thus preventing their accumulation (39,50). We can also highlight the DNA repair enzymes methionine sulfoxide reductase and phospholipase A2, which cleave oxidized phospholipids from the membrane (51). Another way of classifying antioxidants that is widely used is from a biochemical point of view, which distinguishes between enzymatic and non-enzymatic antioxidants.

Enzymatic antioxidants include SOD, GPx, and Cat whereas non-enzymatic antioxidants include glutathione, vitamin C and E, and certain polyphenols.

1.3.4.1 Superoxide dismutase

This name encompasses a family of metalloproteins widely distributed in nature, present in all cells that use oxygen in their metabolism, and even in some anaerobic bacteria (52). Its activity was first described by McCord and Fridovich in 1969. SOD transforms the superoxide radical into H_2O_2, constituting the first natural means of defense of the organism (53).

$$O_2^{\bullet -} + O_2^{\bullet -} + 2H^+ \xrightarrow{SOD} H_2O_2 + O_2$$

There are two types of manganese-containing superoxide dismutases (MnSOD). One of them is found mostly in the mitochondrial matrix and to a lesser extent in the cytosol (54,55). Its transcription takes place from mtDNA and its presence in the mitochondria is of great importance since, as has been pointed out previously, the mitochondrial respiratory chain is one of the main sources of free radicals in cells (56). Thus, MnSOD constitutes one of the barriers against oxidative damage caused by free radicals. The other MnSOD is found in bacteria such as Escherichia coli and Streptococcus mutans (57).

Another form of SOD is the copper- and zinc-dependent superoxide dismutase (Cu-SOD, Zn-SOD), which is found in most eukaryotic cells. It is a soluble protein containing copper and zinc ions as cofactors. It exists in many isomeric forms differing in metal ion content (58). The most abundant of the isomeric forms is located mostly in the cytosol, and to a lesser extent in the nucleus (59), although it can also be found in other locations. Its transcription is carried out from nuclear DNA. This isoform is found in high concentrations in the liver, brain and testes, and in smaller proportions in erythrocytes, lung and pancreas.

1.3.4.2 Glutathione peroxidase

GPx converts H_2O_2 and lipid peroxides into harmless molecules. There are two types of glutathione peroxidase, both of which require reduced glutathione as a reducing equivalent donor (60).

Selenium-dependent glutathione peroxidase, a tetrameric protein with four selenium atoms, catalyzes the reduction of H_2O_2 and organic peroxides. It is also involved in other processes such as the elimination of xenobiotics. Its activity is strongly affected by the selenium content of the diet (61,62)

Non-selenium-dependent glutathione peroxidase only has the ability to reduce organic peroxides. Most of the glutathione peroxidase activity is found in the cytosol, although it is also present in the mitochondrial matrix (61).

$$H_2O_2 + 2\ GSH \rightarrow 2\ H_2O + GSSG$$

$$ROOH + 2\ GSH \rightarrow ROH + H_2O + GSSG$$

1.3.4.3 Catalase

Catalase participates in the detoxification of H_2O_2, giving rise to water and an oxygen molecule (60).

$$2\ H_2O_2 \longrightarrow 2\ H_2O + O_2$$

It is also capable of catalyzing certain peroxidation reactions in the presence of H_2O_2, using certain alcohols, aldehydes and organic acids as substrates (60).

$$2\ H_2O_2 + AH_2 \longrightarrow 2\ H_2O + A$$

Catalase is mainly found in peroxisomes, although in recent years some catalase activity has been reported to be found also in mitochondria (63,64).

1.4 Polyphenols

Polyphenols are a group of chemical substances that are synthesized mainly in plants as a product of their secondary metabolism and are structurally characterized by having one or more hydroxyl groups attached to one or more benzene rings. Polyphenols are abundant in nature and extremely diverse. To this day more than 8000 different polyphenols have been identified (65), which can be subdivided into phenolic acids, stilbenes, phenolic alcohols, lignans, and flavonoids (66). Polyphenols are the most abundant antioxidants in our diet as they are widely distributed in fruits, vegetables, cereals, as well as in beverages such as tea, coffee, and wine (66). Polyphenols exert multiple regulatory effects, including the activation and

inhibition of enzymes, protection of cellular components against oxidative damage, and interaction with cellular receptors and signal transduction pathways (67–70).

1.4.1 Resveratrol

Resveratrol (3,5,4'-trihydroxystilbene) is a natural polyphenol with a stilbene structure formed by two phenolic rings bound by a double styrene bond. Due to this double bond, resveratrol exists as both *cis*- and *trans*-isomers (figure 4) (71). The *trans*-isomer is the most naturally stable form, as *cis*-isomerization occurs when the *trans*-isoform is exposed to artificial light, solar or ultraviolet radiation at wavelengths of 254 or 366 nm (71).

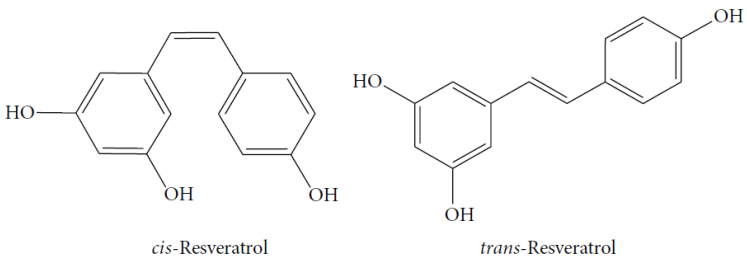

Figure 4. Chemical structure of cis- and trans-resveratrol.

From Gambini, et al. 2015 (71).

Resveratrol is present in numerous plants and fruits, such as blueberries and blackberries, nuts, grapes, and red wine, which is the largest contribution to resveratrol intake in the Mediterranean diet (72,73). Resveratrol has received great interest in the scientific community due to its many biological properties including anti-inflammatory, antioxidant, antiplatelet, anticarcinogenic, neuroprotective, and lipolysis regulatory effects (71,74,75). It is one of the most studied red wine molecules and is considered responsible for what is known as the "French-paradox", which describes the low risk of cardiovascular disease despite a diet rich in saturated fats and cholesterol (71). This suggests that resveratrol can provide an essential role in lipidic metabolism. Indeed, resveratrol has been found to have multiple metabolic effects in relation to insulin resistance, especially in improving energy metabolism in diabetes-induced mice (76). Additionally, resveratrol has been proven to increase lipidic oxidation in healthy, aged mice by inhibiting fatty acid synthesis and activating fatty acid degradation and mobilization (77).

1.4.1 Piceid

Piceid, or polydatin, is a natural glycosylated derivative of resveratrol found in red wine, cocoa-containing products, grapes, lentils, and peanuts (figure 5). Previous studies have shown that

piceid exhibits various beneficial effects, mainly due to its antioxidant and anti-inflammatory activity (78).

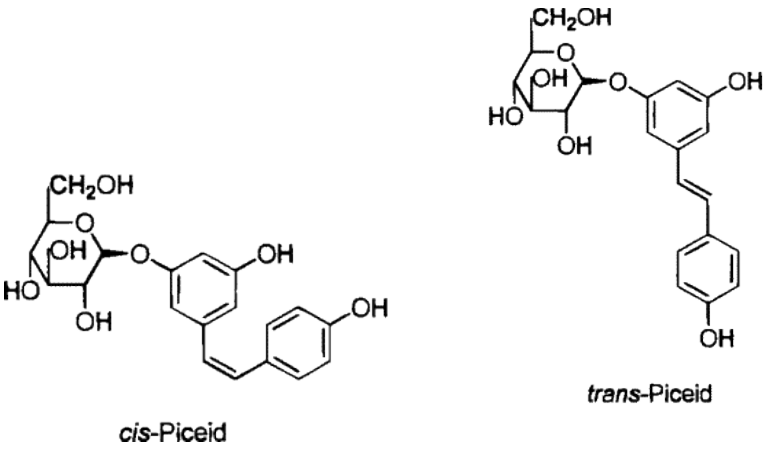

Figure 5. Chemical structures of piceid isomers.

From J. Agric. Food Chem. 1999, 47, 4, 1533-1536 (79).

According to studies published by Hollman et al. in 1995 and Paganga and Rice-Evans in 1997, the absorption of some phenols in diet is enhanced by conjugation with glucose (80,81). If glucosides facilitate absorption, piceid could be more efficiently absorbed than resveratrol in its aglycone form (79).

There are many studies published on the beneficial effects of piceid, including protection against myocardial ischemia, endometriosis, small intestine injury during hemorrhagic shock,

mitochondrial- dependent apoptosis, liver inflammation, diabetic renal fibrosis, and spinal cord injury (82–88).

1.4.2 Hydroxytyrosol

Hydroxytyrosol is a polyphenol with a phenylethyl-alcohol structure found in olive oil and olive leaves (figure 6) (89). It is an amphipathic compound that is both liposoluble and highly soluble in water, it is stable in the free form and penetrates readily into tissues (90). Hydroxytyrosol is usually absorbed in the intestine through bi-directional passive diffusion with an efficiency between 75% to 100%. The absorption depends on the composition of the food matrix through which hydroxytyrosol is administered, as its absorption is greater in its natural form present in extra virgin olive oil as opposed to when added in refined olive oil (91,92).

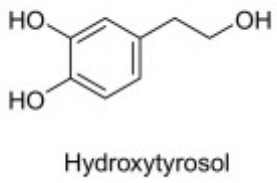

Figure 6. Chemical structure of hydroxytyrosol.

From Roleira et al. (93)

Hydroxytyrosol has been found to have anti-inflammatory and antioxidant effects through multiple signaling pathways (92). Is has also been shown to exert protective effects against a number of conditions. Some of them include endothelial dysfunction, ulcerative colitis, coronary heart disease, steatosis and hypertriglyceridemia, and Covid-19 (94–97).

1.5 Harmol

Harmol is a β-carboline alkaloid present in coffee and alcoholic beverages (figure 7) (98). It has been found to exert a variety of bioactivities, including antifungal, antiviral, and antioxidant, properties (99–101). Additionally, it has been reported to improve object recognition in mice (102). Furthermore, harmol inhibits human monoamine oxidase, which has been linked to depression (103).

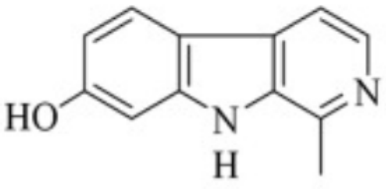

Figure 7. Chemical structure of harmol.

From Xu et al. 2002 (104).

In a recent study, harmol was described as a mitohormetic compound, which are defined as mild mitochondrial stressors that trigger compensatory responses that ultimately results in an improvement of mitochondrial function (105).

1.6 Drosophila Melanogaster

1.6.1 *Drosophila Melanogaster* as a model for longevity

The fly *Drosophila melanogaster* has become widely accepted for the analysis of developmental genetics in the recent years. However, it has been used for decades for longevity studies. The extrapolation of the results obtained to higher systems such as mammals has been found to be possible. In the year 2000, an important advance was made, as the complete genome sequence was published, which can be found in databases such as http://flybase.bio.indiana.edu.

The main advantages of *Drosophila melanogaster* as a model organism are centered on its easy handling due to its small size, its abundant offspring, and its relatively short lifespan.

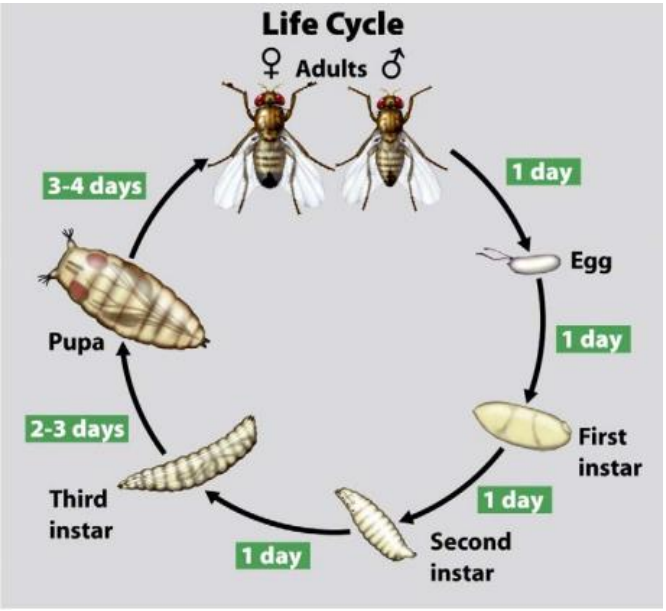

Figure 8. Life cycle of *Drosophila melanogaster*.

The length of the stages is approximate and refers to a cultivation at 25 °C. Illustration taken from "Genetics: A Conceptual Approach, Third Edition."

Drosophila melanogaster is a holometabolous insect, meaning it has larval and adult stages separated by a pupal stage, during which complete metamorphosis takes place (Figure 8).

The life cycle of *Drosophila* lasts about 10 days at 25ºC. The *Drosophila* egg forms over a period of about 3.5 days. Once fertilized, the mother deposits it outside, initiating embryogenesis. After about 24 hours, the embryo hatches into a

free-living larva that will pass through 3 larval stages (LI, LII and LIII). After 5 days the larva pupates and metamorphosis begins, hatching approximately 5 days later. After a few hours of life, the adult fly is fertile, and the life cycle begins again (106). Once the progeny is obtained, males and females can be separated, and a survival curve can be initiated.

1.6.2 Longevity curve

Among the animals used for longevity studies in research we can find, from less to greater biological complexity, yeast, worms, flies, mice, rats, rabbits, and primates. Each of these individuals has advantages and disadvantages for this type of study.

For example, a longevity study in yeast can give results in a matter of a few weeks, although the biological relevance applied to humans is very low. At the other extreme, a survival study in primates has a very high relevance for application to humans, however the time taken to obtain results is also very high, so that during the experimental period the probability of an external event affecting the experiment, such as an infection, is large, which could affect the results.

The advantages of longevity studies in *Drosophila melanogaster* flies are the duration of the experiment, since the lifespan of the

adult fly is around 3-4 months. You can also use a high number of individuals to make the curves. For example, populations of 1000 flies can be used without any difficulties. Additionally, the genetic homology of the fly compared to humans is about 75% (107). Therefore, although the *Drosophila melanogaster* are not mammals, due to the high homology extrapolations to higher systems might not be so remote.

1.7 Frailty

In recent decades, life expectancy has significantly increased in industrialized countries. This is primarily attributed to new pharmacological therapies and the adoption of a healthy lifestyle by the population. Among these factors, nutritional habits have played a particularly relevant role. Consequently, there has been a shift in the population pyramid. It is estimated that by 2030, 1 in 6 people will be over 60 years of age and the number of people aged 80 years or older is expected to triple by 2050, reaching 425 million people worldwide (108).

This shift has posed a new challenge for society as it has led to an increase in the percentage of individuals facing physical and mental limitations caused by age-related diseases. Among these, frailty, associated with age, is a geriatric syndrome that describes

vulnerable individuals with increasing disability, institutionalization, and even death, as first described by Linda Fried et al. in 2001 (109).

1.7.1 Frailty parameters in humans

Linda Fried defined frailty in 2001 as a geriatric syndrome involving declines in physiologic complexity or reserve in other systems, leading to loss of homeostatic capability to withstand stressors and resulting vulnerabilities. Frailty confers a high risk for adverse health outcomes, including mortality, institutionalization, falls, and hospitalization.

It was here that Fried and her team designed an assessment guideline to determine if the patient was in a state of frailty, pre-frailty, or remained far from this risk, which they defined as robust. The patients are characterized as frail when they present at least 3 of the following 5 situations: unintentional weight or muscle loss, self-reported exhaustion or poor endurance, slow walking speed, muscle weakness, and low level of physical activity. Those presenting one or two characteristics are identified as prefrail and those not presenting any characteristics are considered robust. This frailty index is defined in terms of the accumulation of deficits, which can be expressed in symptoms,

signs, diseases, and disabilities. Furthermore, it is clinically characterized by multisystem pathophysiological processes, such as chronic inflammation, autoimmune activation, dysregulation of the musculoskeletal and endocrine systems, oxidative stress, energy imbalances, mitochondrial dysfunction, and sarcopenia (110).

1.7.2 Frailty in *Drosophila Melanogaster*

Models determining frailty in other animal models, such as mice, rats, and dogs have been established (111). In 2017, a team of researchers in our laboratory in Valencia, Spain, developed a frailty assessment in experimental animals, based on Linda Fried's frailty phenotype, using male mice (C57Bl/6J) with the modification of the criteria as follows: 1) involuntary weight loss (as is), 2) low endurance (running time), 3) slowness (running speed), 4) weakness (grip strength), and 5) low activity level (motor coordination) (112).

However, models in flies that allow studying frailty have not yet been described. Thus, we have developed a proposal to study frailty in *Drosophila Melanogaster*, based on the parameters used in humans described by Linda Fried (Table 1).

Table 1. Frailty parameters in *Drosophila Melanogaster*.

Frailty parameters by Linda Fried	**Frailty parameters in *Drosophila Melanogaster***
Walking speed	Speed of flight
Low physical activity	Poor phototaxis
Muscle weakness	Poor geotaxis
Poor endurance or exhaustion	Decreased fertility
Weight or muscle loss	Decreased food ingestion

OBJECTIVES

2.1 General objective

The objective of this thesis was to study the effects of nutritionally relevant doses of different compounds present in diet, and specifically the mediterranean diet, on mitochondrial function, oxidative stress, and longevity.

2.2 Specific objectives

To achieve our objective, we set the following objectives:

1. Study the effects of resveratrol at different doses and treatment duration of resveratrol on lipid catabolism
2. Study the effects of nutritionally relevant concentrations of resveratrol on mitochondrial respiration and oxidative stress in C2C12 cells
3. Analyze the role of androgen receptors in the signaling pathway of resveratrol and determine the location of these receptors
4. Study the effects of different doses of hydroxytyrosol on mitochondrial function and oxidative stress in C2C12 cells
5. Study the effects of nutritionally relevant concentrations of piceid on longevity and frailty parameters in *Drosophila Melanogaster*

6. Study the effects of harmol on longevity and frailty parameters in *Drosophila Melanogaster*

MATERIAL AND METHODS

3.1 Material

Spectrophotometers:

- Spectrophotometer 7315 (Jenway)
- Cary 3500 Multicell UV-Vis Spectrophotometer (Agilent)
- Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific)

Fluorometer:

- LS 50B (Perkin Elmer).

Thermocyclers:

- T100 PCR Thermal Cycler (Bio-Rad).
- QuantStudio® 5 Real-Time PCR Systems (Applied Biosystems).

Western blot:

- Mini-PROTEAN Tetra Handcast Systems (Bio-Rad)
- Mini Trans-Blot® Electrophoretic Transfer Cell (Bio-Rad)
- PowerPac Basic power supply (Bio-rad)

Seahorse:

- Seahorse XFe96 Analyzer (Agilent)

Autoclave:

- Autester-G (Selecta)

Incubators:

- 5415IR, CO2 System (Napco)
- AGP-570-HR (Rodiber S.Q.)

Centrifuges:

- Mikro 220 R (Hettich, 2205)
- Sorvall Legend XTR Centrifuge (ThermoScientific)
- Centrifuge MPW-352R (MPW Med. Instruments)
- Microcentrifuge 5430 (Eppendorf)

Microscopes:

- Inverted microscope Oxion Inverso OX.2053-PL (Euromex)

Laminar flow hoods:

- Ductless Fume Hood Model 1100-G A (CRUMA)
- OR-ST 1200 Fume cupboard (Burdinola)
- Vertical laminar Cell culture hood B100 (Cultair)

Digital imaging equipment:

- ImageQuant™ LAS 4000 imaging system (GE Healthcare)

Biosciences)

Scales:

- Precision balance 6110 Tecator (Sartorius)
- Precision scale AHZ series (Gram)

Water baths:

- WB01 Thermostatic water bath (Ibx instruments, Labbox)

Hot plate stirrer:

- Multiposition hot plate stirrer, SB162-3 (Stuart)

3.2 Reagents

Cell culture

- Low Glucose Dulbecco's Modified Eagle Medium (Biowest)
- High Glucose Dulbecco's Modified Eagle Medium (Biowest)
- Bovine fetal serum (Capricorn)
- Horse serum (Capricorn)
- Phosphate buffered saline (Biowest)
- Trypsin-EDTA 1X in solution w/o Calcium w/o Magnesium w/ Phenol Red (Biowest)
- Resveratrol (sigma)
- Piceid (Sigma)
- Bicalutamide (Sigma)
- Hydroxytyrosol (Sigma)
- Alcohol 96% (Guinama)

Drosophila Melanogaster

- Harmol hydrochloride dihydrate, 98% (Fisher Scientific)
- Piceid (Sigma)
- Methylparaben (Sigma)
- Propionic acid (Sigma)
- Phosphoric acid (Sigma)
- CO_2

Gene expression

- TRIzol™ Reagent (Invitrogen)
- Chloroform (Sigma)
- 2-propanol (Sigma)
- Absolute ethanol (Scharlau)
- Nuclease free H_2O (Invitrogen)
- High-capacity cDNA Reverse Transcription Kit (Applied Biosystems)
- Maxima SYBR Green/ROX qPCR Master Mix (Thermo Scientific)
- RNase AWAY (Thermo Scientific)

Protein analysis

- Lysis buffer: 76,5mM TRIS, 10mL Glycerol, 2g SDS
- Sodium orthovanadate (Sigma)
- Protease inhibition cocktail (Sigma)
- AMPKα-1 Antibody (Invitrogen)
- Phospho-AMPKα (Thr172) Antibody (Invitrogen)
- Anti-GAPDH antibody produced in rabbit (Sigma)
- Anti-rabbit IgG HRP linked (Cell Signaling)
- Lowry reagent (Sigma)
- Folin & Ciocalteu's phenol reagent (Sigma)
- Running Buffer 10x: 0.25 M Tris, 2 M glycine, and 1% SDS dissolved in distilled H_2O

- Transfer Buffer 1x: 25mM Tris and 0.2 M glycine dissolved in distilled H_2O and 20% methanol
- PBS-T: PBS, pH 7.2-7.5, 0.05% Tween® 20
- TBS-T: Tris: 20 mM, NaCl: 150 mM, Tween® 20: 0.1% (v/v)
- Bovine Serum Albumin (BSA) (Pancreac)
- Acrylamide/Bis Solution, 29:1 (40% w/v), 3.3% C (Serva)
- Sodium dodecyl sulfate (SDS) (Sigma)
- Ammonium persulfate (APS) (Sigma)
- N,N,N′,N′-Tetramethylethylenediamine (TEMED) (Sigma)
- Bromophenol blue (Sigma)
- 2-mercaptoethanol (Sigma)
- PageRuler Plus Prestained Protein Ladder (Thermo Scientific)
- Immobilon Classico Western HRP substrate (Millipore)

Determination of hydrogen peroxide production

- Peroxidase from horseradish (type X) (Sigma)
- Homovanillic acid (Sigma)
- Hydrogen peroxide buffer: EGTA 0.1 mM, KH_2PO_4 5 mM, $MgCl_2$ 3 mM, KCl 145 mM, HEPES 30 mM. Dissolved in Milli-Q water and pH adjusted to 7.4 with 20% KOH.
- Glycine-EDTA solution: Glycine 2 mM, EDTA 50 mM, NaOH 2.2 mM dissolved in Milli-Q water. The pH is adjusted to ≥ 11 with 20% KOH.

Mitochondrial respiration

- XF DMEM medium pH 7.4 (Agilent)
- XF 1.0 M Glucose Solution (Agilent)
- XF 100 mM Pyruvate Solution (Agilent)
- XF 200 mM Glutamine Solution (Agilent)
- Antimycin A (Sigma)
- Oligomycin (Sigma)
- Carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (Sigma)
- Rotenone A (Sigma)

Ultra-performance liquid chromatography

- Thiobarbituric acid
- Phosphoric acid
- Formic acid
- Acetonitrile

3.3 Cell culture

Cell cultures are the product of the collection of animal cells from different organs, maintained under special conditions favorable for their survival and multiplication, thus preserving all their metabolic functions in a manner similar to those found in the host.

When the culture comes from cells that have been obtained from a recently sacrificed animal, it is called primary culture. Continuous Cell Lines or secondary cultures are made up of cells that differentiate genetically and morphologically from the cells from which they originated. They can come from cells that are derived from tumors, or from a transformation process of a primary culture altered to proliferate indefinitely and differentiate.

In our studies, we used C2C12, a myoblast cell line derived from murine satellite cells (113), which is widely used as an *in vitro* model due to their ability to rapidly differentiate from a proliferative phase into myofibers (114,115).

3.3.1 Cell treatment

3.3.1.1 Concentration and time curve

The cells were seeded according to AMERICAN TYPE CELL CULTURE (ATCC), in low glucose Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS). For our experiments, the cells were seeded in 6-well plates at a confluency of 8000 cells/cm^2. When the cells reached 80% confluency, they were introduced to the differentiation medium, consisting of high glucose DMEM supplemented with 2% horse serum (HS). After 5 days of differentiation, the media was changed to non-FBS-supplemented low glucose DMEM. The cells were then treated with 1 nM, 10 nM, 50 nM, and 100 nM of resveratrol and incubated for 24 hours in 5% CO_2 at 37°C utilizing DMSO as vehicle and control. Each condition was triplicated and reproduced on three different days (n = 9). The treatments had previously been dissolved and diluted in DMSO at 1000x its final concentration. RNA was then isolated, and PCRs were performed to analyze the optimal treatment concentration and time as described in detail in the paragraphs 3.5.1 to 3.5.3. We concluded that a treatment of 24 hours was optimal, and the lowest effective dose was 10 nM. The subsequent experiments were therefore performed treating the differentiated myotubes with 10 nM resveratrol for 24 hours.

3.3.1.2 Treatment with resveratrol, piceid, hydroxytyrosol, and bicalutamide

C2C12 cells were seeded in 6-well plates at a confluency of 8000 cells/cm^2 with low glucose DMEM + 2% FBS. When the cells reached 80% confluency, the cells were introduced to the differentiation medium, consisting of high glucose DMEM supplemented with 2% horse serum (HS). After 5 days of differentiation, the media was changed to non-FBS-supplemented low glucose DMEM and the cells were treated with 10 nM resveratrol or piceid alone or in combination with 1 μM androgen receptor, bicalutamide, and incubated for 24 hours in 5% CO_2 at 37°C. A control group treated with the vehicle DMSO, and a group treated with bicalutamide alone were also included. For the experiments using hydroxytyrosol, the cells were treated with 10 nM, 100 nM, and 10 μM before being incubated for 24 hours. Each condition for all of the treatment groups was triplicated and reproduced on three different days (n = 9).

3.4 Drosophila Melanogaster

The fly *Drosophila melanogaster* is a well-established longevity model, useful for mapping genetic and nutritional interventions

(116). They functionally conserve the majority of the genes present in mammals, as 65-70% of human genes can be found in *Drosophila* (117). Due to their short life cycle and the body size, it is possible to manage a number of animals, gaining facilitating reproducibility and statistical analysis.

The strain of *Drosophila Melanogaster* used for all following experiments were Oregon R, obtained from the Genetics Department at the Faculty of Biological Sciences at the University of Valencia.

The flies were incubated at 25 °C and 85% humidity with a period of light of 12 hours and 12 hours darkness. Wild flies feed on fruits, yeasts and insect carcasses, but prefer a yeast-based diet (118). In the laboratory, they can be maintained on a diet consisting of corn flour, sugar, yeast, and agar as a thickening agent. To avoid the proliferation of fungi, we add the antifungal agent nipagin (methylparaben) and the antibacterial propionic acid.

To prepare 1 liter of food we proceeded as follows:

1. In a pot we add
    - 900 mL tap water
    - 25 g yeast
    - 6 g agar

- 40 g corn flour
- 40 g wheat flour
- 50 g sugar

2. Boil for 5 minutes while stirring constantly
3. When the mixture has cooled down to about 60 °C add
   - 1.8 g nipagin dissolved in 10 mL ethanol
   - 5 mL mix of phosphoric (H_3PO_4) and propionic acid (CH_3CH_2COOH)
4. Mix well and portion quickly into vials while liquid. The food will thicken as it cools.

3.4.1 Experimental groups

3.4.2 Separation of frail and robust flies

The first step in obtaining the flies for the experiments 20 males and 20 females were introduced to a stock bottle of 200 mL with food and left for three days. This gives the females time to lay their eggs after being fertilized. The eggs will then go through the various stages of development until reaching the adult stage. The newly hatched adults are anesthetized with CO_2 and collected within 48 hours. After 10 days we performed a test combining a geotaxis assay and a phototaxis assay to separate the frail flies from the robust. A large crystal tube with openings on both ends was placed on top of the bottle containing the flies,

with another empty bottle placed at the bottom. After 30 seconds, the flies that reached the bottle at the top of the tube were considered robust and the ones remaining in the bottom bottle were considered frail. The separation was performed in a dark place, with a single source of light placed above the vial containing the animals to optimize the positive phototaxis of the fly. The separated flies were put to sleep and separated into experimental vials. 400 flies were used for each group (robust control, frail control, robust piceid, frail piceid, robust harmol, frail harmol), in total 2400 flies, separated into 20 flies per vial.

3.4.3 Treatment

Each vial of 30 cm with 20 flies were filled with approximately 5 mL food as follows:

- Control: Standard food
- Piceid: Standard food with 10 nM piceid
- Harmol: Standard food with 10 nM harmol

The treatments were prepared at 1000 times more concentrated than the final concentration and dissolved in 22% ethanol, resulting in a final concentration of 0.2% ethanol. The addition of alcohol was corrected by adding less during the preparation of the food.

3.4.4 Longevity curve

100 flies per condition were used for the longevity curve. The treatment was performed as in paragraph 3.4.3 the dead flies were noted down each 2-3 days and interpolated to an Excel spreadsheet where the percentage of survival was calculated and represented together with the life span.

3.4.5 Geotaxis assay

Drosophila Melanogaster have a negative geotaxis, meaning they tend to move against the force of gravity. Geotaxis assays can be used to assess fitness in a fly, by measuring their ability to move upwards.

The test consists of putting the flies in a vial to which is given a light tap to bring the flies to the bottom. The middle of the tube is marked with a line. The flies above the line are marked as having successfully passed the test.

The size of the tube varies as the flies age. For the tests performed at 80% survival, when the flies are still considered young, a tube with 45 cm length was used. For the tests performed on the flies

at 50% and 10% survival a smaller tube of 20 cm was used to compensate for the general weakness of the flies accompanied by age.

3.4.6 Phototaxis assay

Drosophila Melanogaster have a positive phototaxis, meaning they tend to move toward the light. A measurement of the ability of the fly to move towards a light source in a dark environment can be used to assess its fitness.

For our studies, the flies were introduced to a tube which was tapped to bring the flies to the bottom and placed horizontally, to prevent any influence of the negative geotaxis of the fly, with a light source placed at one end. The flies having passed the midline of the tube after 15 seconds were marked as having successfully passed the test.

As with the geotaxis assay, a larger vial was used at the 80% survival mark and a smaller vial for the flies at 50% and 10% survival.

3.4.7 Determination of gene expression

When reaching the desired survival percentage (80%, 50% and 10%), the flies were put to sleep on ice and nine flies per treatment group were separated into three microtubes. 100 mL of Trizol was added and the flies were homogenized and then centrifuged at 20,000 G at 4°C for 10 minutes. Total RNA was then isolated following the steps outlined in paragraph 3.5.1. The gene expression was analyzed in all the experimental groups after obtaining cDNA following the procedure detailed in the paragraphs 3.5.2 and 3.5.3.

3.4.8 Food ingestion

To ensure that none of the parameters analyzed were affected by difference in food consumption or caloric restriction, an analysis of the food ingested in each group was performed.

A new deposition was performed and 10 days after the hatching the separation of frail and robust was executed as described in paragraph 3.1.1. The flies were then introduced to their respective treatment, with 20 flies per experimental group. The flies were incubated with their treatment for 10 days, and on the 11^{th} day 0.5% (w/v) erioglaucine disodium salt was added to the food. Erioglaucine disodium salt, also known as Brillian Blue, dyes the fly´s stomachs content, allowing us to measure the food

intake by spectrophotometry. After 24 hours incubation, the flies were anesthetized on ice and homogenized with 500 µL phosphate buffered saline (PBS) per 20 flies and centrifuged at 20,000 G for 10 minutes at 4°C. 400 µL of the supernatant was collected and diluted with 400 µL PBS and measured at 625 nanometers, the maximum absorption for brilliant blue, with a spectrophotometer. The results were corrected with the amount of protein in the fly homogenate as detailed in paragraph 3.6.1.

3.5 Gene expression

3.5.1 RNA isolation

3.5.1.1 Principle

For the isolation of RNA from the cells and flies TRIzol™ (Invitrogen) reagent was used. Invitrogen™ TRIzol™ Reagent is a monophasic solution of phenol, guanidine isothiocyanate, RNase inhibitors, and other components designed to isolate high quality total RNA from cell and tissue samples of human, animal, plant, yeast, or bacterial origin.

After homogenizing the sample with TRIzol™ Reagent, chloroform is added, allowing the homogenate to separate into a clear upper aqueous layer containing RNA, an interphase, and a red lower organic layer containing the DNA and protein.

3.5.1.2 Procedure

Following the instructions of the TRIzol™ user guide, the RNA was isolated as follows:

1. Add 1 mL of TRIzol™ per 50-100 mg of fly homogenate or per 1×10^5—1×10^7 cells directly to the culture dish to lyse the cells
2. Incubate for 5 minutes to allow complete dissociation of the nucleoprotein complex
3. If working with cells, collect the lysate with a micropipette and bring to a 1.5 mL tube
4. Add 0.2 mL of chloroform close the tube and mix by shaking
5. Incubate for 2-3 minutes
6. Centrifuge the samples for 15 minutes at 12,000 G at 4°C to separate the sample into a lower phenol-chloroform, an interphase, and a colorless upper aqueous phase
7. Transfer the aqueous phase containing the RNA to a new tube
8. Add 0.5 mL of isopropanol to the aqueous phase and incubate for 10 minutes on ice

9. Centrifuge for 10 minutes at 12,000 G at 4 °C to form a gel-like pellet with RNA precipitate at the bottom of the tube.
10. Discard the supernatant and resuspend the pellet in 1 mL of 75% ethanol. Vortex the sample briefly and centrifuge for 5 minutes at 7500 G for 5 minutes at 4 °C.
11. Discard the supernatant and air-dry pellet for 5-10 minutes.
12. Resuspend the pellet in 20-50 μL RNase-free water
13. Incubate on heat block set at 55-60 °C for 10-15 minutes

3.5.1.3 RNA quantification

The RNA concentration is quantified by spectrophotometric measurement at 260 nm with NanoDrop 2000 Spectrophotometer. A measurement at 280 nm is performed to verify the purity of the sample. The ratio between the 260/280 nm is calculated and values between 1.8 and 2.0 indicate pure RNA was obtained.

3.5.2 cDNA reverse transcription

3.5.2.1 Principle

Reverse transcription consists of obtaining cDNA from an mRNA. This requires DNA polymerases, called reverse transcriptases. The reverse transcriptase used for our experiments is MultiScribe™ Reverse Transcriptase (Applied Biosystems). These enzymes derive from retroviruses, which are viruses that have RNA as their genome, rather than DNA. To express their proteins, they have to pass the information to DNA. For the synthesis of cDNA from RNA, we use random primers as anchor points that will be used by the reverse transcriptase to initiate the synthesis.

3.5.2.2 Procedure

For our experiments, we used The Applied Biosystems™ High-Capacity cDNA Reverse Transcription Kit, which contains all the reagents needed for reverse transcription of total RNA to single-stranded cDNA (119,120).

Following the user guide of The Applied Biosystems™ High-Capacity cDNA Reverse Transcription Kit, the reverse transcription was executed as follows:

1. Allow the kit components to thaw on ice

2. Calculate the amount of each component for the 2x reverse transcription master mix as in table 2.

Table 2. Components for 2x reverse transcription master mix.

Component per sample	Volume
Nuclease-free H_2O	3.2 µL
10x RT Buffer	2 µL
10x RT Random Primers	2 µL
MultiScribe™ Reverse Transcriptase	1 µL
25x dNTP Mix (100 nM)	0.8 µL

3. Dilute the samples in nuclease free water to obtain 1 µg total RNA in 10 µL
4. Add 10 µL master mix to each sample and centrifuge tubes to spin down the contents and eliminate air bubbles
5. Place the tubes in a T100 PCR Thermal Cycler (Bio-Rad)
6. Run the thermocycler programmed with the conditio detailed in table 3:

Table 3. Thermal cycle program for reverse transcription.

Settings	Step 1	Step 2	Step 3	Step 4
Temperature	25°C	37°C	85°C	4°C

Time	10 minutes	120 minutes	5 minutes	∞ hold

3.5.3 Quantitative polymerase chain reaction (RT-qPCR)

3.5.3.1 Principle

Once the cDNA is synthesized, we amplify it by polymerase chain reaction (PCR). This method allows us to selectively amplify specific DNA sequences in a targeted manner. The PCR method is based on the synthesis of a complementary strand of DNA, using a single strand as a template. The PCR uses two short DNA fragments (oligonucleotides) as primers for the synthesis. These primers bind specifically to sequences flanking the region to be amplified, one on each strand of DNA. The requirements of the reaction are deoxynucleotides (dNTPs) that provide both the energy and the units of synthesis, a DNA polymerase, primers, the template cDNA and a magnesium-containing buffer. The process is carried out in three steps that are repeated successively (figure 9):

- Denaturation: separation of the complementary DNA strands.

- Annealing: binding of the specific primers to their complementary sequences. The binding temperature is characteristic of each pair of primers.
- Extension: synthesis of the complementary strand from the respective primer.

The repetition of this cycle produces an exponential increase in the amount of the DNA in the target region, which is given by the expression 2n (where n is the number of cycles) until a point is reached at which the efficiency of the enzyme decreases, and the reaction ceases to be exponential.

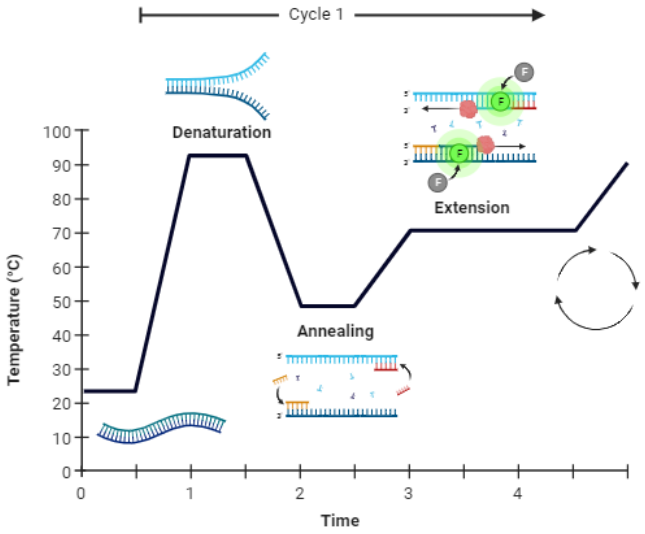

Figure 9. Graphical illustration of the qPCR cycle steps.

Created with BioRender.com

Real-time polymerase chain reaction (RT-qPCR) is currently the most sensitive and accurate method for determining messenger RNA (mRNA) levels in cells and tissues. In contrast to semiquantitative methods, this method uses fluorescent compounds that only emit fluorescence when the DNA is in a double-stranded form, allowing the amount of product formed to be determined by measuring the increase in fluorescence during the exponential phase of the reaction. The fluorescent substance used in our experiments was SYBR Green I, which binds to DNA and emits fluorescence only when the two complementary DNA strands are joined together. This method allows us to follow the reaction as it progresses through the increase in fluorescence and allows us to control the reaction at any time and increase the number of cycles, repeat cycles, or pause if necessary. Figure 10 shows the amplification curves obtained using this method.

In quantitative PCR, the measurement parameter for the expression of a given gene is not fluorescence, but the cycle in which amplification begins to be exponential, called the threshold cycle (Ct) (figure 10). The threshold cycle values decrease linearly as the amount of starting cDNA increases. This is because the more copies of the starting mRNA of the gene being studied, the more cDNA is obtained in reverse transcription before the amplification begins to be exponential.

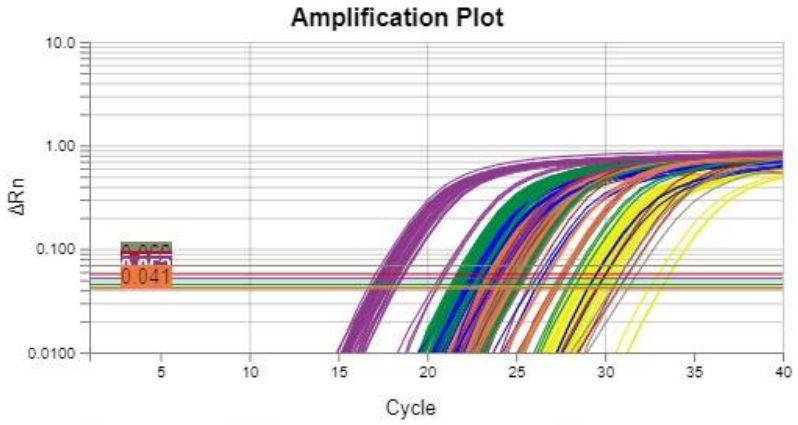

Figure 10. Amplification curve obtained with RT-qPCR with threshold of each gene indicated.

3.5.3.2 Procedure

Following the protocol outlined by ThermoFisher for the use of SYBR Green I the qPCR was executed as follows:

1. Dilute samples to obtain 250 ng/μL
2. Prepare master mix by adding the following components detailed in table 4:

Table 4. qPCR master mix components.

Components	Volume per reaction
Maxima SYBR Green qPCR Master Mix (2X)	12.5 μL

Forward primer	300 nM
Reverse primer	300 nM
Nuclease-free water	To 9 µL
Total volume	9 µL

3. Mix the master mix thoroughly
4. Dispense 9 µL of master mix into each well of a 384-well plate
5. Add 1 µL template DNA (250 ng)
6. Cover plate and centrifuge briefly at 1500 G for 30 seconds
7. Program the thermo cycler on QuantStudio™ 5 system according to the instructions below, insert the plate and start the program as indicated in table 5

Table 5. qPCR cycle program configuration.

Step	Temperature	Time	Number of cycles
UDG pre-treatment	50 °C	2 min	1
Initial denaturation	95 °C	10 min	1
Denaturation	95 °C	15 s	40
Annealing/Extension	60 °C	60 s	

The primers used for our experiments were designed using GCG and IDT PrimerQuest™ Tool with genetic sequences obtained by

PubMed´s GenBank. All the primers were designed to have an annealing temperature of 60 ± 1°C. The nucleotide sequence for each gene is detailed in table 6.

Table 6. Nucleotide sequences for qPCR primers.

Gene	Forward primer	Reverse primer
Dmel-RP49	5'-ATCGGTTACGGATCGAACAA-3´	5'-GACAATCTCCTTGCGCTTCT-3´,
Dmel-Attacin C	5´-GTCAGTTCCAGGCCGTGTCC-3´	5´-CGCTCCACTCCCGTAACCAG-3´
Dmel-Crecropin C	5´-CCAATGCGCTCGATTCTCTTG-3´	5´-CGATTCCATCAGCATTGGACAAT-3´
Dmel-Catalase	5´-TTCCTGGATGAGATGTCGCACT-3´,	5´-TTCTGGGTGTGAATGAAGCT GG-3´,
Dmel-MnSOD	5´-GCAGATATGTTCGTGGCCCGTA-3´	5´-AGTTGCAGTTTGCCCGACTTCT-3´
Dmel-Diptericin	5´-CCACCGCAGTACCCACTCAAT-3´	5´-CGATGACTGCAAAGCCAAAACCA-3´
Mmu-Gapdh	5´-TGCTGAGTATGTCGTGGAGT-3´	5´-AGATGATGACCCGTTTGGCT-3´
Mmu-Cpt1	5´- TCA AAG GAG GAG GTA AGA CTA C-3´	5´-CCT TGG CTG CGG TAA GAC TA-3´
Mmu-Acc1	5´- CGT CAG CTC AGA TAC ACT TTC T-3´	5´-AGG TGC AAG CCA GAC ATG CT-3´
Mmu-Cact	5´- GAT TCC AGA CTG CAC CTC CT-3´	5´-CAA GGA AAC AGG CAG CGT TG-3´

Mmu-Ampkα-1	5′- ACT GTA GTG AAT CGT GTT GCT TC-3′	5′-ACC AAA GAC ATT CAA CAC TGG CT-3′
Mmu-Acsl	5′- CCT TCC AAC CAA CAC CCT CA-3′	5′-GAC TGC ATG GAG AGG TCA CA-3′
Mmu-Echs1	5′- TCT CTA CAA ATG CAG TCA TCC C-3′	5′-AGC CTT TGA GAT GAC GTT AAC AG -3′
Mmu-Tfam	5′-AAAGGATGATTCGGCTC AGGG-3′	5′-TCGTTTCACACTTCGACG GAT-3′
Mmu-Nrf-1	5′-AGAAACGGAAACGGC CTCAT-3′	5′-ATCCAACGTGGCTCTGAGT-3′
Mmu-Pgc1α	5′-CGGAAATCATATCCAAC CAG-3′	5′-TGAGGACCGCTAGCAAG TTTG-3′
Mmu-Mnsod	5′-GCA CAT TAA CGC GCA GAT CA-3′	5′-AGC CTC CAG CAA CTC TCC TT-3′
Mmu-Gpx	5′-CCT CAA GTA CGT CCG ACC TG-3′	5′-CAA TGT CGT TGC GGC ACA CC-3′
Mmu-Cat	5′-GCA GAT ACC TGT GAA CTG TC-3′	5′-GTA GAAT GTC CGC ACC TGA G-3′

The standard curve method is used to determine relative mRNA quantity of the target genes in each sample, depending on their Ct. The quantity of each sample is then normalized by the quantity of an endogenous control, in our case GAPDH. Additionally, by loading a standard curve, we are able verify that the amplification was successful. This is done by calculating the

slope of the curve, where a slope of -3.322 indicate 100% efficiency, utilizing the following calculation:

$$\textbf{Efficiency} = [10\ (\frac{1}{slope})] - 1$$

3.6 Protein analysis

Western blot is used in research to separate and identify proteins. In this technique proteins are separated based on molecular weight, and thus by type, through gel electrophoresis. For the execution of this technique, it is crucial that the proteins are completely denatured. To this end, we use two denaturing agents, sodium dodecyl sulfate (SDS) as a detergent and β-mercaptoethanol as a reducing agent, which causes the disulfide bridges of the protein to break. These proteins are then transferred to a membrane producing a band for each protein (121). The membrane can then be incubated with antibodies specific to the protein of interest, creating bands visualized by binding the proteins and antibodies with a chemiluminescence. The amount of protein can be quantified depending on the thickness of its band on the membrane. A loading control is used to assure the same amount of protein was loaded in each sample.

3.6.1 Protein extraction

To be able to separate the proteins during electrophoresis, the proteins must be lysed and denatured as follows:

1. Add 100 μL lysis buffer containing 76.5 mM Tris, 10% Glycerol and 2% SDS, 1% protease inhibitor cocktail, and 1% sodium orthovanadate to cells or fly homogenate
2. Incubate samples for 10 minutes on ice
3. Incubate samples at 95°C for 10 minutes to ensure denaturation of the proteins

3.6.2 Protein quantification

To quantify the cytosolic extracts, we used the Lowry-Folin method. The method is based on the reaction of proteins with Folin's reagent, giving a colored complex. This reagent is a solution of sodium tungstate and sodium molybdate in phosphoric and hydrochloric acid. The mechanism of the process is as follows: $Cu^{2+,}$ in an alkaline medium, forms a complex with the peptide bonds of the complexes with the peptide bonds of proteins and is reduced to Cu^{+}. This ion, as well as the R-groups of the tyrosine and tryptophan residues of the proteins, react with Cu^{+} proteins, react with Folin's reagent, initially producing an unstable product that is reduced to form a colored compound

(122). The intensity of the color depends on the amount of these aromatic amino acids present in the proteins, which can be measured by spectrophotometry at λ = 660nm and will be proportional to the concentration of proteins in the solution. A standard curve of known concentrations is used to extrapolate the concentration of the samples.

3.6.3 Western blotting

3.6.3.1 Electrophoresis

To detect the different proteins a 5 μL PageRuler Plus Prestained Protein Ladder (Thermo Scientific) and 30 μg of proteins were stained with bromophenol blue and loaded onto a gel of 1.5 mm thickness made with 12,5% acrylamide/bisacrilamide (29:1) and 0.1% SDS. The gels were covered with a running buffer (25 mM Tris, 200 mM glycine, 0.1% SDS, pH 8.3) and connected to a power supply at constant voltage of 20 mAmps until the proteins reached the bottom of the gel (approximately 1.5 hours).

3.6.3.2 Electrophoretic transfer

Once the electrophoresis had terminated, the gels were transferred to a PVDF membrane through an electrophoretic

transfer. The gel and membrane were placed in between filter paper as illustrated in figure 11 and placed in a Mini-PROTEAN™ tank transfer system (Bio-Rad). The cuvette was filled with transfer buffer (25 mM Tris, 192 mM glycine, and 20% methanol (v/v), pH 8.3) and connected to a power supply at 100 mAmps for 2 hours at 4 °C.

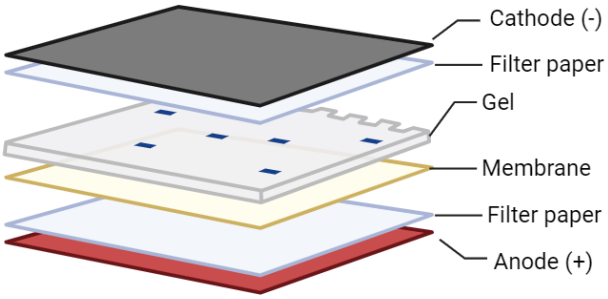

Figure 11. Gel and membrane set up for tank transfer.

Created with BioRender.com

3.6.3.3 Antibody incubation

The membranes were incubated with the antibodies of interest following the guidelines of the Sigma and Invitrogen:

1. Block membrane for 60 minutes at room temperature in blocking buffer made of 5% BSA in TBS-T (20 mM Tris, 137 mM NaCl, 0.1% Tween-20®, pH 7.6)
2. Incubate the membrane with 10 mL of the primary antibodies as indicated in table 7 overnight at 4°C with gentle shaking
3. Wash membrane three times for 10 minutes with TBS-T
4. Incubate the membrane with HRP-linked anti-igG secondary antibody for 60 minutes with gentle shaking at room temperature

Table 7. Antibody dilutions for Western Blot.

Antibody	Molecular weight	Host	Dilution
Phospho-AMPKα-1	62 kD	Rabbit	1:1000
AMPKα-1	62 kD	Rabbit	1:1000
GAPDH	36 kD	Rabbit	1:10,000
HRP-linked Anti-igG	N/A	Rabbit	1:1000

3.6.3.4 Chemiluminescence Detection

1. Place the membrane on a clear plastic sheet
2. Cover the membrane with Immobilon Classico Western HRP substrate (Millipore) and incubate for 2 – 5 minutes at room temperature

3. Drain the excess substrate and cover the membrane with another clear plastic sheet
4. Expose the blot using ImageQuant™ LAS 4000 imaging system

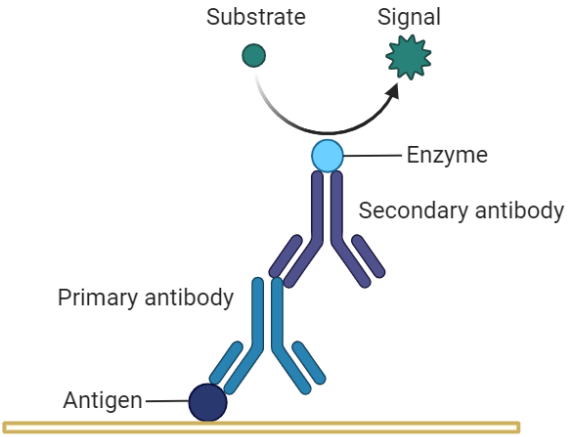

Figure 12. Visual illustration of the molecular principle of antibody probing.

Created with BioRender.com

3.6.3.5 Result quantification

The images obtained by the ImageQuant™ LAS 4000 imaging system were exported to ImageLab for quantification. The amount of protein was calculated depending on the width of each band and the amount of phospho-AMPKα-1 was relativized by the amount of AMPKα-1 and GAPDH.

3.6.4 Carbonylated proteins

Free radicals and other reactive species cause carbonyl groups to be introduced into protein side chains by site-specific mechanisms. The OxyBlot™ Kit allows for immunodetection of these carbonyl groups, which is a hallmark of the oxidation status of proteins. The carbonyl groups in the protein side chains are derivatized to 2,4-dinitrophenylhydrazone by a reaction with 2,4-dinitrophenylhydrazine (DNPH). The DNP-derivatized protein samples can then be separated by gel electrophoresis followed by Western blotting (123).

3.6.4.1 Protein derivatization

1. Transfer 5 μL of 15-20 μg protein to a 0.5 mL Eppendorf tube.
2. Denature each aliquot of protein by adding 5 μL of 12% SDS for a final concentration of 6%
3. Derivatize the sample by adding 10 μL of 1X DNPH Solution to the tubes.
4. Incubate tubes for 15 minutes at room temperature
5. Add 7.5 μL Neutralization Solution to tubes.

3.6.4.2 Electrophoresis and transfer

Follow the instructions for electrophoresis and transfer as detailed in paragraphs 3.6.3.1 and 3.6.3.2

3.6.4.3 Antibody incubation and chemiluminescence detection

1. Incubate the membrane in blocking buffer for 1 hour with gentle shaking at room temperature
2. Dilute the primary antibody stock 1:150 in blocking buffer and incubate the membrane for 1 hour with gentle shaking at room temperature
3. Rinse the membrane twice with PBS-T (0.05% Tween-20 in phosphate buffered saline). Wash the membrane with PBS-T once for 15 minutes, then twice for 5 minutes each at room temperature
4. Dilute the secondary antibody stock 1:300 in blocking buffer and incubate for 1 hour with gentle shaking at room temperature
5. Wash membrane as in step 3.
6. Expose blot as indicated in paragraph 3.6.3.4

3.7 Determination of hydrogen peroxide levels

To measure the levels of H_2O_2 in cells, we used a modified protocol of the method developed by Barja et al. in 1999. This technique is based on the measurement of the fluorescence emitted by the dimer formed between H_2O_2 produced by the cells, and homovanillic acid (4-hydroxy-3-methylphenylacetic acid). This dimer, when excited by a beam of light of a wavelength of 312 nm, is capable of emitting a maximum of fluorescence at 420 nm, which is detected through fluorimetry. The catalysis of the dimerization reaction is carried out by the enzyme peroxidase, which uses H_2O_2 as its substrate to produce dimerization (124).

1. Prepare H_2O_2 mix by mixing 5 μL peroxidase (6U/mL), 20 μL homovanillic acid (1mM) and hydrogen peroxide buffer (EGTA 0.1 mM, KH_2PO_4 5 mM, $MgCl_2$ 3 mM, KCl 145 mM, HEPES 30 mM. Dissolved in Milli-Q water with the pH adjusted to 7.4 with 20% KOH) until 2 mL per sample and warm to 37 °C
2. Remove cell culture medium and wash cells twice with PBS
3. Add 2 mL of the H_2O_2 mix directly to the cell plate and incubate for 10 minutes at 37 °C

4. Add 1 mL Glycine-EDTA solution (Glycine 2 mM, EDTA 50 mM, NaOH 2.2 mM dissolved in Milli-Q water with the pH is adjusted to ≥ 11 with 20% KOH) to stop the reaction
5. Scrape cells and transfer to tubes and centrifuge for 15,000 G for 15 minutes
6. Measure the supernatant by fluorimetry with an emission of 420 nm and excitation of 312 nm

The concentration of H_2O_2 produced by the cells is calculated by comparing the fluorescence emitted by the cells against those emitted by samples of known concentrations of H_2O_2. The standard curve is prepared by adding concentrations ranging from 1 μM to 100 μM of H_2O_2 to the same mix used for the measurements of the cells.

To calculate the exact concentration of the H_2O_2 stock a dilution of $1/10^3$ is prepared and measured by spectrophotometer at λ = 320 nm, corresponding to the molar extinction coefficient of H_2O_2 (ε 230 = 72,4 mM^{-1} x cm^{-1}). The concentration of H_2O_2 can be calculated by the following equation:

$$\frac{A_{230}\, x\, H_2O_2\, x\, 10^4}{\varepsilon_{H_2O_2}\, x\, 1000\, \mu L} = nmoles\ H_2O_2/mL$$

Extrapolating the standard curve, we obtain the value of H_2O_2 in nmol/mL for each of our samples. This is divided by the amount of proteins in mg/mL, thus obtaining the final result in nmol H_2O_2/mg of proteins.

3.8 Mitochondrial respiration

To measure mitochondrial respiration, we used the Mito Stress Test by Agilent Seahorse XF, which allows for a direct measurement of oxygen consumption rate (OCR) in real time. The assay uses injection ports on sensor cartridges to add modulators of the electron transport chain (ETC) to observe key parameters of the mitochondrial function. The modulators utilized in this assay include Oligomycin, Rotenone and Antimycin A, and Carbonyl cyanide-4 (trifluoromethoxy) phenylhydrazone (FCCP).

Oligomycin inhibits the complex V of the electron transport chain (ATP synthase), thereby inducing a decrease in OCR, which allows us to measure ATP-linked respiration and proton leak. FCCP in an uncoupling agent that disrupts the mitochondrial membrane potential and collapses the proton gradient. This results in an uninhibited electron flow though the

ETC and thereby allows us to measure the maximal respiration capacity of the cells as well as the cells spare respiratory capacity, which is an indicator of the cells ability to respond under stress or to an increased energy demand. The final injection is a mixture of rotenone and antimycin A, which inhibit complex I and III respectively. This combination results in a complete shutdown of mitochondrial respiration and therefore allows us to measure nonmitochondrial respiration (125).

3.8.1 Day prior to assay

1. Turn on Agilent Seahorse XF Analyzer and let it warm up overnight
2. Plate C2C12 cells at a density of 7000 cells/cm^2 on a 96XF Cell Culture Microplate in DMEM + 10% FBS. Leave the corner wells without cells as background control
3. Hydrate sensor cartridge with 200 µL Seahorse XF Calibrant overnight in a non-CO_2 incubator

3.8.2 Day of assay

1. Prepare assay medium by supplementing Seahorse XF DMEM with 10 mM glucose, 2 mM glutamine, and 1 mM pyruvate and adjust pH to 7.4
2. Warm the assay medium to 37 °C in water bath
3. Wash cells twice with PBS, add 180 µL of complete assay medium and incubate the cells in a non-CO_2 incubator for 45 – 60 minutes
4. Prepare 3 mL of 15 µM oligomycin, 20 µM FCCP, and 10 µM Rotenone/Antimycin A in complete assay medium
5. Load 20 µL of the oligomycin solution to port A for a final concentration of 1,5 µM
6. Load 22 µL of the FCCP solution to port B for a final concentration of 2 µM
7. Load 25 µL of the Rotenone/Antimycin A solution to port C for a final concentration of 1 µM
8. Place the calibration plate with the loaded sensor cartridge on the instrument tray and initiate calibration
9. When prompted, load the cell culture microplate and run assay using the following injection protocol:
    - Basal: 3 cycles of 3 min *Mix* and 3 min *Measure*
    - Inject port A followed by 3 cycles of 3 min *Mix* and 3 min *Measure*
    - Inject port B followed by 3 cycles of 3 min *Mix* and 3 min *Measure*

- Inject port C followed by 3 cycles of 3 min *Mix* and 3 min *Measure*

When the assay is complete, export the results to the Seahorse XF Mito Stress Test Report Generator, which exports the data to Excel and automatically calculates the parameters used in the assay

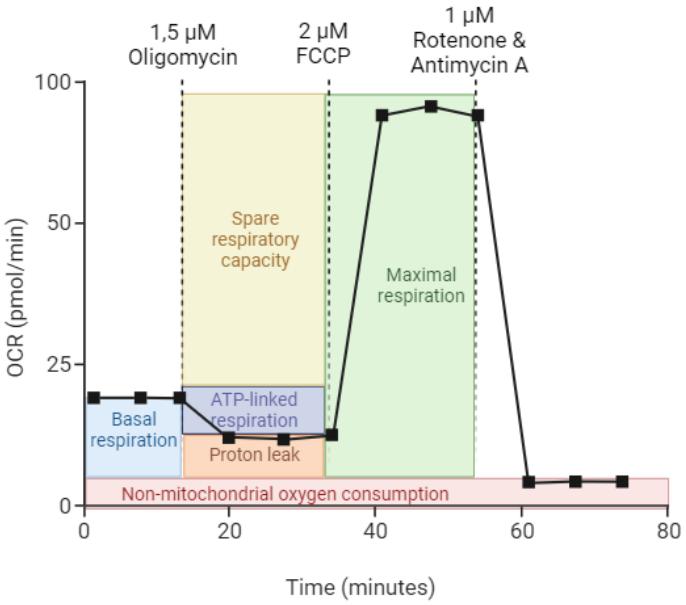

Figure 13. Summary of the injection strategy for Seahorse XF Mito Stress Test.

Created with BioRender.com

3.9 Ultra performance liquid chromatography

Malondialdehyde (MDA) was used as a marker for lipid peroxidation, which was detected using ultra performance liquid chromatography (UPLC) following a method described previously (126). This method is based on the hydrolysis of lipoperoxides and the subsequent formation of an adduct between 2-thiobarbituric acid and MDA. This adduct was detected using UPLC in reverse phase and quantified at 532 nm. The chromatographic technique was performed under isocratic conditions, with the mobile phase being an aqueous mixture of H_2O + phosphoric acid (0.3%) and acetonitrile at an 80:20 ratio. The results were normalized by protein quantification and the results presented as μmol MDA/mg protein.

3.10 Statistical analysis

The statistical analysis of this thesis was performed with GraphPad Prism 10 using one-way ANOVA.

Many statistical tests make assumptions about the data being studied, one of them being the assumption of normality. Shapiro-Wilk's test was performed to ensure Gaussian distribution of the samples. If a group did not pass the normality test, we performed

a Kruskal-Wallis test with two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli Post-Hoc test.

The one-way ANOVA assumes that all the populations have the same standard deviation and thus the same variance. This assumption is not very important when all the groups have the same or similar number of subjects but is very important when sample sizes differ. The Brown-Forsythe test and Bartlett's test can be performed to analyze equality of variance. If either test showed a difference in variance, the Welch ANOVA test was used to analyze the difference in the means of each group.

For the experiments with hydroxytyrosol, the mean of each group was compared with the control. In the experiments of resveratrol, piceid, and harmol the mean of each group was compared to the mean of every other experimental group. Correction of multiple comparisons was performed utilizing Tukey test.

Outliers were removed using robust regression and outlier removal using the ROUT method. This method was developed to identify outliers from nonlinear regression. It first fits a model to the data using a robust method where outliers have little impact. It then uses a new outlier detection method, which is based on the false discovery rate, to decide which points are too far from the prediction of the model and be identified as outliers.

Survival analysis provides special techniques that are required to compare the risks for death associated with different treatments or groups. Longevity in *Drosophila melanogaster* was analyzed using Kaplan-Meier, which provides a method to estimate a survival curve (127). Log rank test was performed to analyze the statistical comparison between groups.

RESULTS

4.1 Resveratrol activates lipid catabolism in C2C12 cells

Previous studies in our laboratory have shown that resveratrol shifts energy metabolism favoring fatty acid oxidation in aged healthy mice (128). However, as previously explained, the signaling pathway of resveratrol is still not clear. Therefore, we set out to study whether resveratrol exerts its beneficial effects through an androgen receptor.

4.1.1 Concentration curve

The first thing we set out to do was study if resveratrol exerts effects on lipid catabolism at nutritionally relevant concentrations *in vitro*, specifically in murine skeletal muscle cells, C2C12. To determine the optimal concentration and treatment duration, we performed a concentration curve and time course. The cells were treated with resveratrol at 1 nM, 10 nM, 50 nM, and 100 nM for 6, 12, 24, and 48 hours. We then measured genes related to lipid transport and fatty acid β-oxidation.

We found that *Ampk* expression increased with treatment of 10 nM, 50 nM, and 100 nM for six hours. *Acsl* increased after treatment with 10 and 100 nM and *Cpt1* increased in the cells

treated with 10 nM for six hours. No differences were found in *Acc1*, *Cact*, or *Ech1* expression in the cells treated for six hours (figure 14).

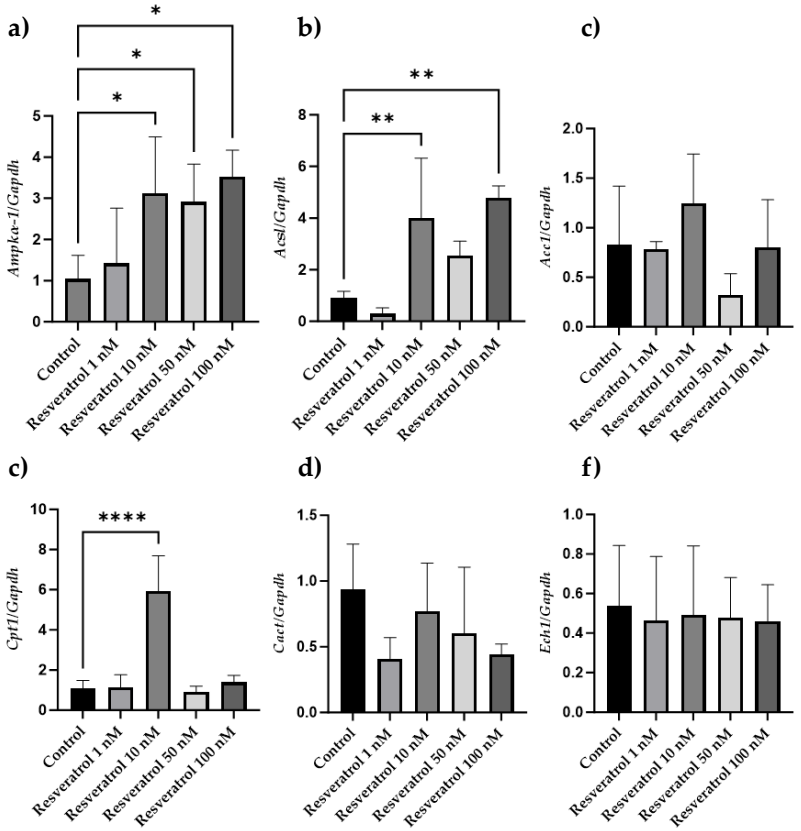

Figure 14. Gene expression of genes related to lipid catabolism in C2C12 cells treated with resveratrol for 6 hours.

mRNA levels of a) *Ampk* b) *Acsl* c) *Cpt1* d) *Acc1* e) *Cact* f) *Ech1*. Each experiment was triplicated and repeated on three different days (n = 9). The data is

represented as mean ± standard deviation. The statistical significance is expressed as * p<0.05, ** p<0.01, **** p<0.0001.

After 12 hours of treatment, we found a significant decrease in *Acc1* in the cells treated with 100 nM resveratrol, although no differences were observed in any other genes at any of the concentrations (figure 15).

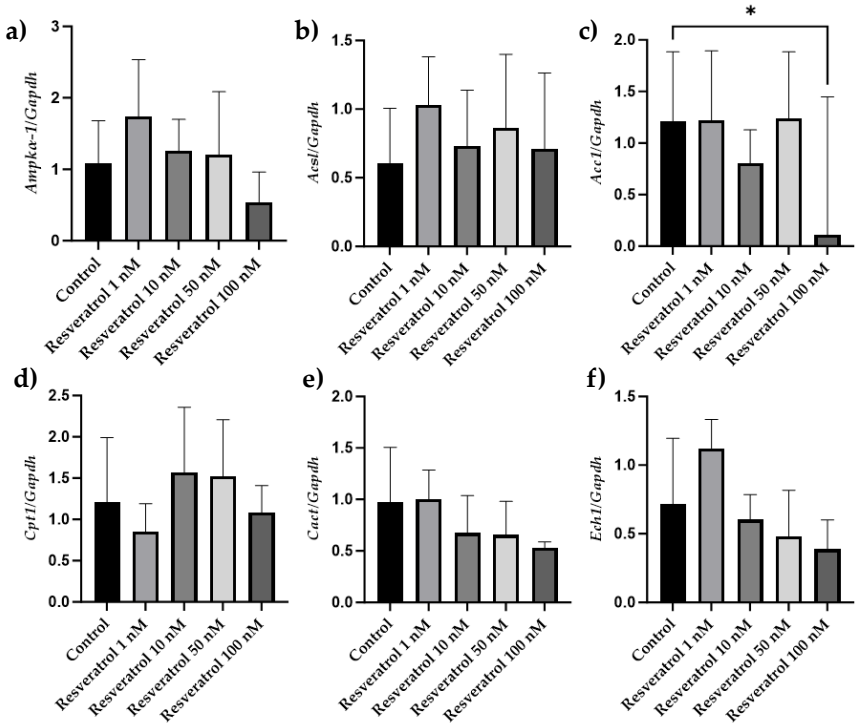

Figure 15. Gene expression of genes related to lipid catabolism in C2C12 cells treated with resveratrol for 12 hours.

mRNA levels of a) *Ampk* b) *Acsl* c) *Cpt1* d) *Acc1* e) *Cact* f) *Ech1*. Each experiment was triplicated and repeated on three different days (n = 9). The data is represented as mean ± standard deviation. The statistical significance is expressed as * $p<0.05$.

The cells treated with 10 nM resveratrol for 24 hours showed a significant upregulation in *Ampk, Acsl, Cpt1, Cact*, and *Ech1*. Additionally, *Acc1* was downregulated in the cells treated with 1 nM, 10 nM, and 50 nM. Furthermore, treatment with 100 nM increased *Ech1* expression after 24 hours (figure 16).

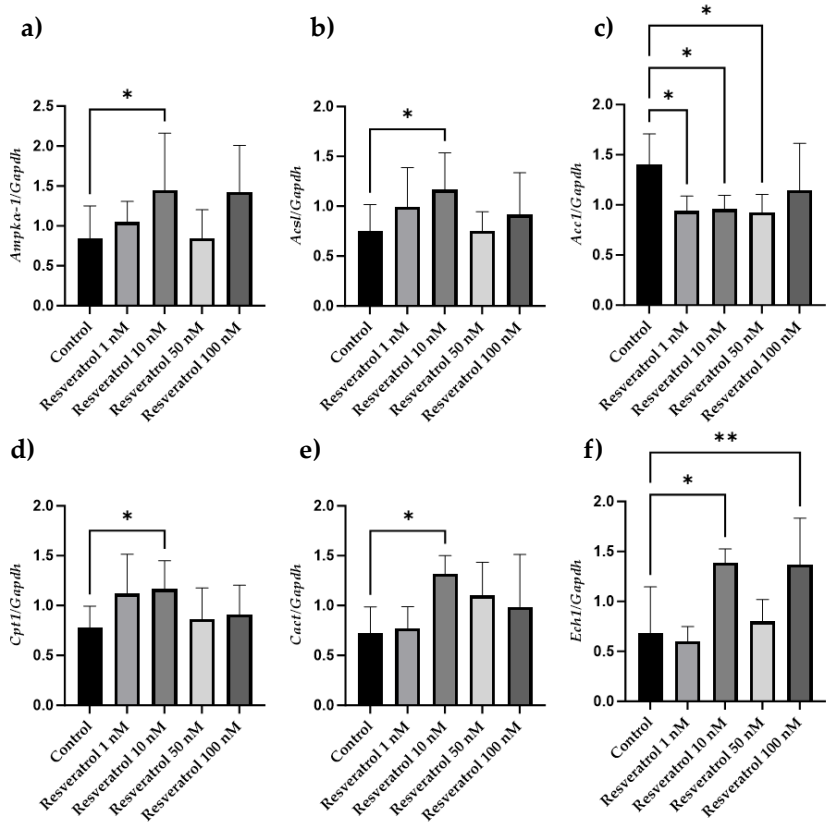

Figure 16. Gene expression of genes related to lipid catabolism in C2C12 cells treated with resveratrol for 24 hours.

mRNA levels of a) *Ampk* b) *Acsl* c) *Cpt1* d) *Acc1* e) *Cact* f) *Ech1*. Each experiment was triplicated and repeated on three different days (n = 9). The data is represented as mean ± standard deviation. The statistical significance is expressed as * $p<0.05$, ** $p<0.01$.

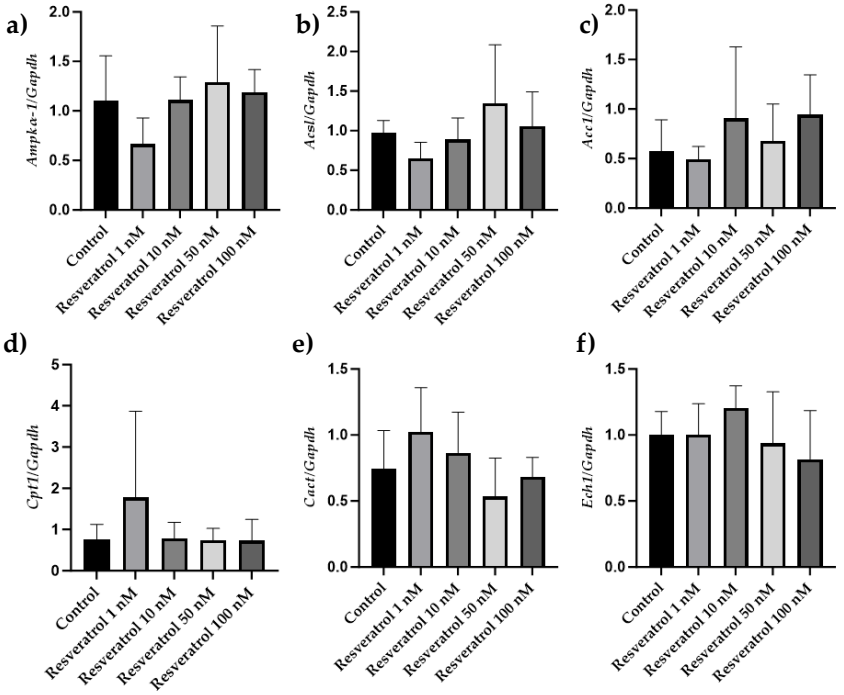

Figure 17. Gene expression of genes related to lipid catabolism in C2C12 cells treated with resveratrol for 48 hours.

mRNA levels of a) *Ampk* b) *Acsl* c) *Cpt1* d) *Acc1* e) *Cact* f) *Ech1*. Each experiment was triplicated and repeated on three different days (n = 9). The data is represented as mean ± standard deviation.

Interestingly, a 48-hour incubation did not significantly affect any of the genes measured at any concentration (figure 17).

We therefore concluded that the optimal treatment concentration is 10 nM, and the most effective incubation time is 24 hours and proceeded with the following experiments with these treatment conditions.

4.2 Resveratrol activates lipid catabolism and improves mitochondrial respiration through androgen receptors

4.2.1 Lipid catabolism

To study if resveratrol activates fatty acid transport to the mitochondria, we treated C2C12 cells with resveratrol and a combination of resveratrol and androgen receptor inhibitor, bicalutamide. We then measured the gene expression of the metabolic sensor AMP-activated protein kinase. We also measured the mitochondrial fatty acid transport enzymes carnitine-palmitoyltransferase 1 and β-oxidation enzyme enoyl-CoA, as well as the phosphorylation of Ampk to verify its activation. Figure 18 shows how *Ampk* and p-AMPK increase in the cells treated with resveratrol, but not in the cells treated with a combination of resveratrol and bicalutamide.

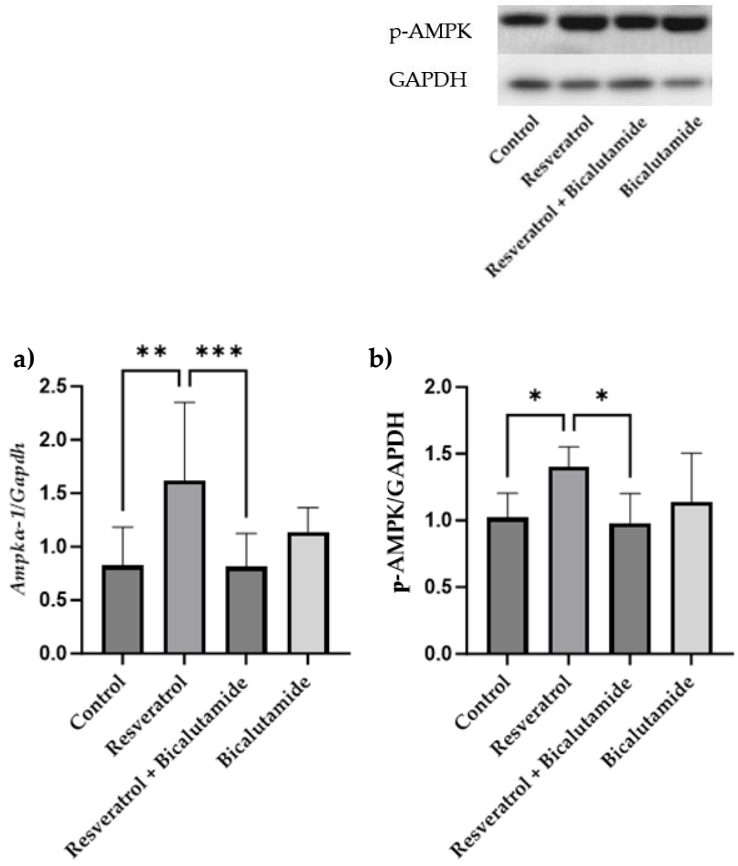

Figure 18. Gene expression of Ampk and p-AMPK protein levels in C2C12 cells treated with resveratrol and bicalutamide.

mRNA levels of a) *Ampk* and b) p-AMPK protein levels in comparison to *Gapdh*. Each experiment was triplicated and repeated on three different days (n = 9). The data is represented as mean ± standard deviation. The statistical significance is expressed as * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

Similarly, gene expression of *Cpt1* and *Ech1* increased in the cells treated with resveratrol, but not in those treated with the combination of resveratrol and bicalutamide (figure 19). These results indicate that resveratrol activates lipid catabolism through androgen receptor.

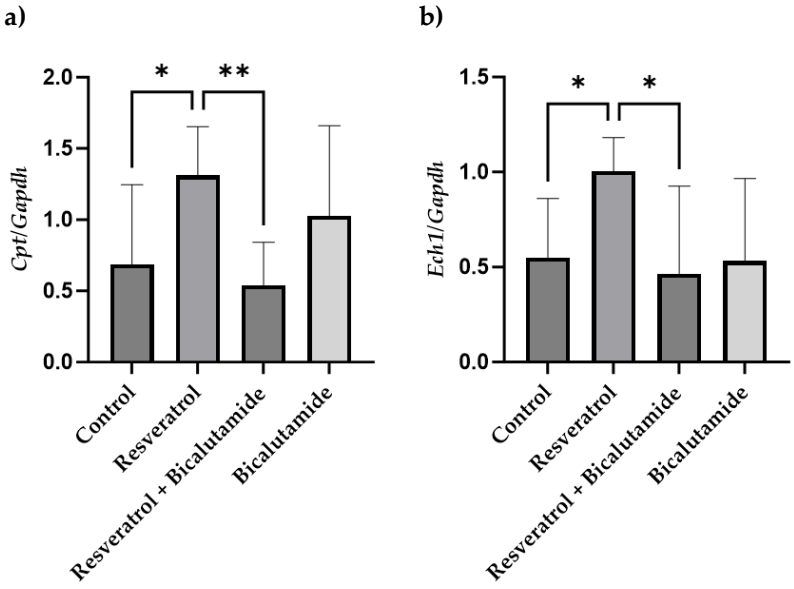

Figure 19. Gene expression of Cpt1 and Ech1 in C2C12 cells treated with resveratrol and bicalutamide.

mRNA levels of a) *Cpt1* and *Ech1* in comparison to *Gapdh*. Each experiment was triplicated and repeated on three different days (n = 9). The data is represented as mean ± standard deviation. The statistical significance is expressed as * $p<0.05$ and ** $p<0.01$.

4.2.2 Mitochondrial respiration

As resveratrol increased gene expression of genes related to lipid catabolism as well as phosphorylation of AMPK, but not in combination with bicalutamide we were interested in studying the translation to physiological outcomes of these results. We set out to study the effects of resveratrol and the implication of bicalutamide in the oxygen consumption of the cells in real-time. To this end, we performed a Mito Stress Test using acute resveratrol and bicalutamide treatment of 30 minutes and measured key parameters of mitochondrial respiration using a Seahorse Xf96. Figure 20 shows that maximal respiration, a parameter to measure the ability of the cells to meet a metabolic challenge, was increased when treated with resveratrol. Interestingly, it was also increased in the cells treated with the combination of resveratrol and bicalutamide, but not with bicalutamide alone.

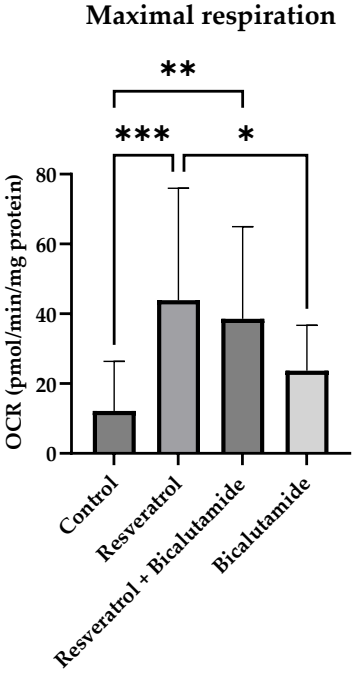

Figure 20. Maximal respiration of C2C12 cells after acute resveratrol and bicalutamide treatment.

Maximal respiration measured as OCR. 23 replicates were used for each experimental group seeded on three different days (n = 69). The data is represented as mean ± standard deviation. The statistical significance is expressed as * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

Next, we measured the spare capacity of the cell, which is used as a parameter to analyze the fitness or flexibility of a cell. As with the maximal respiration, we also found that the spare capacity was increased in the cells treated with resveratrol alone

and in combination with bicalutamide, but not in those treated with bicalutamide alone (figure 21).

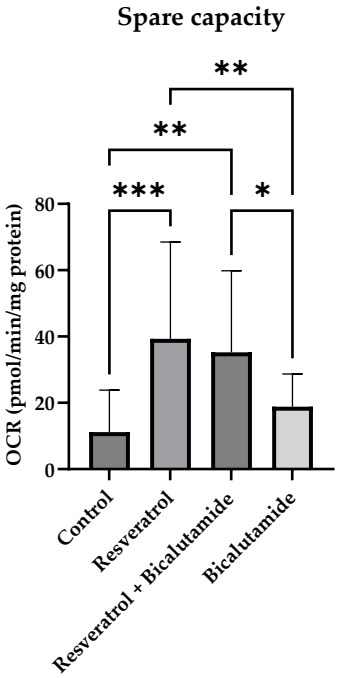

Figure 21. Spare respiratory capacity of C2C12 cells after acute resveratrol and bicalutamide treatment.

Spare respiratory capacity measured as OCR. 23 replicates were used for each experimental group seeded on three different days (n = 69). The data is represented as mean ± standard deviation. The statistical significance is expressed as * $p<0.05$, ** $p<0.01$, *** $p<0.001$

Proton leak is defined as the remaining basal respiration not coupled to ATP production. An increased proton leak can be a sign of mitochondrial damage. Figure 22 b) shows how acute resveratrol treatment alone, but not in combination with bicalutamide, reduces the proton leak of the cells. Interestingly, we found no difference in ATP production in any of the experimental groups (figure 22 a).

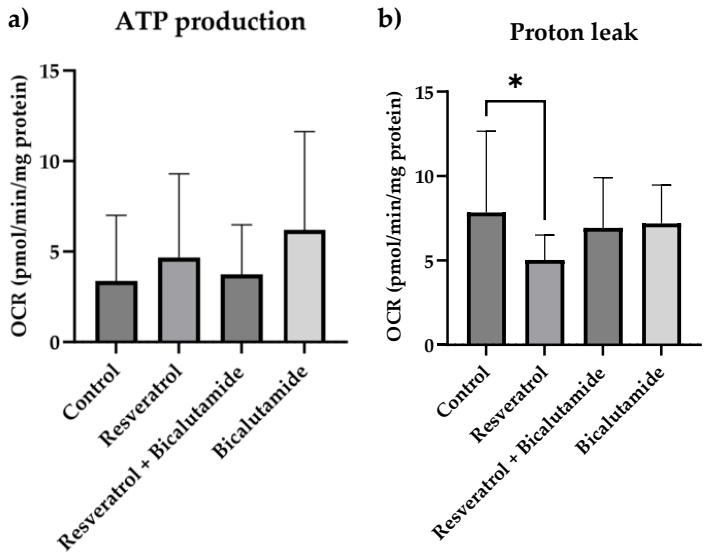

Figure 22. ATP production and proton leak in C2C12 cells treated with resveratrol and bicalutamide.

a) ATP production and b) proton leak measured as OCR. 23 replicates were used for each experimental group seeded on three different days (n = 69). The data is represented as mean ± standard deviation. The statistical significance is expressed as * $p<0.05$.

4.3 Resveratrol reduces oxidative stress in C2C12 cells through androgen receptors

As resveratrol has been found to have antioxidant properties (129), we set out to study if also these were mediated by an androgen receptor.

4.3.1 Antioxidant genes

To study if bicalutamide affects the antioxidant properties of resveratrol, we analyzed mRNA expression of the antioxidant genes catalase, glutathione peroxidase (*Gpx*), and Manganese-dependent superoxide dismutase (*MnSOD*) in comparison to the housekeeping gene *Gapdh.*

Figure 23 shows that neither resveratrol nor bicalutamide had any effects on the expression of *MnSOD* or *Cat*. However, the expression of *Gpx* was found to be significantly increased in the cells treated with resveratrol, but not in those treated with resveratrol in combination with bicalutamide.

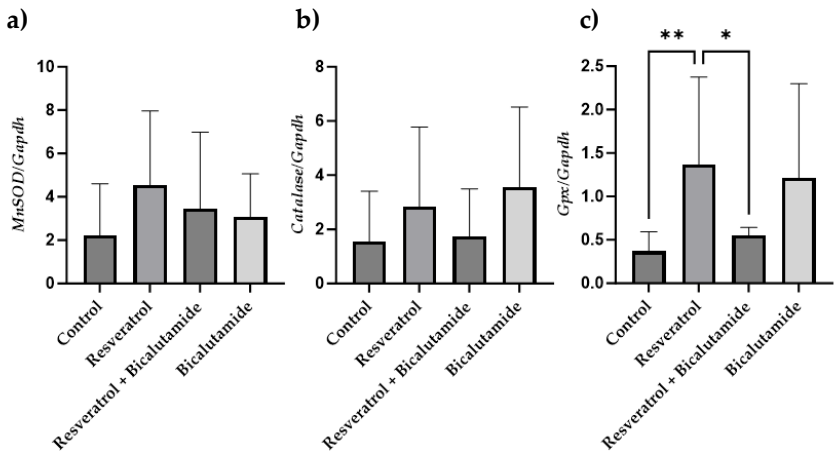

Figure 23. Expression of antioxidant genes in C2C12 treated with resveratrol and bicalutamide.

mRNA levels of a) *MnSOD,* b) *catalase,* and c) *Gpx* comparison to *Gapdh*. Each experiment was triplicated and repeated on three different days (n = 9). The data is represented as mean ± standard deviation. The statistical significance is expressed as * $p<0.05$ and ** $p<0.01$.

4.3.2 Hydrogen peroxide levels

To analyze if bicalutamide influences the endogenous ROS status of the cells, we measured H_2O_2 levels in cells treated with resveratrol alone and in combination with bicalutamide.

We found that the cells treated with resveratrol showed significantly lower levels of H_2O_2 in comparison to the control and resveratrol + bicalutamide group (figure 24).

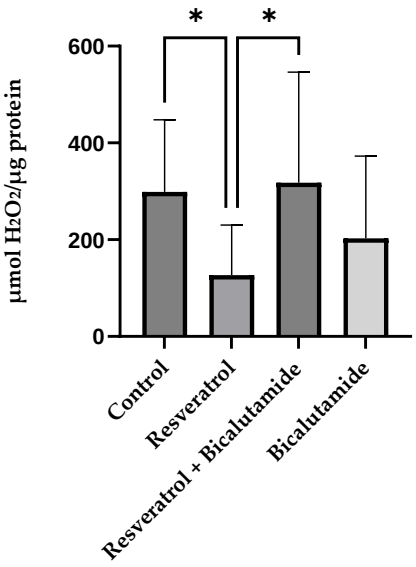

Figure 24. Hydrogen peroxide levels in C2C12 cells treated with resveratrol and bicalutamide.

Each experiment was triplicated and repeated on three different days (n = 9). The data is represented as mean ± standard deviation. The statistical significance is expressed as * $p<0.05$.

4.3.3 Lipid and protein oxidation

To analyze the effects of resveratrol and bicalutamide on lipid and protein oxidation, we measured carbonylated proteins and MDA as a marker for lipid peroxidation.

Figure 25 shows that neither resveratrol alone or in combination with bicalutamide affected protein carbonylation nor MDA levels.

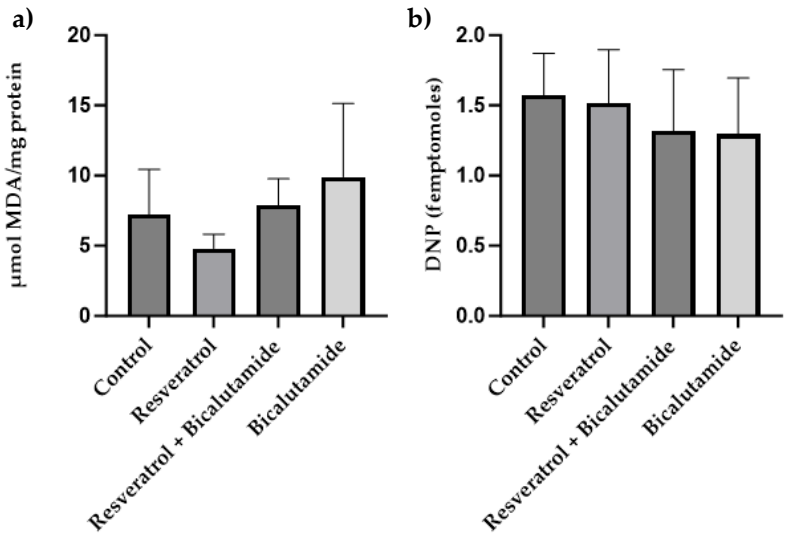

Figure 25. Lipid and protein oxidation in C2C12 cells treated with resveratrol and bicalutamide.

a) MDA levels b) levels of protein carbonylation. Each experiment was triplicated and repeated on three different days (n = 9). The data is represented as mean ± standard deviation.

4.4 Resveratrol activates lipid catabolism and reduces oxidative stress through nuclear androgen receptors in C2C12 cells

To study if resveratrol binds to membrane or nuclear receptor, we treated the cells with piceid alone and in combination with bicalutamide. As piceid is a glycosylated derivative of resveratrol, it will not be able to readily enter the cell membrane, and we can therefore assess the location of the receptor by comparing the effects of piceid to those of resveratrol.

4.4.1 Genes related to fatty acid transportation

Treatment with piceid significantly increased expression of *Ampkα-1* but did not affect the expression of *Acc1* or the mitochondrial enzymes *Cpt1*, *Cact*, or *Ech1* (Figure 26). However, the expression of *Ech1* was found to be overexpressed in the cells treated with piceid in combination with bicalutamide, in comparison to the control and bicalutamide group (Figure 26 e)).

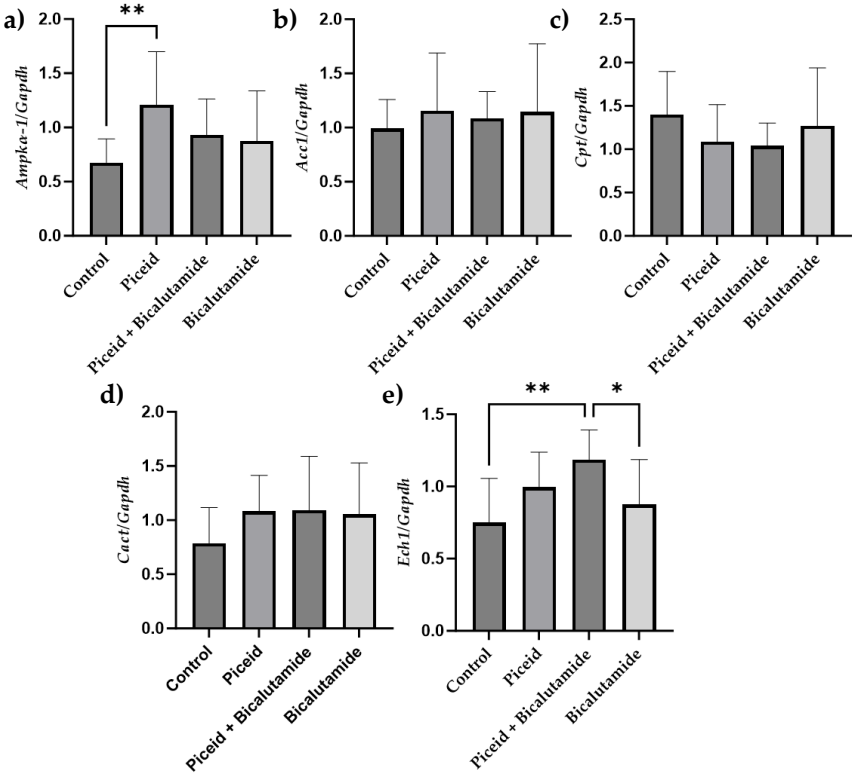

Figure 26. Expression of genes related to fatty acid transport and oxidation in C2C12 treated with piceid and bicalutamide.

mRNA levels of a) *Ampkα-1,* b) *Acc1,* c) *Cpt1* d) *Cact,* and e) *Ech1* in comparison to *Gapdh.* Each experiment was triplicated and repeated on three different days (n = 9). The data is represented as mean ± standard deviation. The statistical significance is expressed as * $p<0.05$ and ** $p<0.01$.

4.4.2 Antioxidant genes and lipid peroxidation

Treatment of piceid was not found to affect the antioxidant enzymes *MnSOD, Gpx,* or *catalase,* nor the levels of MDA as a marker for lipid peroxidation. Interestingly, *MnSOD* was upregulated in the cells treated with bicalutamide and *catalase* expression increased in the cells treated with bicalutamide alone and in combination with piceid (figure 27).

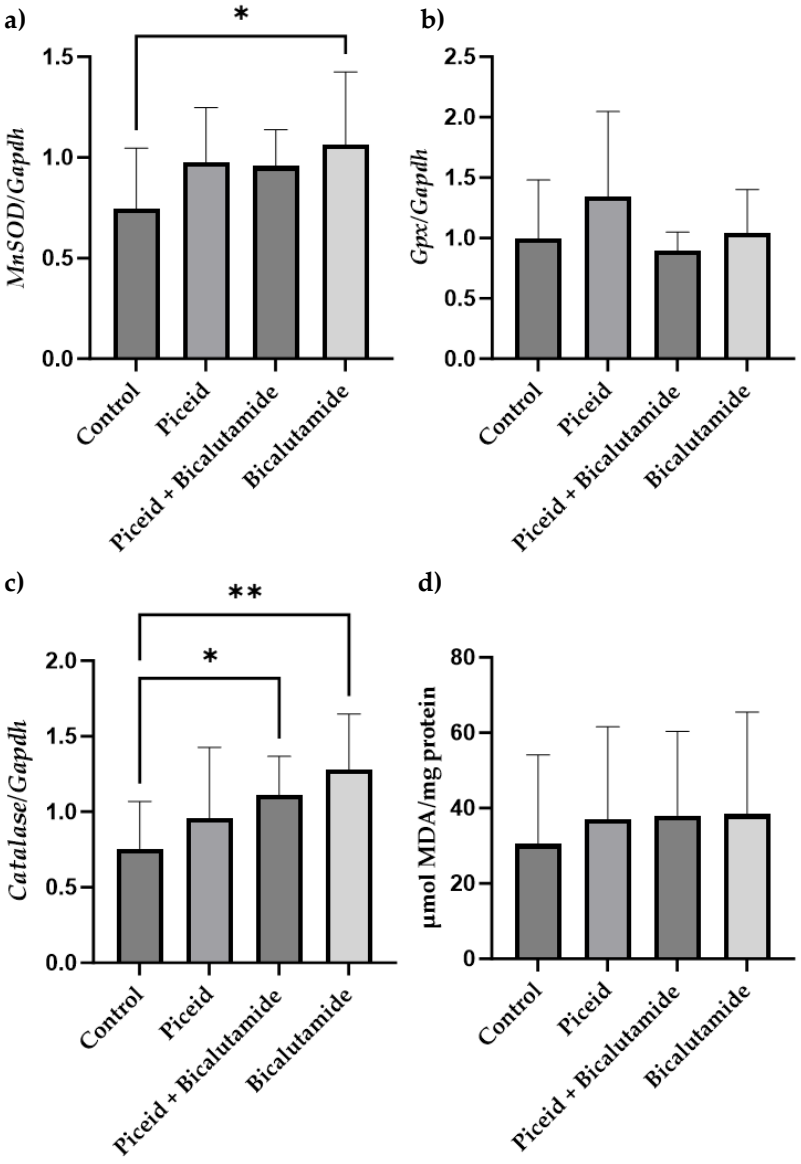

Figure 27. Expression of antioxidant enzymes and lipid peroxidation levels in C2C12 treated with piceid and bicalutamide.

mRNA levels of a) *MnSOD,* b) *Gpx,* and c) *catalase* in comparison to *Gapdh* and d) MDA levels as a marker for lipid peroxidation. Each experiment was

triplicated and repeated on three different days (n = 9). The data is represented as mean ± standard deviation. The statistical significance is expressed as * $p<0.05$ and ** $p<0.01$.

4.5 The effect of hydroxytyrosol on mitochondrial function and oxidative stress in C2C12 cells

As previously described, hydroxytyrosol has been found to exert multiple protective effects. However, most of the studies published are performed with concentrations only available as supplements. Therefore, we set out to study if hydroxytyrosol at nutritionally relevant concentrations could exert the same effects as previously reported at high concentrations.

4.5.1 The effects of hydroxytyrosol on mitochondrial function of C2C12 cells

4.5.1.1 Genes related to mitochondrial biogenesis

To study the effects of hydroxytyrosol on mitochondrial biogenesis, we analyzed the gene expression of *Pgc-1α*, *Nrf1*, and *Tfam*. We found no differences in the expression of *Nrf1* and *Tfam* in any of the experimental groups. However, treatment with 100 nM and 10 μM increased expression of *Pgc-1α* (figure 28).

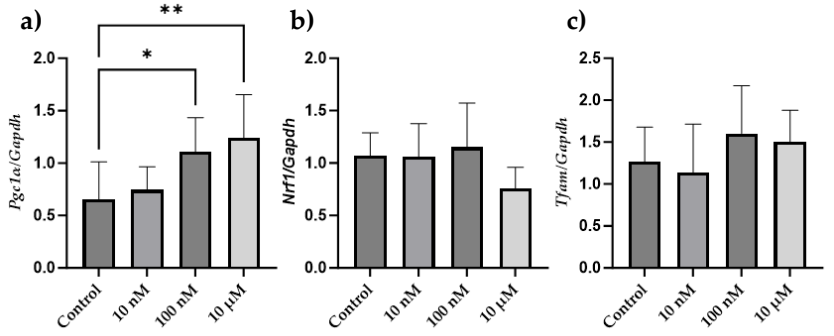

Figure 28. Expression of genes related to mitochondrial biogenesis in C2C12 treated with hydroxytyrosol.

mRNA levels of a) *Pgc-1α,* b) *Nrf1,* and c) *Tfam* in comparison to *Gapdh*. Each experiment was triplicated and repeated on three different days (n = 9). The data is represented as mean ± standard deviation. The statistical significance is expressed as * $p<0.05$ and ** $p<0.01$.

4.5.1.2 Genes related to fatty acid transport and β-oxidation to the mitochondria

To assess the effects of hydroxytyrosol on fatty acid transport and β-oxidation, we measured the expression of *Ampkα-1,* and the mitochondrial enzymes *Cpt1* and *Ech1.* Figure 29 shows how hydroxytyrosol at 10 nM significantly increased expression of *Ampkα-1* and treatment with 10 and 100 nM increased *Cpt1* expression. No differences were found in *Ech1* expression in any of the experimental groups.

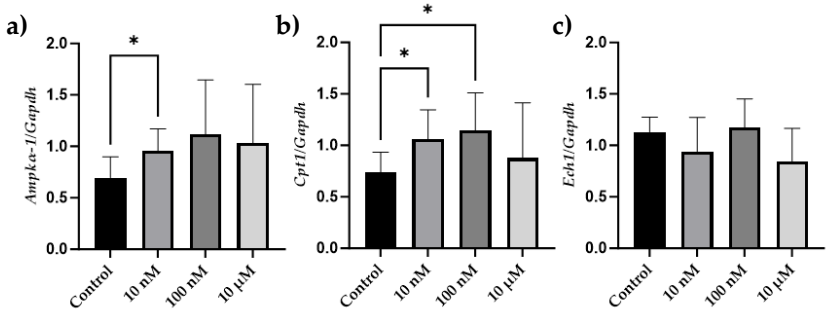

Figure 29. Expression of genes related to fatty acid transport and β-oxidation in C2C12 treated with hydroxytyrosol.

mRNA levels of a) *Ampkα-1,* b) *Cpt1,* and c) *Ech1* in comparison to *Gapdh*. Each experiment was triplicated and repeated on three different days (n = 9). The data is represented as mean ± standard deviation. The statistical significance is expressed as * $p<0.05$.

4.5.2 Mitochondrial respiration

To study the effects of acute hydroxytyrosol treatment on oxygen consumption of the cells in real-time, we performed a Mito Stress Test to measure key parameters of mitochondrial respiration using a Seahorse XF96.

We found that maximal respiration, a parameter used to measure the ability of the cells to meet a metabolic challenge, increased significantly after treatment with 10 nM, 100 nM, and 10 µM hydroxytyrosol (figure 30).

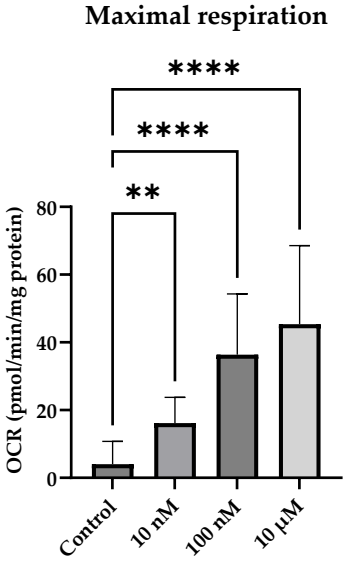

Figure 30. Maximal respiration of C2C12 cells after acute hydroxytyrosol treatment.

Maximal respiration measured as OCR. 23 replicates were used for each experimental group seeded on three different days (n = 69). The data is represented as mean ± standard deviation. The statistical significance is expressed as ** $p<0.01$ and **** $p<0.0001$.

Spare capacity, as a parameter for the fitness and flexibility of the cells (figure 31), and ATP production (figure 32) was also found to be significantly increased in all treatment groups.

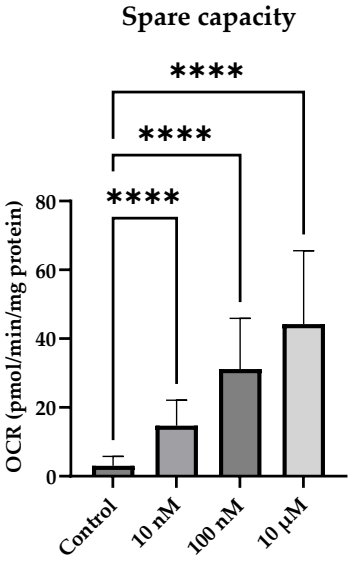

Figure 31. Maximal respiration of C2C12 cells after acute hydroxytyrosol treatment.

Maximal respiration measured as OCR. 23 replicates were used for each experimental group and repeated on three different days (n = 69). The data is represented as mean ± standard deviation. The statistical significance is expressed as **** $p<0.0001$.

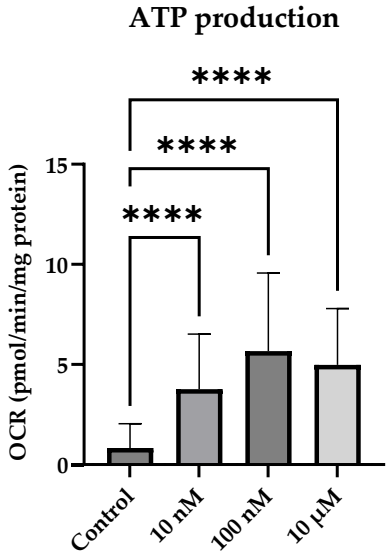

Figure 32. ATP production of C2C12 cells after acute hydroxytyrosol treatment.

ATP production measured as OCR. 23 replicates were used for each experimental group and repeated on three different days (n = 69). The data is represented as mean ± standard deviation. The statistical significance is expressed as **** $p<0.0001$.

No differences were found between the experimental groups in proton leak, a parameter used to measure potential mitochondrial damage (figure 33).

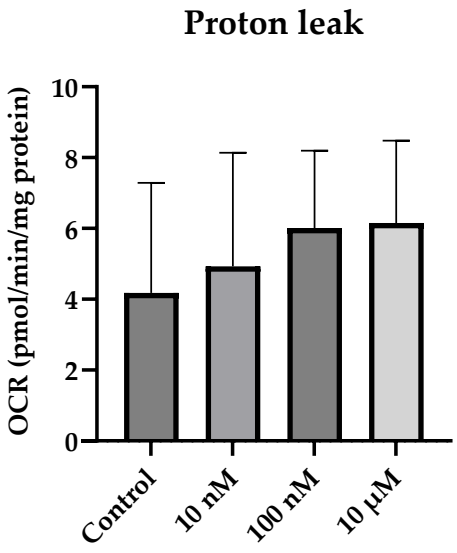

Figure 33. Proton leak of C2C12 cells after acute hydroxytyrosol treatment.

Proton leak measured as OCR. 23 replicates were used for each experimental group and repeated on three different days (n = 69). The data is represented as mean ± standard deviation.

4.6 The effects of hydroxytyrosol on the redox state of C2C12 cells

4.6.1 Antioxidant genes

To study if hydroxytyrosol could affect the antioxidant defense system, we analyzed the mRNA expression of the antioxidant enzymes *MnSOD, catalase,* and *Gpx*. We found that 10 μM of

hydroxytyrosol significantly increased *catalase* expression, although no differences were found in *MnSOD* and *Gpx* expression (figure 34).

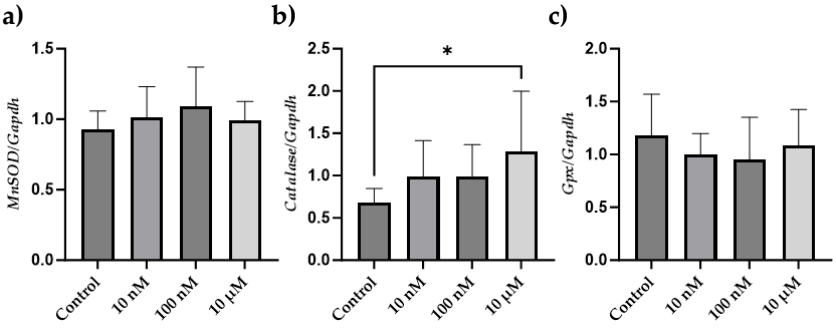

Figure 34. Expression of antioxidant genes in C2C12 treated with hydroxytyrosol.

mRNA levels of a) *MnSOD,* b) *catalase,* and c) *Gpx* comparison to *Gapdh*. Each experiment was triplicated and repeated on three different days (n = 9). The data is represented as mean ± standard deviation. The statistical significance is expressed as * $p<0.05$.

4.6.2 Hydrogen peroxide levels

To analyze whether hydroxytyrosol could have an effect on endogenous ROS levels, we measured the levels of hydrogen of the cells. We found no significant differences between the experimental groups (figure 35).

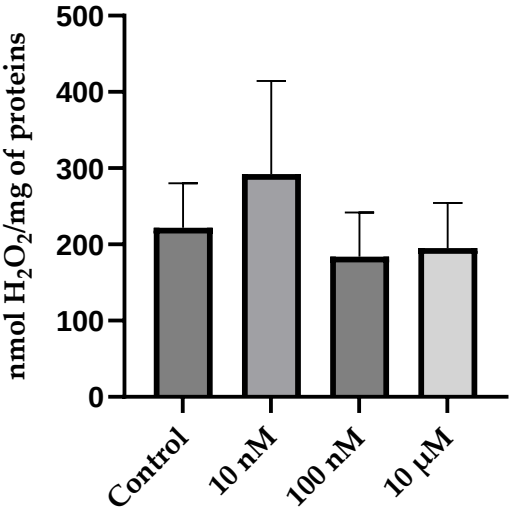

Figure 35. Hydrogen peroxide levels in C2C12 cells treated with hydroxytyrosol.

Each experiment was triplicated and repeated on three different days (n = 9). The data is represented as mean ± standard deviation.

4.6.3 Protein and lipid oxidation

To study if hydroxytyrosol could influence oxidation of proteins and lipids, we measured protein carbonylation and MDA as a marker for lipid peroxidation.

We found that both MDA levels and protein carbonylation decreased in the cells treated with 10 μM hydroxytyrosol (figure 36).

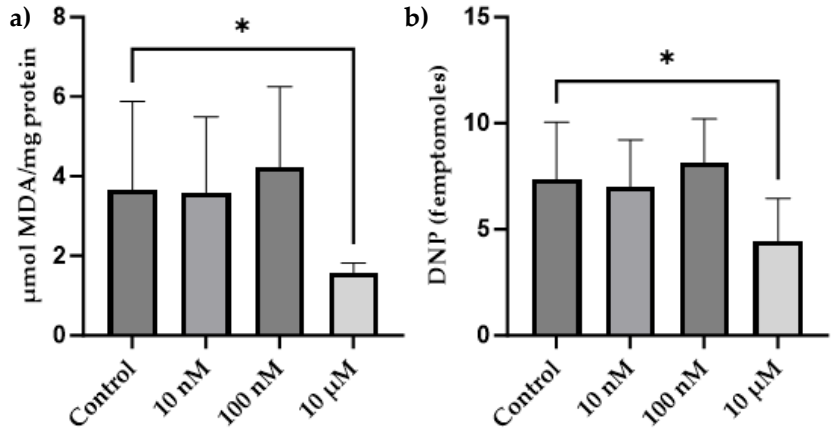

Figure 36. Lipid and protein oxidation in C2C12 cells treated with hydroxytyrosol.

a) MDA levels b) levels of protein carbonylation. Each experiment was triplicated and repeated on three different days (n = 9). The data is represented as mean ± standard deviation. The statistical significance is expressed as * $p<0.05$.

4.7 The effects of piceid treatment on frailty and longevity of Drosophila Melanogaster

4.7.1 Phenotypic parameters

4.7.1.1 Longevity curve

To analyze the possible effects of piceid treatment on longevity, we performed a survival curve with *Drosophila Melanogaster*. The experimental groups were separated into a robust control group and a group of robust flies receiving 10 nM piceid treatment. The frail flies were also separated into a control group and a group receiving 10 nM piceid in their feed. Figure 37 shows the survival curve of the flies along their lifespan. We found that the robust control group had a 13% increase in median survival in comparison to the frail control group. The robust piceid group also showed a 10% increase in median survival in comparison to frail piceid group. No statistical differences were found between the treatment groups and their respective controls.

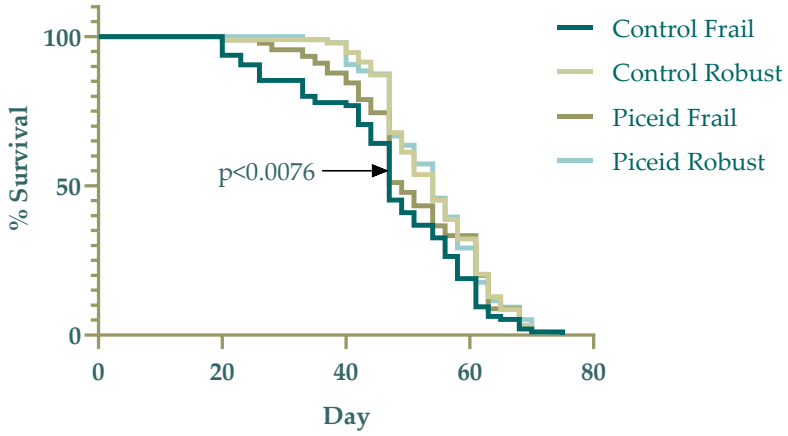

Figure 37. Longevity curve of *Drosophila Melanogaster* treated with piceid.

Longevity curve of frail control flies (dark blue), robust control flies (beige), frail flies treated with piceid (brown), and robust flies treated with piceid (light blue). The statistical difference was calculated with Kaplan-Meier and log-rank and is shown as p<0.0076 between the frail and robust control group. n = 100 per group.

4.7.1.2 Geotaxis assay

Geotaxis assays were performed to analyze whether piceid treatment could affect the fitness or activity levels of the flies. This assay is performed taking into consideration the negative geotaxis of the fly. The assay was performed at 80%, 50%, and 10% survival. We found no differences in the number of flies being

able to pass the test between any of the experimental groups at any of the time points (figure 38).

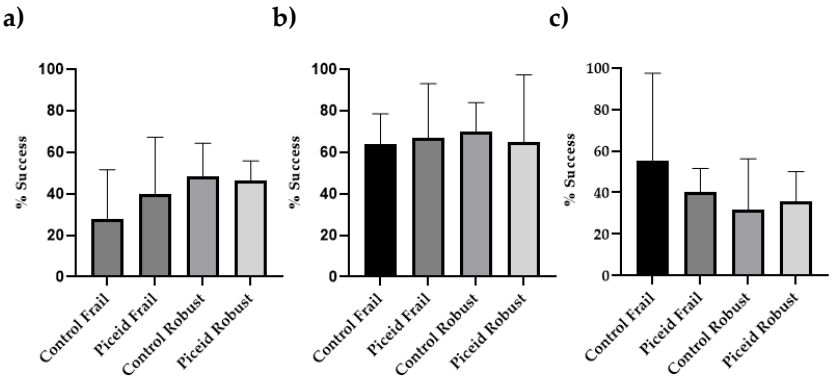

Figure 38. Geotaxis assay in *Drosophila Melanogaster* treated with piceid.

The number of flies that successfully passed the geotaxis test represented as percentage of total number of participants. The figures show the results of the assay at a) 80% of longevity, b) 50% of longevity, and c) 10% of longevity. The data is represented as mean ± standard deviation.

4.7.1.3 Phototaxis assay

We analyzed phototaxis as another parameter of fitness of the fly. This assay takes advantage of the natural instinct of the fly to follow a light source. The assay was performed at 80%, 50%, and 10% of survival. As shown in figure 39, we found a significant difference between the control frail and control robust group, as

well as the piceid frail and piceid robust group at 80% and 50% survival. No differences were found between the controls and their respective treatment groups.

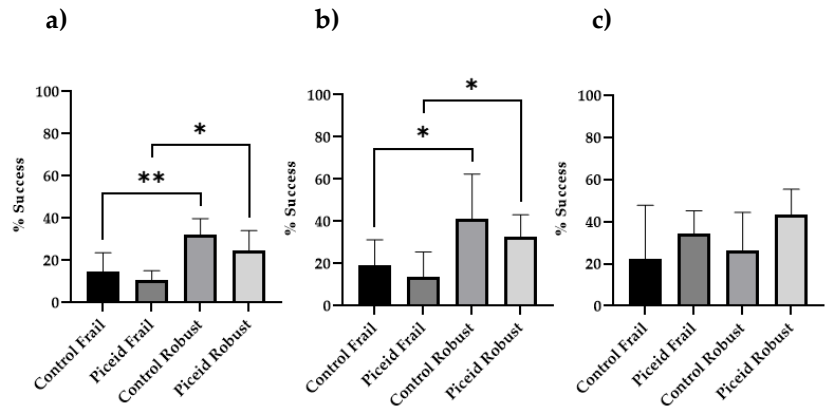

Figure 39. Phototaxis assay in *Drosophila Melanogaster* treated with piceid.

The number of flies that successfully passed the phototaxis test represented as percentage of total number of participants. The figures show the results of the assay at a) 80% of longevity, b) 50% of longevity, and c) 10% of longevity. The data is represented as mean ± standard deviation. The statistical significance is expressed as * $p<0.05$ and ** $p<0.01$.

4.7.1.4 Food ingestion

To ensure that none of the results were due to calorie restriction or overconsumption of food, we measured the food ingestion in each experimental group. As shown in table 8, we found no

difference in the amount of food ingested between the experimental groups.

Table 8. Food ingestion of Drosophila Melanogaster consuming piceid.

Frail Control	Robust Control	Frail Piceid	Robust Piceid
3.744	3.648	3.592	3.656

Comparison between the consumption of food between the flies following the standard control diet and piceid presented as absorption of erioglaucine blue at 625 nm/mg protein.

4.7.2 Biochemical parameters

4.7.2.1 Genes related to longevity

Even though piceid treatment did not improve lifespan or the activity levels of the flies, we wanted to research if there could be a possible improvement in the expression of genes related to longevity. We set out to measure three genes related to the immune system, as well as 16s rRNA, which has been positively correlated with improved longevity curves in *Drosophila melanogaster*. At 80% of lifespan, we found no differences in the expression of *Attacin C, Cecropin C, Diptericin, nor 16s rRNA* (figure 40).

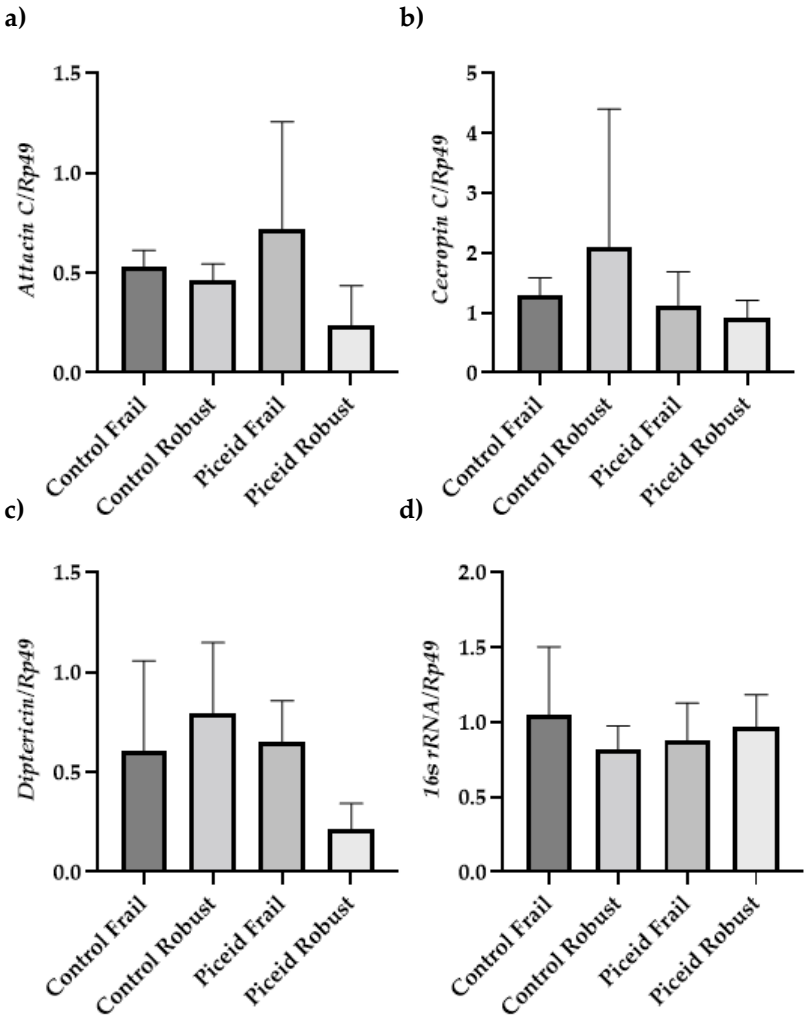

Figure 40. Gene expression of immune system-related genes in *Drosophila melanogaster* treated with piceid at 80% longevity.

mRNA levels of a) *Attacin C* b) *Cecropin C* c) *Diptericin,* and d) *16s rRNA* in comparison to control gene *Rp49*. Each experiment was performed with three samples of three flies each (n = 9). The data is represented as mean ± standard deviation.

However, as the flies age, we did observe an improvement in gene expression. At 50% of lifespan, we found an upregulation of *Attacin C* and *Diptericin* in the frail treated group in comparison to the frail controls. We also found an upregulation of *Cecropin* in the robust flies administered piceid in comparison to the control robust group (figure 41).

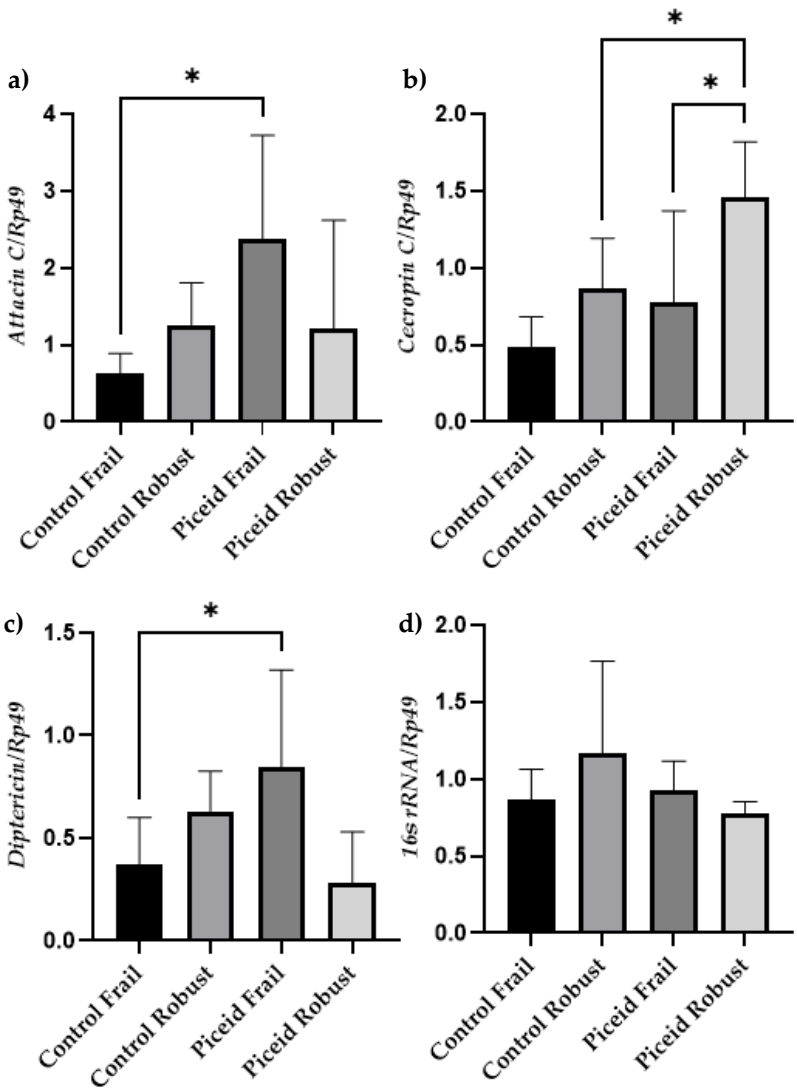

Figure 41. Gene expression of immune system-related genes in *Drosophila melanogaster* treated with piceid at 50% longevity.

mRNA levels of a) *Attacin C* b) *Cecropin C* c) *Diptericin,* and d) *16s rRNA* in comparison to control gene *Rp49*. Each experiment was performed with three samples of three flies each (n = 9). The data is represented as mean ± standard deviation. The statistical significance is expressed as * $p<0.05$.

Interestingly, at 10% longevity we found significantly higher levels of all the genes measured in the piceid-treated frail flies in comparison to the frail control group. Additionally, *Diptericin* was upregulated in the robust flies treated with piceid in comparison to the robust controls (figure 42).

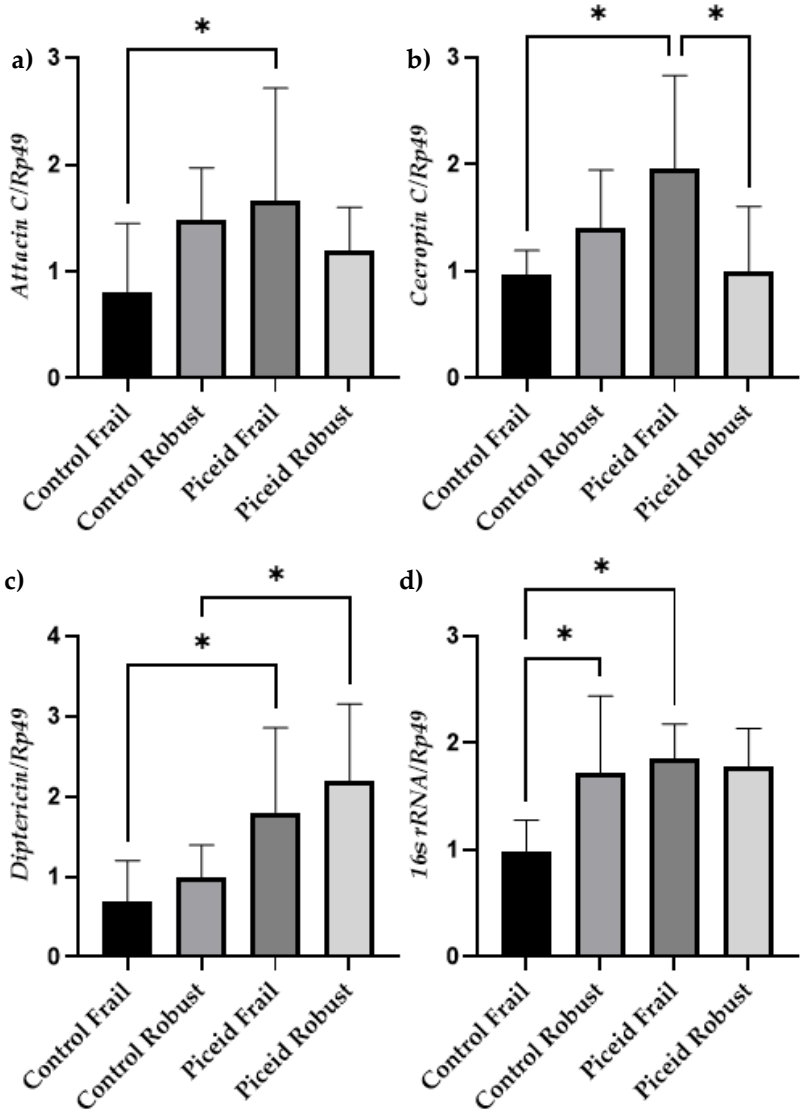

Figure 42. Gene expression of immune system-related genes in *Drosophila melanogaster* treated with piceid at 10% longevity.

mRNA levels of a) *Attacin C* b) *Cecropin C* c) *Diptericin* and d) *16s rRNA* in comparison to control gene *Rp49*. Each experiment was performed with three

samples of three flies each (n = 9). The data is represented as mean ± standard deviation. The statistical significance is expressed as * $p<0.05$.

4.7.2.2 Antioxidant genes

We were also interested to determine whether piceid treatment could protect the flies against oxidative stress. We therefore analyzed the expression of the antioxidant enzymes *catalase*, *MnSOD*, as well as *p53*, due to its ability to induce expression of pro-survival genes with antioxidant properties (130).

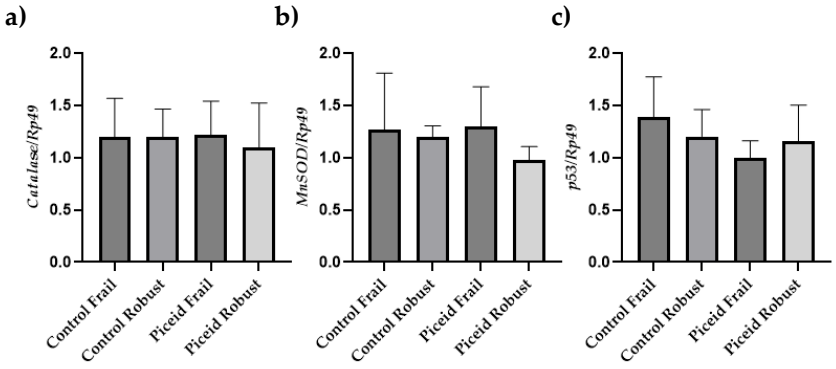

Figure 43. Gene expression of antioxidant genes in *Drosophila melanogaster* treated with piceid at 80% longevity.

mRNA levels of a) *Catalase* b) *MnSOD* c) *p53* in comparison to control gene *Rp49*. Each experiment was performed with three samples of three flies each (n = 9). The data is represented as mean ± standard deviation.

As shown in figure 43, we found no differences in the expression of any of the measured genes in the flies at 80% longevity.

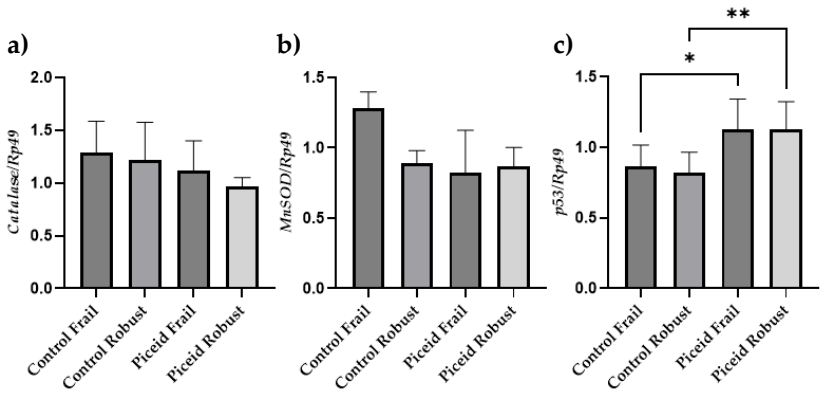

Figure 44. Gene expression of antioxidant genes in *Drosophila melanogaster* treated with piceid at 50% longevity.

mRNA levels of a) *Catalase* b) *MnSOD* c) *p53* in comparison to control gene *Rp49*. Each experiment was performed with three samples of three flies each (n = 9). The data is represented as mean ± standard deviation. The statistical significance is expressed as * $p<0.05$ ** $p<0.01$.

However, at 50% longevity, we found an increased expression in *p53* in both the frail and robust flies treated with piceid in comparison to their respective controls (figure 44). Interestingly, this protective effect of piceid is lost as the flies age. No changes in antioxidant enzyme expression were found at 10% longevity (figure 45).

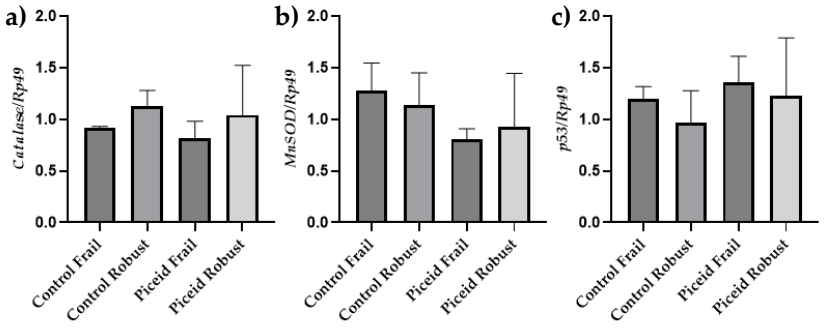

Figure 45. Gene expression of antioxidant genes in *Drosophila melanogaster* treated with piceid at 10% longevity.

mRNA levels of a) *Catalase* b) *MnSOD* c) *p53* in comparison to control gene *Rp49*. Each experiment was performed with three samples of three flies each (n = 9). The data is represented as mean ± standard deviation.

4.8 The effects of harmol treatment on frailty and longevity of Drosophila Melanogaster

Harmol, an alkaloid present in coffee has recently been found to increase longevity in *Drosophila Melanogaster* and delay aging in mice (105). We were therefore interested in studying whether harmol at lower concentrations would be able to improve frailty parameters and improve lifespan in *Drosophila Melanogaster.*

4.8.1 Phenotypic parameters

4.8.1.1 Longevity curve

We found that treatment with 10 nM harmol increased median survival of the frail flies with 16%. We also found a 13% increase in median survival in the robust control flies in comparison to the frail controls (figure 46).

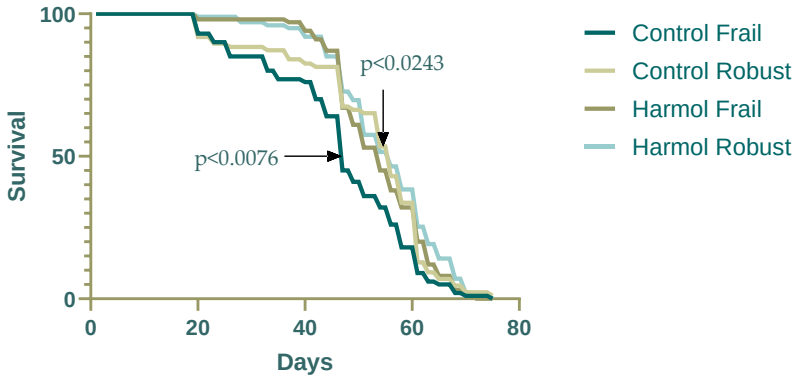

Figure 46. Longevity curve of *Drosophila melanogaster* treated with harmol.

Longevity curve of frail control flies (dark blue), robust control flies (beige), frail flies treated with harmol (brown), and robust flies treated with harmol (light blue). The statistical difference was calculated with Kaplan-Meier and log-rank and is shown as $p<0.0243$ between the frail control and frail harmol-treated flies and $p<0.0076$ between the frail and robust control group. n = 100 per group.

4.8.1.2 Geotaxis assay

To assess the activity levels and fitness of the flies as the flies aged, we performed a geotaxis assay at 80%, 50%, and 10% survival. As shown in figure 47, we found no differences in the flies able to pass the test any of the experimental groups at any time point.

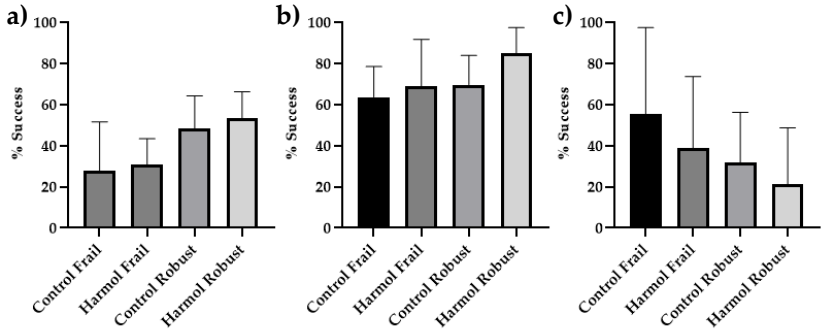

Figure 47. Geotaxis assay in *Drosophila melanogaster* treated with harmol.

The number of flies that successfully passed the geotaxis test represented as percentage of total number of participants. The figures show the results of the assay at a) 80% of longevity, b) 50% of longevity, and c) 10% of longevity. The data is represented as mean ± standard deviation.

4.8.1.3 Phototaxis assay

To further asses the activity level of the flies, we performed phototaxis assays at 80%, 50%, and 10% survival. We found a significant increase in the flies able to pass the test of the robust groups in comparison to the frail flies of the same treatment (figure 48 a). At 50% survival we only found a significant improvement in the robust controls in comparison to the frail controls. At 10% survival, a tendency of improvement can be observed in both harmol groups, although this was not statistically significant (figure 4 c).

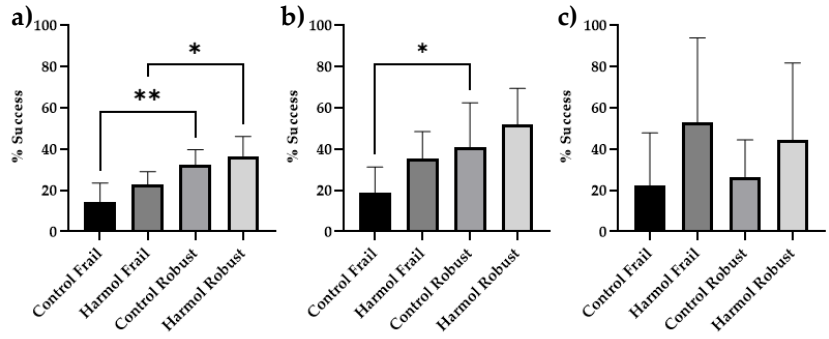

Figure 48. Phototaxis assay in *Drosophila melanogaster* treated with harmol.

The number of flies that successfully passed the geotaxis test represented as percentage of total number of participants. The figures show the results of the assay at a) 80% of longevity, b) 50% of longevity, and c) 10% of longevity. The data is represented as mean ± standard deviation. The statistical significance is expressed as * $p<0.05$ and ** $p<0.01$.

4.8.1.4 Food ingestion

To ensure that none of the results were due to calorie restriction or overconsumption of food, we measured the food ingestion in each experimental group. As shown in table 9, we found no difference in the amount of food ingested between the experimental groups.

Table 9. Food ingestion of *Drosophila melanogaster* consuming harmol.

Frail Control	Robust Control	Frail Harmol	Robust Harmol
3.744	3.648	3.696	3.616

Food ingestion of *Drosophila Melanogaster* following the standard control diet and harmol presented as absorption of erioglaucine blue at 625 nm/mg protein.

4.8.2 Biochemical parameters

4.8.2.1 Genes related to the immune system

As harmol treatment improved lifespan of the flies, we set out to analyze if this increase was due to an improved immune system. For this, we analyzed three genes related to the immune system in the *Drosophila Melanogaster, Attacin C, Cecropin C, Diptericin.* Additionally, we analyzed the expression of *16s rRNA,* a gene present in the gut microbiota and related to longevity in *Drosophila melanogaster.* The four genes were analyzed at 80%, 50%, and 10% survival.

We found no differences in the expression of *attacin C, diptericin, or 16s rRNA.* However, we found a decrease in *cecropin c* in the harmol-treated robust group compared to the robust control group (figure 49).

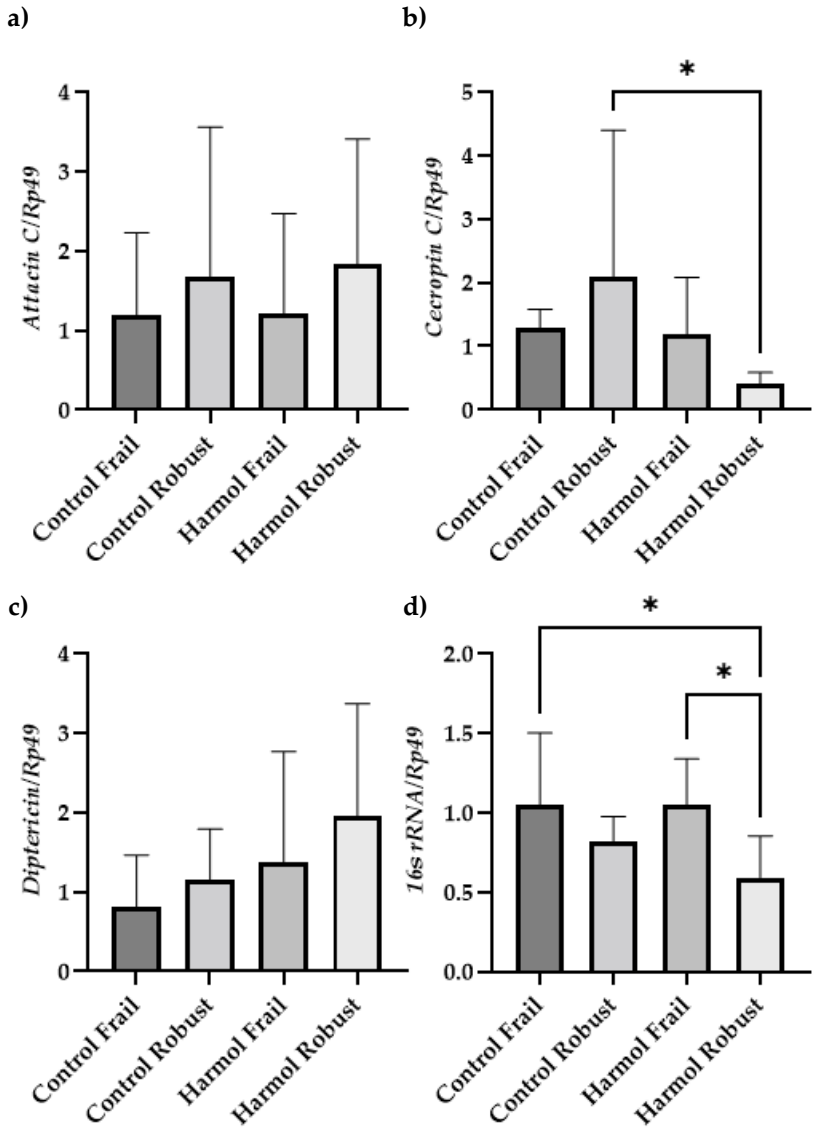

Figure 49. Gene expression of immune system-related genes in *Drosophila Melanogaster* treated with harmol at 80% longevity.

mRNA levels of a) *Attacin C* b) *Cecropin C* c) *Diptericin,* and d) *16s rRNA* in comparison to control gene *Rp49*. Each experiment was performed with three

samples of three flies each (n = 9). The data is represented as mean ± standard deviation. The statistical significance is expressed as * $p<0.05$.

Similarly, at 50% survival, we found a decrease in the levels of *attacin C* in the robust harmol-treated flies in comparison to the robust control group. Harmol treatment did however succeed in activating *diptericin* and *16s rRNA* in the frail flies. Although interestingly, *16s rRNA* was downregulated in the robust harmol group in comparison to the frail flies administered harmol treatment (figure 50).

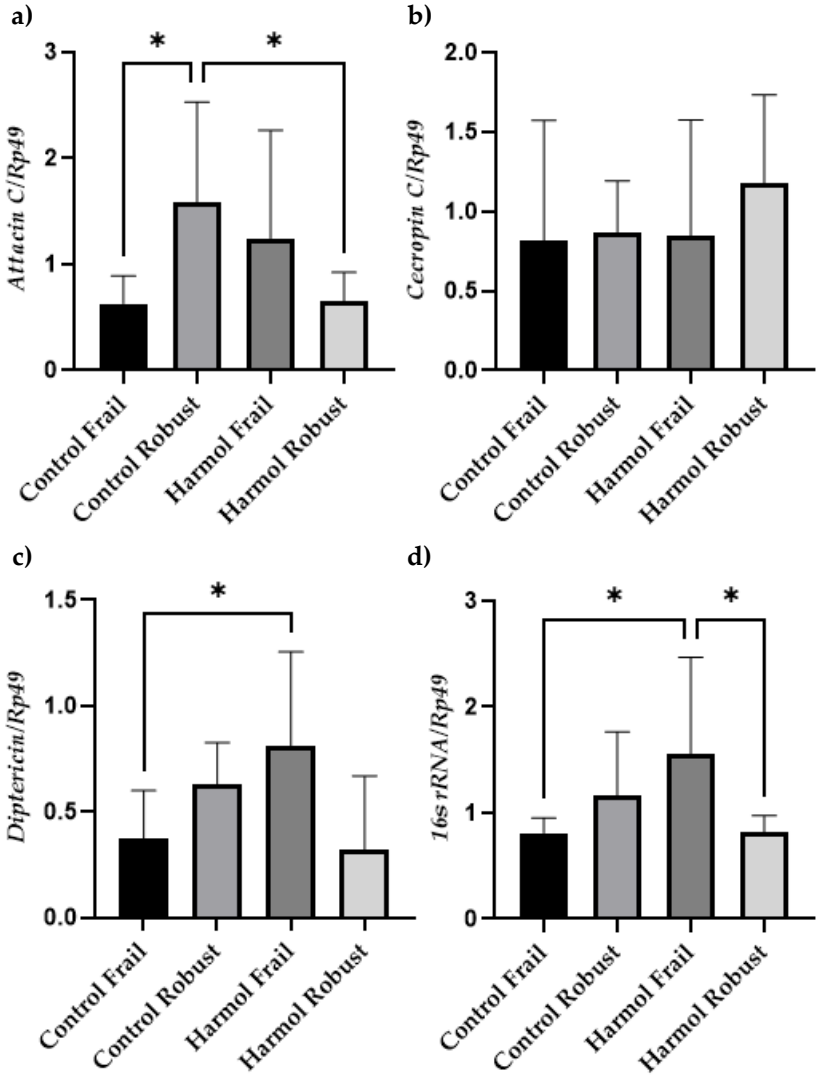

Figure 50. Gene expression of immune system-related genes in *Drosophila melanogaster* treated with harmol at 50% longevity.

mRNA levels of a) *Attacin C* b) *Cecropin C* c) *Diptericin,* and d) *16s rRNA* in comparison to control gene *Rp49*. Each experiment was performed with three samples of three flies each (n = 9). The data is represented as mean ± standard deviation. The statistical significance is expressed as * $p<0.05$.

No differences were found in the expression of any of the genes measured at 10% survival (figure 51).

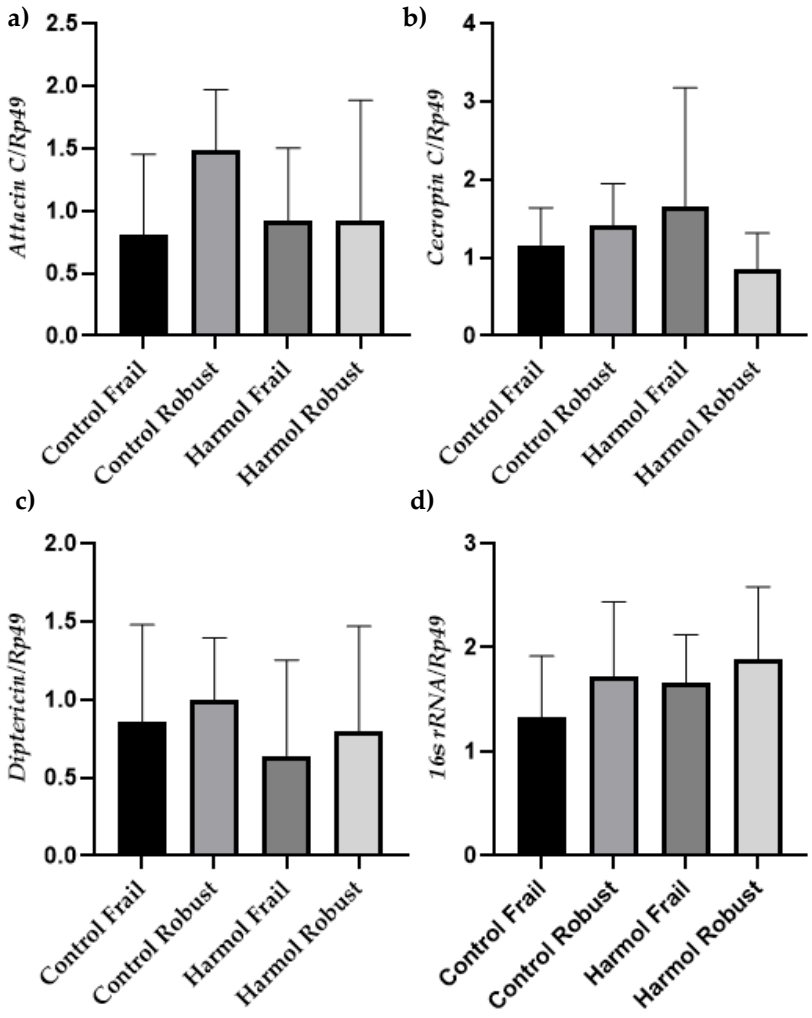

Figure 51. Gene expression of immune system-related genes in *Drosophila melanogaster* treated with harmol at 10% longevity.

mRNA levels of a) *Attacin C* b) *Cecropin C* c) *Diptericin,* and d) *16s rRNA* in comparison to control gene *Rp49*. Each experiment was performed with three samples of three flies each (n = 9). The data is represented as mean ± standard deviation. The statistical significance is expressed as * $p<0.05$.

4.8.2.2 Antioxidant genes

To analyze whether the increase in longevity found in the harmol treated flies were due to a decrease in oxidative stress, we measured the expression of the antioxidant enzymes *catalase*, *MnSOD,* as well as *p53* at 80%, 50%, and 10% survival.

However, no differences were found in the expression of any of the genes at any of the time points (figure 52, 53, 54).

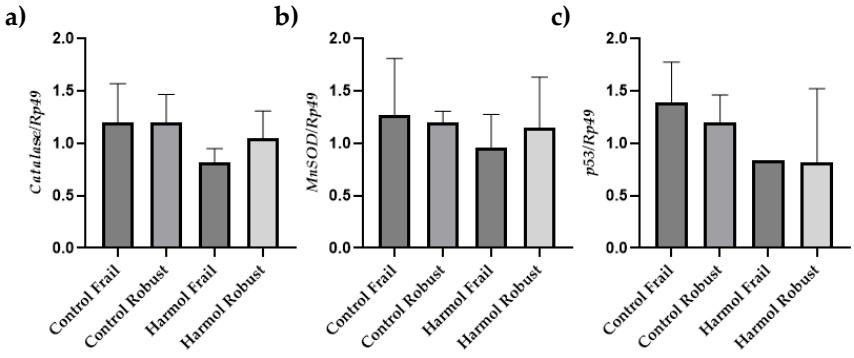

Figure 52. Gene expression of antioxidant genes in *Drosophila melanogaster* treated with harmol at 80% longevity.

mRNA levels of a) *Catalase* b) *MnSOD* c) *p53* in comparison to control gene *Rp49*. Each experiment was performed with three samples of three flies each (n = 9). The data is represented as mean ± standard deviation.

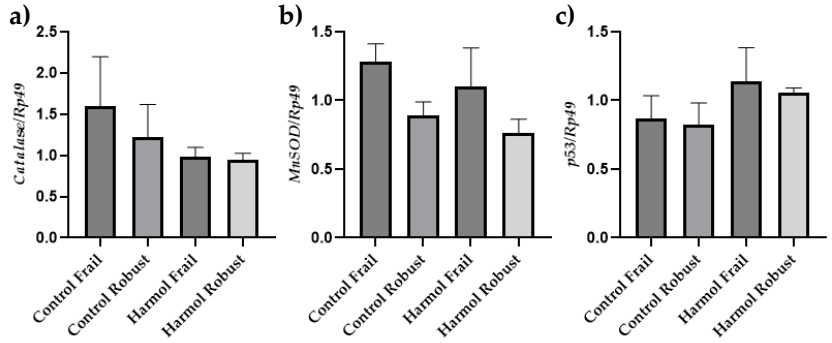

Figure 53. Gene expression of antioxidant genes in *Drosophila melanogaster* treated with harmol at 50% longevity.

mRNA levels of a) *Catalase* b) *MnSOD* c) *p53* in comparison to control gene *Rp49*. Each experiment was performed with three samples of three flies each (n = 9). The data is represented as mean ± standard deviation.

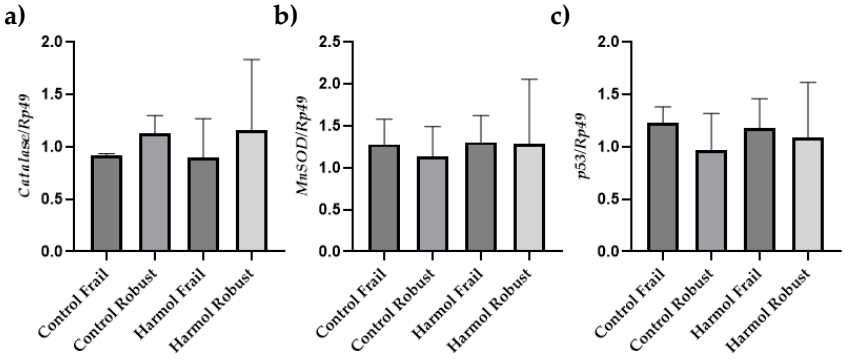

Figure 54. Gene expression of antioxidant genes in *Drosophila melanogaster* treated with harmol at 10% longevity.

mRNA levels of a) *Catalase* b) *MnSOD* c) *p53* in comparison to control gene *Rp49*. Each experiment was performed with three samples of three flies each (n = 9). The data is represented as mean ± standard deviation.

DISCUSSION

DISCUSSION

5.1 Resveratrol activates lipid catabolism, improves mitochondrial function, and exerts protective effects against oxidative stress at nutritionally relevant concentrations.

As previously mentioned, there are numerous studies on the beneficial effects of resveratrol. However, the majority of these studies are performed utilizing doses only obtainable through supplements and studies showing the effects of resveratrol at dosis achievable through diet are scarce.

In a study published by our laboratory show that resveratrol at 2.3 μg/kg/day, equating approximately 100 nM, induces lipid catabolism in old mice (128). Therefore, we were interested in understanding the mechanism behind this change and to see if the same effects were replicated at even lower concentrations.

Our experiments *in vitro* show that the lowest effective concentration to activate genes related to lipid catabolism was 10 nM with a 24-hour incubation. We therefore set out to study the effects of 10 nM resveratrol treatment on mitochondrial respiration and the antioxidant defense system.

We found that resveratrol at 10 nM increased maximal respiration and spare capacity of our cells, while also decreasing proton leak. This activation of the mitochondria was

accompanied by an activation of the oxidant defense system through an upregulation of *Gpx* and a reduction in H_2O_2 levels.

Although resveratrol significantly increased maximal respiration and spare capacity, it did not affect ATP production. This could be do to a dissipation of the proton gradient. The transfer of electrons from substrates to oxygen in the respiratory chain is coupled to translocation of protons across the membrane (131). Mitochondrial uncoupling occurs when uncoupling proteins (UCP) causes an increase in the permeability of protons to the inner mitochondrial membrane. This causes protons to return to the mitochondrial matrix without passing through ATP synthase, thereby causing an increase in temperature instead of ATP production. In fact, previous studies have shown resveratrol increases protein expression of UCP2, thereby increasing energy dissipation, causing a decrease in body-fat in rats (132). Further studies on the action mechanism to determine whether UCP could have caused the lack of ATP production found in our experiments.

The concentration of resveratrol used in our studies could be achieved through diet by consuming foods rich in resveratrol. However, the bioavailability of polyphenols remains a challenge in the confirmation of their effectiveness. In this context, it is

worth highlighting studies on the absorption and bioavailability of resveratrol.

A study published in 2004 shows that more than 70% of resveratrol is absorbed. In this study, six healthy volunteers were administered 25 mg resveratrol before plasma levels were measured. They reported peak plasma levels of 491 ng/mL of resveratrol and its metabolites and 5 ng/mL of unchanged resveratrol (133). These doses equal approximately 2 μM and 22 nM respectively. To obtain 10 nM unchanged resveratrol based on this uptake rate, we would need to consume around 11 mg resveratrol. This could be achieved by consuming 300 mL of red wine, 200 grams of lingonberries, and 100 grams of peanuts (134–136). However, if you take into consideration the concentrations of resveratrol with its metabolites found in plasma, one would only need to consume 0.11 mg of resveratrol.

Another study performed on men with well-controlled type 2 diabetes found a prolonged intake of resveratrol to be much more bioavailable. The participants were administered 150 mg resveratrol per day for 30 days and found plasma levels of unchanged resveratrol as high as 378 ng/mL, which equates to 1.66 μM. To obtain the 10 nM used in our experiments, based on these plasma concentrations, we would only need to consume 0.9 mg resveratrol. This would equate to less than 100 mL of red

wine, 30 grams of lingonberries, or 80 grams of peanuts a day. We do have to take into account that the uptake of resveratrol was measured after 30 days of daily intake. As resveratrol is a liposoluble molecule, a repeated intake of resveratrol allows it to be stored in the fat, thereby generating a reservoir, which results in an enhanced bioavailability. Therefore, it could be possible to achieve these concentrations by following a diet rich in these resveratrol-containing foods.

Once the doses and treatment time were established, we set out to study the pharmacodynamics of resveratrol. Seeing as resveratrol was able to exert effects at concentrations as low as 10 nM, we speculated that this effect could be mediated by a receptor, and specifically a hormone receptor. We therefore set out to study whether resveratrol is mediated by an androgen receptor, as we know they are abundant in skeletal muscle cells.

5.2 Resveratrol activates lipid catabolism and reduces oxidative stress through nuclear androgen receptor

Resveratrol is thought to activate mitochondrial fatty acid β-oxidation through the activation of AMPK (77), although the exact signaling pathway is still unknown. Resveratrol additionally stimulates Ca2+/Calmodulin-dependent Protein

Kinase Kinase 2 (CaMKK2), which phosphorylates and thereby activates AMPK in response to cytoplasmic Ca^{2+} increase (137,138). Interestingly, it has been proven that CaMKK2 is transcriptionally regulated by androgen receptor (139). Testosterone deficiency has been found to promote insulin resistance in skeletal muscle, at least partially, of both males and females via an androgen receptor-dependent mechanism involving a decrease in PGC1α-mediated oxidative and insulin-sensitive muscle fibers (140). Whether resveratrol is mediated by androgen receptors and acts through the same pathway as cAMKK2 remains to be found. We therefore proposed the following signaling pathway, indicating the non-documented parts (figure 55).

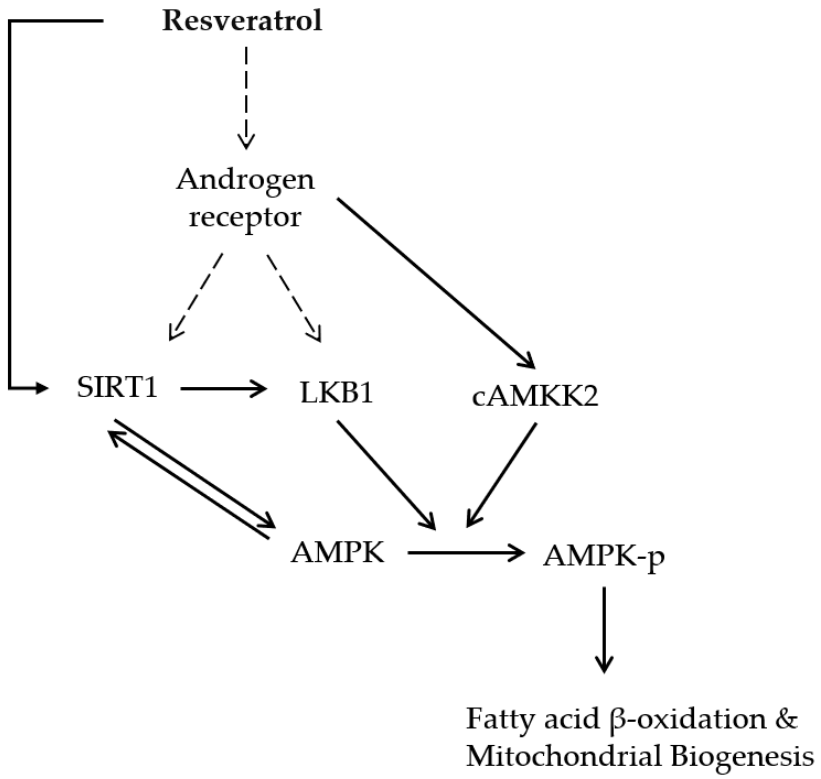

Figure 55. Simplified signaling pathway of resveratrol.

Arrows indicate activation. Dotted lines indicate that the correlation has yet to be reported.

There are many studies published on the effects of high concentrations of resveratrol on androgen receptor-dependent transcriptional activity in cancer cells. A study published in 2010, reported that 10 μM and 50 μM resveratrol decreased prostate-specific antigen expression, whereas 1 μM was suggested to increase androgen receptor transcriptional activity in androgen-dependent LNCaP cells, but not in its androgen-independent

sub-clone, C4-2 cells (141). These results are confirmed by a similar study on the effects of resveratrol on prostate cancer. They reported that the activation of androgen receptor-driven gene expression following low-dose resveratrol treatment was dependent on the presence of androgen. Therefore, in prostate cells containing high levels of endogenous androgen, 1 - 10 μM resveratrol was found to stimulate, whereas 100 μM was found to inhibit luciferase activity. Interestingly, concentrations of 0.1 μM or lower were reported to have no effect on luciferase activity or androgen receptor protein levels (142).

Despite these findings, there is a lack of studies on the effects of resveratrol and its correlation with androgen receptors in other cell types and at lower concentrations. Our experiments show that resveratrol at nutritionally relevant concentrations activates lipid catabolism and improves certain parameters of oxidative stress, and most importantly that these effects are not produced when the cells are treated with resveratrol in combination with the androgen receptor inhibitor bicalutamide.

Thus, in this doctoral thesis, we show for the first time that resveratrol acts similarly to a hormone by activating lipid catabolism and reducing oxidative stress through androgen receptors at nutritionally relevant concentrations in skeletal muscle cells.

It is well known that various androgen receptors exist. We therefore set out to determine the location of the receptor through which resveratrol acts. By studying the effects of piceid and comparing them to those of resveratrol, we could analyze whether the receptor is intracellular or on the cell membrane. As piceid is glycosylated it cannot readily enter the cell without the presence of a specific transporter. Our analysis of the effects of piceid on genes related to lipid catabolism and antioxidant enzymes, as well as MDA as a marker for lipid peroxidation, showed that piceid does not exert the same effects as resveratrol. This indicates that resveratrol acts through intracellular androgen receptors.

After determining the effects of resveratrol and androgen receptor on lipid catabolism, we were interested in analyzing whether resveratrol could increase mitochondrial respiration at nutritionally relevant concentrations and if this increase could be produced by activating androgen receptors. We found that resveratrol at 10 nM increased maximal respiration and spare capacity while decreasing proton leak. However, maximal respiration and spare capacity were also increased in the cells treated with resveratrol in combination with bicalutamide, although not when treated with bicalutamide alone. These results indicate that resveratrol improves mitochondrial respiration through an androgen-independent pathway.

Although it is important to take into account that this experiment was run using an acute treatment of 30 minutes and not 24 hours as with the other parameters analyzed.

5.3 The effects of hydroxytyrosol on mitochondrial function and oxidative stress

Hydroxytyrosol is a polyphenol abundant in olive oil, which is widely consumed in the Mediterranean Diet. Hydroxytyrosol has been reported to exert protective effects against endothelial dysfunction in human aortic endothelial cells (94). This study reported that both free hydroxytyrosol and its metabolites were effective in the reduction of the endothelial dysfunction biomarkers at physiological doses. We were therefore interested in researching the potential effects of this polyphenol on mitochondrial function at low doses in skeletal muscle cells.

In regard to mitochondrial biogenesis and fatty acid transport hydroxytyrosol upregulated *pgc-1α, ampk-1α,* and *cpt-1* at nutritionally relevant concentrations. Similar results have been found in both 3T3-L1 adipocytes and hepatocytes (143,144). We also found that mitochondrial respiration was significantly increased in the cells treated with hydroxytyrosol of all the doses studied, suggesting 10 nM is sufficient to effectively increase mitochondrial respiration and ATP production. To our knowledge, ours is the first study analyzing the effects of hydroxytyrosol on mitochondrial respiration in real time.

As previously explained, the mitochondria are one of the largest sources of endogenous oxidants in the body. Therefore, it is

important that the activation of the mitochondria is accompanied by an activation of the antioxidant system of the body. Unfortunately, the redox status of our cells did not seem to improve in the majority of the parameters measured, and the few defense systems found were only present at 10 μM. Hydroxytyrisol has been found to exert antioxidant effects in previous studies both *in vitro* and *in vivo* (145,146). However, these studies were performed with considerably higher doses than the ones used in our studies. This suggests that hydroxytyrosol might not be able to combat oxidant stressors following an increase in mitochondrial respiration at nutritionally relevant concentrations in skeletal muscle cells.

5.4 Nutritional relevance of hydroxytyrosol

In this doctoral thesis, we show that hydroxytyrosol at doses obtainable through diet can exert beneficial effects on mitochondrial function. However, as with all nutrients, and especially polyphenols, we have to take into consideration its absorption and bioavailability.

A recent study on the bioavailability of hydroxytyrosol found plasma concentrations up to 3.79 ng/mL after a 5 mg intake, which would equate 24.6 nM (147). This study also showed how

the bioavailability of hydroxytyrosol depended on the food matrix, showing how oily matrices favor absorption. This is important to take into consideration when planning our diet to obtain the maximum benefits of this phenolic compound.

Based on these findings, the concentrations of 10 nM, 100 nM, and 10 μM in this study could be achieved by consuming 2 mg, 20 mg, and 2000 mg respectively. Studies have found concentrations of hydroxytyrosol in olives as high as 413 mg/100 g, 3.47 mg/100 mL olive oil, and 0.96 mg/100 mL red wine (148–150). This indicates we could achieve both 10 nM and 100 nM hydroxytyrosol easily following a diet rich in these compounds. To achieve 10 nM of hydroxytyrosol, one could consume 200 mL of red wine or less than 60 mL of olive oil, and for 100 nM one could consume 4.84 grams of black olives.

Another study administrating volunteers with 25 mL extra-virgin olive oil reported plasma concentrations of hydroxytyrosol of 4.40 ng/mL (151). This concentration equates approximately 28 nM, and one could therefore achieve 10 nM with one tablespoon, and 100 mM with 90 mL extra-virgin olive oil. Interestingly, only trace amounts were found in the subjects consuming ordinary olive oil, indicating that the quality of the oil is highly correlated with the bioavailability of hydroxytyrosol.

However, a study from 2013 comparing the difference in bioavailability of hydroxytyrosol in men and women reported greater plasma levels of hydroxytyrosol in men than in women after 9.7 mg supplementation. The mean peak concentration was reported to be 28 ng/mL in women, with values ranging from 9-94 ng/mL. Whereas in men, the mean peak plasma concentrations were 117 ng/mL, with values ranging between 42-325 ng/mL (152). This would indicate that women would need to consume 534 mg of hydroxytyrosol, equating to around 130 grams of olives to achieve plasma concentrations of 10 μM. In comparison, men would need to consume 30 grams of olive to achieve this same concentration. Although, based on these findings, both sexes would be able to achieve both 10 nM and 100 nM by regularly including red wine, olive oil, or olives in their diet.

Interestingly, the difference in bioavailability between sexes does not seem to translate to rats. In a study published in 2015, plasma concentrations of hydroxytyrosol were found to be higher in female rats in comparison to males (153). Further studies on bioavailability in all animal models should be performed to confirm the differences in sexes in this regard.

5.5 Piceid induces immune gene expression but does not affect longevity parameters

In a study previously performed in our laboratory have shown that moderate red wine consumption can increase longevity in *Drosophila melanogaster* (154). We were interested in studying if this effect could be partly due to the presence of piceid. To this end, the first thing we did was perform a longevity curve and evaluate parameters associated with frailty, adapted from those published by Linda Fried.

Under optimal conditions of life, it is in the last stage of life that the probability of death increases. It is important to keep in mind that aging is not a disease and must be separated from the causes of death due to old age. Aging refers to post-maturational processes that lead to a decrease in homeostasis and an increase in the vulnerability of the organism, which leads to a greater predisposition to suffer from different pathologies that end with the death of the individual.

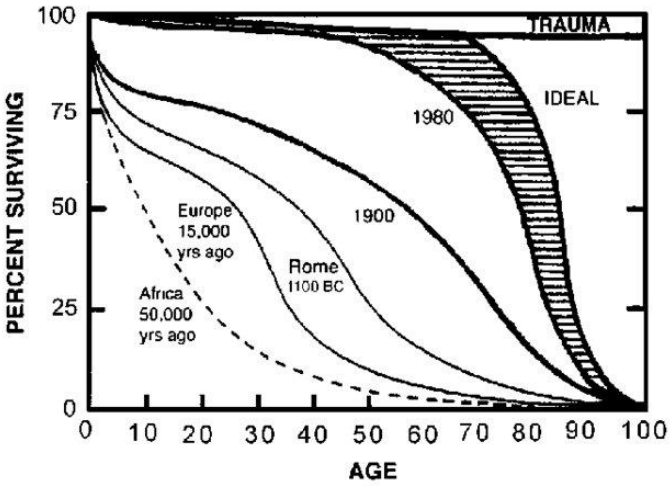

Figure 56. Survival curve for humans at different times in history.

Bruce R Troen, 2003 (155).

Frailty however is considered a geriatric syndrome occurring in vulnerable individuals with increased disabilities and institutionalization. The Medical Research Council Manual Muscle Testing is widely used in patients to evaluate weakness and can be effective in differentiating true weakness from imbalance or poor endurance. In this way, overcoming gravity to perform a movement, with or without resistance, will determine muscular strength of the patient. Similarly, the fly will have less ability to ascend the test tube in frail individuals where muscle weakness is present (156). We correlated low physical activity with phototaxis, as the fly is naturally attracted to light and

would instinctively fly towards sources of light. Their reluctance or inability to do so could therefore indicate low levels of activity. Similarly, measuring sarcopenia or weight loss in the fly would be complicated. We therefore opted for a measurement of food consumption of the fly to evaluate this parameter. Although piceid did not improve any of the frailty parameters, we did find a significant increase in both longevity and phototaxis in the robust groups in comparison to the frail flies, indicating that the methods used to evaluate frailty are effective.

To this day, various theories of aging exist, among them the immunological theory of aging. The immunological theory of aging states that the immune system progressively deteriorates over time as antibodies lose their effectiveness. This increases vulnerability to infectious disease, which causes cellular stress and eventually death (157). The immune system has proven to play a critical role in various age-related disease and in the year 2000 the term "inflamm-aging" was coined. This phenomenon refers to a global reduction in the capacity to cope with stressors during aging, leading to a concomitant progressive increase in pro-inflammatory status (158,159).

In light of this, we set out to study the effects of nutritionally relevant doses of piceid on immune system related genes in *Drosophila melanogaster*. At 80% survival we found no differences

in the expression of the genes of interest. This was expected, as the flies at this time point are still quite young. At 50% survival we did find an increased expression in *attacin C* and *diptericin* in the fragile group supplemented with piceid in comparison to the control. Additionally, *cecropin C* was overexpressed in the robust piceid group in comparison to both the robust controls and the fragile group treated with piceid. At 10% of survival piceid activated all of the genes of interest in the fragile individuals as well as *diptericin* in the robust flies. This upregulation in the fragile individuals suggests that piceid could exert a protective effect against infections in aged flies. The lack of differences in the control and piceid treated robust individuals was also expected, as the robust individuals have been shown to have greater longevity and would therefore already have a more active immune system.

Another well described theory of the decreased health status occurring as we age is the free radical theory of aging. This term was coined by Denham in 1956 where he proposed that the effects of free radicals on cell constituents cause the damage and degenerative diseases associated with aging. It has therefore been proposed that the administration of antioxidant could prevent this damage caused by these radicals and therefore prevent age-related degenerative diseases (160). In later years this theory has been proposed to be a theory causing frailty

rather than aging as it increases the risk of disability (161). Therefore, we were interested in studying the possible effects of piceid on antioxidant enzymes. Unfortunately, no changes were found in any of the treatment groups.

Piceid at 10 nM induced expression of immune system genes in aged flies and could therefore protect flies from infections as they age. However, at the doses used in this project, piceid was not able to reduce oxidative stress or improve frailty parameters nor longevity of the flies. As higher doses of piceid have been found to reduce oxidative stress in other models (162,163), further studies should be performed with piceid at other concentrations to better determine its function on frailty parameters and longevity in *Drosophila melanogaster*.

5.6 Harmol increases longevity but not frailty parameters in *Drosophila melanogaster*

Harmol is a scantly studied alkaloid, with only two studies published on its effects in *Drosophila melanogaster*. Out of these two studies, one analyzed its effects on longevity. This study by Costa-Machado, Luis Filipe et al. found that harmol at 25 μg/ml increased longevity in *Caenorhabditis Elegans* and *Drosophila melanogaster* (105). We were therefore interested in studying if low doses of harmol could replicate these effects, as well as analyze its effect on frailty parameters in aged flies.

Although longevity was improved by a significant increase in median survival in fragile flies treated with harmol, no significant differences were found in the parameters we proposed to measure frailty. However, a clear tendency is shown in the phototaxis assay at 10% survival rate, suggesting harmol might exert some protective effects against frailty at the later stages of life.

Interestingly, at 50% survival, *16s rRNA* was improved in the frail group treated with harmol. This ribosomal mitochondrial transcript has been reported to correlate with the shape of the longevity curve in male flies, preceding the decrease in survival (164). This supports our findings of improvement of median

survival rate, but not life span, as this gene is not upregulated at 10% survival. However, some of the genes related to the immune system are downregulated in the robust flies treated with harmol. Taking this together with the fact that no improvements were found in the expression antioxidant enzymes or on the frailty parameters indicates that although harmol positively affects longevity, this increase is not accompanied by an improved health status of the aged flies.

It is important to take into consideration that these studied were performed exclusively in male *Drosophila melanogaster* populations. Further studies should be performed to determine the effects of these compounds on female flies.

CONCLUSIONS

Based on the results presented in this doctoral thesis, we can draw the following conclusions:

1. Resveratrol activates lipid catabolism and exerts protective effects against oxidative stress at nutritionally relevant concentrations in murine skeletal muscle cells
2. Resveratrol exerts its beneficial effects on mitochondrial function and redox status through intracellular androgen receptors
3. Hydroxytyrosol activates lipid catabolism and augments mitochondrial respiration, but does not activate antioxidative systems at nutritionally relevant concentrations
4. Piceid activates genes related to the immune system but does not improve longevity or frailty or redox status of aged male *Drosophila melanogaster* at nutritionally relevant concentrations
5. Harmol improves longevity, but not frailty parameters or health status of male *Drosophila melanogaster*

REFERENCES

REFERENCES

1. Liu W, Chen G. Regulation of energy metabolism in human pluripotent stem cells. Cell Mol Life Sci. 1 de diciembre de 2021;78(24):8097-108.

2. Fredholm BB, Johansson S, Wang YQ. Chapter 3 - Adenosine and the Regulation of Metabolism and Body Temperature. En: Jacobson KA, Linden J, editores. Advances in Pharmacology [Internet]. Academic Press; 2011 [citado 28 de febrero de 2024]. p. 77-94. (Pharmacology of Purine and Pyrimidine Receptors; vol. 61). Disponible en: https://www.sciencedirect.com/science/article/pii/B9780123855268000035

3. Bonet ML, Ribot J, Palou A. Lipid metabolism in mammalian tissues and its control by retinoic acid. Biochim Biophys Acta. enero de 2012;1821(1):177-89.

4. Hajri T, Abumrad NA. FATTY ACID TRANSPORT ACROSS MEMBRANES: Relevance to Nutrition and Metabolic Pathology. Annu Rev Nutr. 1 de julio de 2002;22(1):383-415.

5. Glatz JFC, Luiken JJFP, van Nieuwenhoven FA, Van der Vusse GJ. Molecular mechanism of cellular uptake and intracellular translocation of fatty acids. Prostaglandins Leukot Essent Fatty Acids. 1 de julio de 1997;57(1):3-9.

6. Killenberg PG, Davidson ED, Webster LT. Evidence for a medium-chain fatty acid: coenzyme A ligase (adenosine monophosphate) that activates salicylate. Mol Pharmacol. mayo de 1971;7(3):260-8.

7. Hesler CB, Olymbios C, Haldar D. Transverse-plane topography of long-chain acyl-CoA synthetase in the mitochondrial outer membrane. J Biol Chem. 25 de abril de 1990;265(12):6600-5.

8. Ramsay RR, Gandour RD, van der Leij FR. Molecular enzymology of carnitine transfer and transport. Biochim Biophys Acta. 9 de marzo de 2001;1546(1):21-43.

9. McGarry JD, Brown NF. The mitochondrial carnitine palmitoyltransferase system. From concept to molecular analysis. Eur J Biochem. 15 de febrero de 1997;244(1):1-14.

10. Murthy MS, Pande SV. Some differences in the properties of carnitine palmitoyltransferase activities of the mitochondrial outer and inner membranes. Biochem J. 15 de diciembre de 1987;248(3):727-33.

11. Bahnson B, Anderson V, Petsko G. Structural Mechanism of Enoyl-CoA Hydratase: Three Atoms from a Single Water Are Added in either an E1cb Stepwise or Concerted Fashion † , ‡. Biochemistry. 1 de marzo de 2002;41:2621-9.

12. Strable MS, Ntambi JM. Genetic control of de novo lipogenesis: role in diet-induced obesity. Crit Rev Biochem Mol Biol. junio de 2010;45(3):199-214.

13. Dowell P, Hu Z, Lane MD. Monitoring energy balance: metabolites of fatty acid synthesis as hypothalamic sensors. Annu Rev Biochem. 2005;74:515-34.

14. Kudo N, Barr AJ, Barr RL, Desai S, Lopaschuk GD. High rates of fatty acid oxidation during reperfusion of ischemic hearts are associated with a decrease in malonyl-CoA levels due to an increase in 5'-AMP-activated protein kinase inhibition of acetyl-CoA carboxylase. J Biol Chem. 21 de julio de 1995;270(29):17513-20.

15. Hardie DG, Scott JW, Pan DA, Hudson ER. Management of cellular energy by the AMP-activated protein kinase system. FEBS Lett. 3 de julio de 2003;546(1):113-20.

16. Hood DA, Adhihetty PJ, Colavecchia M, Gordon JW, Irrcher I, Joseph AM, et al. Mitochondrial biogenesis and the role of the protein import pathway. Med Sci Sports Exerc. 1 de enero de 2003;35(1):86-94.

17. Nilsen J, Chen S, Irwin RW, Iwamoto S, Brinton RD. Estrogen protects neuronal cells from amyloid beta-induced apoptosis via

regulation of mitochondrial proteins and function. BMC Neurosci. 3 de noviembre de 2006;7(1):74.

18. Alberts B, Johnson A, Lewis J, Raff M, Roberts K, Walter P. The Mitochondrion. En: Molecular Biology of the Cell 4th edition [Internet]. Garland Science; 2002 [citado 27 de marzo de 2024]. Disponible en: https://www.ncbi.nlm.nih.gov/books/NBK26894/

19. Jornayvaz FR, Shulman GI. Regulation of mitochondrial biogenesis. Essays Biochem. 2010;47:10.1042/bse0470069.

20. Baker MJ, Frazier AE, Gulbis JM, Ryan MT. Mitochondrial protein-import machinery: correlating structure with function. Trends Cell Biol. septiembre de 2007;17(9):456-64.

21. Hardie DG. AMP-activated/SNF1 protein kinases: conserved guardians of cellular energy. Nat Rev Mol Cell Biol. octubre de 2007;8(10):774-85.

22. Virbasius JV, Scarpulla RC. Activation of the human mitochondrial transcription factor A gene by nuclear respiratory factors: a potential regulatory link between nuclear and mitochondrial gene expression in organelle biogenesis. Proc Natl Acad Sci U S A. 15 de febrero de 1994;91(4):1309-13.

23. 9.10: Molecular Orbital Theory Predicts that Molecular Oxygen is Paramagnetic - Chemistry LibreTexts [Internet]. [citado 26 de febrero de 2024]. Disponible en: https://chem.libretexts.org/Courses/Pacific_Union_College/Quantum_Chemistry/09%3A_Chemical_Bonding_in_Diatomic_Molecules/9.10%3A_Molecular_Orbital_Theory_Predicts_that_Molecular_Oxygen_is_Paramagnetic

24. Cheeseman KH, Slater TF. An introduction to free radical biochemistry. Br Med Bull. julio de 1993;49(3):481-93.

25. Gough DR, Cotter TG. Hydrogen peroxide: a Jekyll and Hyde signalling molecule. Cell Death Dis. octubre de 2011;2(10):e213-e213.

26. Halliwell B. Reactive oxygen species in living systems: Source, biochemistry, and role in human disease. Am J Med. 30 de septiembre de 1991;91(3, Supplement 3):S14-22.

27. Ames BN, Shigenaga MK, Hagen TM. Oxidants, antioxidants, and the degenerative diseases of aging. Proc Natl Acad Sci U S A. 1 de septiembre de 1993;90(17):7915-22.

28. Fenton HJH. LXXIII.—Oxidation of tartaric acid in presence of iron. J Chem Soc Trans. 1 de enero de 1894;65(0):899-910.

29. Phaniendra A, Jestadi DB, Periyasamy L. Free Radicals: Properties, Sources, Targets, and Their Implication in Various Diseases. Indian J Clin Biochem. enero de 2015;30(1):11-26.

30. Ahmad MH, Fatima M, Hossain M, Mondal AC. Evaluation of naproxen-induced oxidative stress, hepatotoxicity and *in-vivo* genotoxicity in male Wistar rats. J Pharm Anal. 1 de diciembre de 2018;8(6):400-6.

31. Halliwell B. Antioxidants in human health and disease. Annu Rev Nutr. 1996;16:33-50.

32. Lee DW, Yu BP. Modulation of free radicals and superoxide dismutases by age and dietary restriction. Aging Clin Exp Res. 1 de diciembre de 1990;2(4):357-62.

33. Halliwell B. Free radicals, antioxidants, and human disease: curiosity, cause, or consequence? The Lancet. 10 de septiembre de 1994;344(8924):721-4.

34. Frei B. Natural antioxidants in human health and disease / edited by Balz Frei. San Diego: Academic Press; 1994.

35. Freeman BA, Crapo JD. Biology of disease: free radicals and tissue injury. Lab Investig J Tech Methods Pathol. noviembre de 1982;47(5):412-26.

36. Lippman RD. Rapid in vivo quantification and comparison of hydroperoxides and oxidized collagen in aging mice, rabbits and man. Exp Gerontol. 1985;20(1):1-5.

37. Shigenaga MK, Hagen TM, Ames BN. Oxidative damage and mitochondrial decay in aging. Proc Natl Acad Sci U S A. 8 de noviembre de 1994;91(23):10771-8.

38. Stadtman ER. Protein oxidation and aging. Science. 28 de agosto de 1992;257(5074):1220-4.

39. Davies KJ, Delsignore ME, Lin SW. Protein damage and degradation by oxygen radicals. II. Modification of amino acids. J Biol Chem. 15 de julio de 1987;262(20):9902-7.

40. Dean RT, Gieseg S, Davies MJ. Reactive species and their accumulation on radical-damaged proteins. Trends Biochem Sci. 1 de noviembre de 1993;18(11):437-41.

41. Shibutani S, Takeshita M, Grollman AP. Insertion of specific bases during DNA synthesis past the oxidation-damaged base 8-oxodG. Nature. 31 de enero de 1991;349(6308):431-4.

42. Halliwell B, Aruoma OI. DNA damage by oxygen-derived species. Its mechanism and measurement in mammalian systems. FEBS Lett. 9 de abril de 1991;281(1-2):9-19.

43. Breen AP, Murphy JA. Reactions of oxyl radicals with DNA. Free Radic Biol Med. junio de 1995;18(6):1033-77.

44. Viguie CA, Frei B, Shigenaga MK, Ames BN, Packer L, Brooks GA. Antioxidant status and indexes of oxidative stress during consecutive days of exercise. J Appl Physiol. agosto de 1993;75(2):566-72.

45. The effect of cornea proteoglycans on liposome peroxidation [Internet]. [citado 28 de febrero de 2024]. Disponible en: https://iris.unipv.it/handle/11571/131529

46. Free Radicals and Inflammation: Protection of Synovial Fluid by Superoxide Dismutase | Science [Internet]. [citado 28 de febrero de 2024]. Disponible en: https://www.science.org/doi/10.1126/science.185.4150.529

47. Halliwell B, Gutteridge JM. The definition and measurement of antioxidants in biological systems. Free Radic Biol Med. enero de 1995;18(1):125-6.

48. Halliwell B, Gutteridge JMC. Free Radicals in Biology and Medicine [Internet]. Oxford University Press; 2015 [citado 28 de febrero de 2024]. Disponible en: https://academic.oup.com/book/40045

49. Halliwell B, Gutteridge JM. Role of free radicals and catalytic metal ions in human disease: an overview. Methods Enzymol. 1990;186:1-85.

50. Pacifici RE, Davies KJA. Protein, Lipid and DNA Repair Systems in Oxidative Stress: The Free-Radical Theory of Aging Revisited. Gerontology. 8 de abril de 2009;37(1-3):166-80.

51. Sevanian A, Davies KJ, Hochstein P. Conservation of vitamin C by uric acid in blood. J Free Radic Biol Med. 1985;1(2):117-24.

52. Hassan HM, Fridovich I. Regulation of the synthesis of superoxide dismutase in Escherichia coli. Induction by methyl viologen. J Biol Chem. 10 de noviembre de 1977;252(21):7667-72.

53. McCord JM, Fridovich I. Superoxide dismutase. An enzymic function for erythrocuprein (hemocuprein). J Biol Chem. 25 de noviembre de 1969;244(22):6049-55.

54. Weisiger RA, Fridovich I. Superoxide dismutase. Organelle specificity. J Biol Chem. 25 de mayo de 1973;248(10):3582-92.

55. Fridovich I. Superoxide dismutases. Adv Enzymol Relat Areas Mol Biol. 1974;41(0):35-97.

56. Boveris A, Chance B. The mitochondrial generation of hydrogen peroxide. General properties and effect of hyperbaric oxygen. Biochem J. julio de 1973;134(3):707-16.

57. Vance PG, Keele BB, Rajagopalan KV. Superoxide dismutase from Streptococcus mutans. Isolation and characterization of two

forms of the enzyme. J Biol Chem. 10 de agosto de 1972;247(15):4782-6.

58. Ischiropoulos H, Nadziejko CE, Kikkawa Y. Effect of aging on pulmonary superoxide dismutase. Mech Ageing Dev. 1 de marzo de 1990;52(1):11-26.

59. Chang LY, Slot JW, Geuze HJ, Crapo JD. Molecular immunocytochemistry of the CuZn superoxide dismutase in rat hepatocytes. J Cell Biol. 1 de diciembre de 1988;107(6):2169-79.

60. Chance B, Sies H, Boveris A. Hydroperoxide metabolism in mammalian organs. Physiol Rev. julio de 1979;59(3):527-605.

61. Ketterer B. Detoxication reactions of glutathione and glutathione transferases. Xenobiotica Fate Foreign Compd Biol Syst. 1986;16(10-11):957-73.

62. Sohn OS, Fiala ES, Upadhyaya P, Chae YH, El-Bayoumy K. Comparative effects of phenylenebis(methylene)selenocyanate isomers on xenobiotic metabolizing enzymes in organs of female CD rats. Carcinogenesis. abril de 1999;20(4):615-21.

63. Tolbert NE, Essner E. Microbodies: peroxisomes and glyoxysomes. J Cell Biol. diciembre de 1981;91(3 Pt 2):271s-83s.

64. Rodríguez AM, Carrico PM, Mazurkiewicz JE, Meléndez JA. Mitochondrial or cytosolic catalase reverses the MnSOD-dependent inhibition of proliferation by enhancing respiratory chain activity, net ATP production, and decreasing the steady state levels of H(2)O(2). Free Radic Biol Med. 1 de noviembre de 2000;29(9):801-13.

65. Dai J, Mumper RJ. Plant phenolics: extraction, analysis and their antioxidant and anticancer properties. Mol Basel Switz. 21 de octubre de 2010;15(10):7313-52.

66. D'Archivio M, Filesi C, Di Benedetto R, Gargiulo R, Giovannini C, Masella R. Polyphenols, dietary sources and bioavailability. Ann Ist Super Sanita. 2007;43(4):348-61.

67. Uchiumi F, Watanabe T, Hasegawa S, Hoshi T, Higami Y, Tanuma S ichi. The effect of resveratrol on the Werner syndrome RecQ helicase gene and telomerase activity. Curr Aging Sci. febrero de 2011;4(1):1-7.

68. Spencer JPE, Rice-Evans C, Williams RJ. Modulation of pro-survival Akt/protein kinase B and ERK1/2 signaling cascades by quercetin and its in vivo metabolites underlie their action on neuronal viability. J Biol Chem. 12 de septiembre de 2003;278(37):34783-93.

69. Leifert WR, Abeywardena MY. Grape seed and red wine polyphenol extracts inhibit cellular cholesterol uptake, cell proliferation, and 5-lipoxygenase activity. Nutr Res N Y N. diciembre de 2008;28(12):842-50.

70. Hussain T, Gupta S, Adhami VM, Mukhtar H. Green tea constituent epigallocatechin-3-gallate selectively inhibits COX-2 without affecting COX-1 expression in human prostate carcinoma cells. Int J Cancer. 10 de febrero de 2005;113(4):660-9.

71. Gambini J, Inglés M, Olaso G, Lopez-Grueso R, Bonet-Costa V, Gimeno-Mallench L, et al. Properties of Resveratrol: In Vitro and In Vivo Studies about Metabolism, Bioavailability, and Biological Effects in Animal Models and Humans. Oxid Med Cell Longev [Internet]. 2015 [citado 28 de enero de 2020];2015. Disponible en: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4499410/

72. Abenavoli L, Boccuto L, Federico A, Dallio M, Loguercio C, Di Renzo L, et al. Diet and Non-Alcoholic Fatty Liver Disease: The Mediterranean Way. Int J Environ Res Public Health [Internet]. septiembre de 2019 [citado 21 de mayo de 2020];16(17). Disponible en: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6747511/

73. Castro-Barquero S, Lamuela-Raventós RM, Doménech M, Estruch R. Relationship between Mediterranean Dietary Polyphenol Intake and Obesity. Nutrients [Internet]. 17 de octubre de 2018 [citado 5 de julio de 2020];10(10). Disponible en: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6213078/

74. Carter LG, D'Orazio JA, Pearson KJ. Resveratrol and cancer: focus on in vivo evidence. Endocr Relat Cancer. junio de 2014;21(3):R209-25.

75. Witte AV, Kerti L, Margulies DS, Flöel A. Effects of resveratrol on memory performance, hippocampal functional connectivity, and glucose metabolism in healthy older adults. J Neurosci Off J Soc Neurosci. 4 de junio de 2014;34(23):7862-70.

76. Chen KH, Cheng ML, Jing YH, Chiu DTY, Shiao MS, Chen JK. Resveratrol ameliorates metabolic disorders and muscle wasting in streptozotocin-induced diabetic rats. Am J Physiol Endocrinol Metab. noviembre de 2011;301(5):E853-863.

77. Gimeno-Mallench L, Mas-Bargues C, Inglés M, Olaso G, Borras C, Gambini J, et al. Resveratrol shifts energy metabolism to increase lipid oxidation in healthy old mice. Biomed Pharmacother. 1 de octubre de 2019;118:109130.

78. Huang B, Liu J, Meng T, Li Y, He D, Ran X, et al. Polydatin Prevents Lipopolysaccharide (LPS)-Induced Parkinson's Disease via Regulation of the AKT/GSK3β-Nrf2/NF-κB Signaling Axis. Front Immunol. 5 de noviembre de 2018;9:2527.

79. Romero-Pérez AI, Ibern-Gómez M, Lamuela-Raventós RM, de la Torre-Boronat MC. Piceid, the Major Resveratrol Derivative in Grape Juices. J Agric Food Chem. 1 de abril de 1999;47(4):1533-6.

80. Hollman P, de Vries J, van Leeuwen S, Mengelers M, Katan M. Absorption of dietary quercetin glycosides and quercetin in healthy ileostomy volunteers. Am J Clin Nutr. 1 de diciembre de 1995;62(6):1276-82.

81. Paganga G, Rice-Evans CA. The identification of flavonoids as glycosides in human plasma. FEBS Lett. 1997;401(1):78-82.

82. Ming D, Songyan L, Yawen C, Na Z, Jing M, Zhaowen X, et al. trans-Polydatin protects the mouse heart against ischemia/reperfusion injury via inhibition of the renin-

angiotensin system (RAS) and Rho kinase (ROCK) activity. Food Funct. 21 de junio de 2017;8(6):2309-21.

83. Li R, Maimai T, Yao H, Liu X, He Z, Xiao C, et al. Protective effects of polydatin on LPS-induced endometritis in mice. Microb Pathog. diciembre de 2019;137:103720.

84. Zeng Z, Chen Z, Xu S, Song R, Yang H, Zhao K seng. Polydatin Alleviates Small Intestine Injury during Hemorrhagic Shock as a SIRT1 Activator. Oxid Med Cell Longev. 2015;2015:965961.

85. Li T, Liu Y, Xu W, Dai X, Liu R, Gao Y, et al. Polydatin mediates Parkin-dependent mitophagy and protects against mitochondria-dependent apoptosis in acute respiratory distress syndrome. Lab Investig J Tech Methods Pathol. junio de 2019;99(6):819-29.

86. Zhao XJ, Yu HW, Yang YZ, Wu WY, Chen TY, Jia KK, et al. Polydatin prevents fructose-induced liver inflammation and lipid deposition through increasing miR-200a to regulate Keap1/Nrf2 pathway. Redox Biol. septiembre de 2018;18:124-37.

87. Gong W, Li J, Chen Z, Huang J, Chen Q, Cai W, et al. Polydatin promotes Nrf2-ARE anti-oxidative pathway through activating CKIP-1 to resist HG-induced up-regulation of FN and ICAM-1 in GMCs and diabetic mice kidneys. Free Radic Biol Med. mayo de 2017;106:393-405.

88. Lv R, Du L, Zhang L, Zhang Z. Polydatin attenuates spinal cord injury in rats by inhibiting oxidative stress and microglia apoptosis via Nrf2/HO-1 pathway. Life Sci. 15 de enero de 2019;217:119-27.

89. Robles-Almazan M, Pulido-Moran M, Moreno-Fernandez J, Ramirez-Tortosa C, Rodriguez-Garcia C, Quiles JL, et al. Hydroxytyrosol: Bioavailability, toxicity, and clinical applications. Food Res Int. 1 de marzo de 2018;105:654-67.

90. Bertelli M, Kiani AK, Paolacci S, Manara E, Kurti D, Dhuli K, et al. Hydroxytyrosol: A natural compound with promising pharmacological activities. J Biotechnol. 10 de febrero de 2020;309:29-33.

91. Peyrol J, Riva C, Amiot MJ. Hydroxytyrosol in the Prevention of the Metabolic Syndrome and Related Disorders. Nutrients. 20 de marzo de 2017;9(3):306.

92. Karković Marković A, Torić J, Barbarić M, Jakobušić Brala C. Hydroxytyrosol, Tyrosol and Derivatives and Their Potential Effects on Human Health. Molecules. 24 de mayo de 2019;24(10):2001.

93. Roleira FMF, Varela CL, Costa SC, Tavares-da-Silva EJ. Chapter 4 - Phenolic Derivatives From Medicinal Herbs and Plant Extracts: Anticancer Effects and Synthetic Approaches to Modulate Biological Activity. En: Atta-ur-Rahman, editor. Studies in Natural Products Chemistry [Internet]. Elsevier; 2018 [citado 29 de febrero de 2024]. p. 115-56. Disponible en: https://www.sciencedirect.com/science/article/pii/B9780444640574000041

94. Catalán Ú, López de Las Hazas MC, Rubió L, Fernández-Castillejo S, Pedret A, de la Torre R, et al. Protective effect of hydroxytyrosol and its predominant plasmatic human metabolites against endothelial dysfunction in human aortic endothelial cells. Mol Nutr Food Res. diciembre de 2015;59(12):2523-36.

95. Elmaksoud HAA, Motawea MH, Desoky AA, Elharrif MG, Ibrahimi A. Hydroxytyrosol alleviate intestinal inflammation, oxidative stress and apoptosis resulted in ulcerative colitis. Biomed Pharmacother Biomedecine Pharmacother. octubre de 2021;142:112073.

96. Ikonomidis I, Katogiannis K, Chania C, Iakovis N, Tsoumani M, Christodoulou A, et al. Association of hydroxytyrosol enriched olive oil with vascular function in chronic coronary disease. Eur J Clin Invest. julio de 2023;53(7):e13983.

97. Dhuli K, Micheletti C, Medori MC, Madeo G, Bonetti G, Donato K, et al. The potential preventive role of a dietary supplement containing hydroxytyrosol in COVID-19: a multi-center study. Eur Rev Med Pharmacol Sci. diciembre de 2023;27(6 Suppl):33-8.

98. Zhao T, He Y qi, Wang J, Ding K min, Wang C hong, Wang Z tao. Inhibition of Human Cytochrome P450 Enzymes 3A4 and 2D6 by β-Carboline Alkaloids, Harmine Derivatives. Phytother Res. 2011;25(11):1671-7.

99. Olmedo GM, Cerioni L, González MM, Cabrerizo FM, Volentini SI, Rapisarda VA. UVA Photoactivation of Harmol Enhances Its Antifungal Activity against the Phytopathogens Penicillium digitatum and Botrytis cinerea. Front Microbiol. 2017;8:347.

100. Quintana VM, Piccini LE, Panozzo Zénere JD, Damonte EB, Ponce MA, Castilla V. Antiviral activity of natural and synthetic β-carbolines against dengue virus. Antiviral Res. octubre de 2016;134:26-33.

101. Moura DJ, Richter MF, Boeira JM, Pêgas Henriques JA, Saffi J. Antioxidant properties of beta-carboline alkaloids are related to their antimutagenic and antigenotoxic activities. Mutagenesis. julio de 2007;22(4):293-302.

102. Moura DJ, Rorig C, Vieira DL, Henriques JAP, Roesler R, Saffi J, et al. Effects of beta-carboline alkaloids on the object recognition task in mice. Life Sci. 26 de octubre de 2006;79(22):2099-104.

103. Herraiz T, González D, Ancín-Azpilicueta C, Arán VJ, Guillén H. beta-Carboline alkaloids in Peganum harmala and inhibition of human monoamine oxidase (MAO). Food Chem Toxicol Int J Publ Br Ind Biol Res Assoc. marzo de 2010;48(3):839-45.

104. Xu J, Ao YL, Huang C, Song X, Zhang G, Cui W, et al. Harmol promotes α-synuclein degradation and improves motor impairment in Parkinson's models via regulating autophagy-lysosome pathway. NPJ Park Dis. 6 de agosto de 2022;8:100.

105. Costa-Machado LF, Garcia-Dominguez E, McIntyre RL, Lopez-Aceituno JL, Ballesteros-Gonzalez Á, Tapia-Gonzalez A, et al. Peripheral modulation of antidepressant targets MAO-B and GABAAR by harmol induces mitohormesis and delays aging in preclinical models. Nat Commun. 15 de mayo de 2023;14(1):2779.

106. Pierce BA. Genetics: A Conceptual Approach. W. H. Freeman; 2008. 840 p.

107. Shahzad U, Taccone MS, Kumar SA, Okura H, Krumholtz S, Ishida J, et al. Modeling human brain tumors in flies, worms, and zebrafish: From proof of principle to novel therapeutic targets. Neuro-Oncol. 5 de mayo de 2021;23(5):718-31.

108. Ageing and health [Internet]. [citado 29 de febrero de 2024]. Disponible en: https://www.who.int/news-room/fact-sheets/detail/ageing-and-health

109. Fried LP, Tangen CM, Walston J, Newman AB, Hirsch C, Gottdiener J, et al. Frailty in Older Adults: Evidence for a Phenotype. J Gerontol Ser A. 1 de marzo de 2001;56(3):M146-57.

110. Perazza LR, Brown-Borg HM, Thompson LV. Physiological Systems in Promoting Frailty. Compr Physiol. 26 de abril de 2022;12(3):3575-620.

111. Heinze-Milne SD, Banga S, Howlett SE. Frailty Assessment in Animal Models. Gerontology. 22 de julio de 2019;65(6):610-9.

112. Gomez-Cabrera MC, Garcia-Valles R, Rodriguez-Mañas L, Garcia-Garcia FJ, Olaso-Gonzalez G, Salvador-Pascual A, et al. A New Frailty Score for Experimental Animals Based on the Clinical Phenotype: Inactivity as a Model of Frailty. J Gerontol A Biol Sci Med Sci. 1 de julio de 2017;72(7):885-91.

113. Yaffe D, Saxel O. Serial passaging and differentiation of myogenic cells isolated from dystrophic mouse muscle. Nature. diciembre de 1977;270(5639):725-7.

114. Kokabu S, Nakatomi C, Matsubara T, Ono Y, Addison WN, Lowery JW, et al. The transcriptional co-repressor TLE3 regulates myogenic differentiation by repressing the activity of the MyoD transcription factor. J Biol Chem. 4 de agosto de 2017;292(31):12885-94.

115. Sigma-Aldrich [Internet]. [citado 5 de julio de 2020]. C2C12 Cell Line from mouse CB_91031101. Disponible en:

https://www.sigmaaldrich.com/catalog/product/sigma/cb_91031101

116. Gambini J, Gimeno-Mallench L, Olaso-Gonzalez G, Mastaloudis A, Traber MG, Monleón D, et al. Moderate Red Wine Consumption Increases the Expression of Longevity-Associated Genes in Controlled Human Populations and Extends Lifespan in Drosophila melanogaster. Antioxidants. 16 de febrero de 2021;10(2):301.

117. Adesola RO, Lawal JT, Oladele OE. Drosophila melanogaster (Meigen, 1830): A potential model for human diseases. World News Nat Sci. 2021;36:42-59.

118. Yang D. Carnivory in the larvae of Drosophila melanogaster and other Drosophila species. Sci Rep. 19 de octubre de 2018;8(1):15484.

119. Reverse Transcription Applications - ES [Internet]. [citado 10 de enero de 2024]. Disponible en: https://www.thermofisher.com/es/es/home/life-science/cloning/cloning-learning-center/invitrogen-school-of-molecular-biology/rt-education/reverse-transcription-applications.html

120. TFS-AssetsLSGmanualsMAN0017977_highcap_cDNA_RT_UG.pdf [Internet]. [citado 10 de enero de 2024]. Disponible en: https://assets.thermofisher.com/TFS-Assets%2FLSG%2Fmanuals%2FMAN0017977_highcap_cDNA_RT_UG.pdf

121. Mahmood T, Yang PC. Western Blot: Technique, Theory, and Trouble Shooting. North Am J Med Sci. septiembre de 2012;4(9):429-34.

122. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. J Biol Chem. noviembre de 1951;193(1):265-75.

123. Kit de detección de oxidación de proteínas OxyBlot The OxyBlot Protein Oxidation Detection Kit provides the reagents to perform the immunoblot detection of carbonyl groups introduced into proteins by oxidative reactions with ozone or oxides of nitrogen or by metal catalyzed oxidation. | Sigma-Aldrich [Internet]. [citado 15 de enero de 2024]. Disponible en: http://www.sigmaaldrich.com/

124. Barja G. Mitochondrial Oxygen Radical Generation and Leak: Sites of Production in States 4 and 3, Organ Specificity, and Relation to Aging and Longevity. J Bioenerg Biomembr. 1 de agosto de 1999;31(4):347-66.

125. Cell mito stress test, Seahorse Cell Mito Stress Test Kit | Agilent [Internet]. [citado 16 de enero de 2024]. Disponible en: https://www.agilent.com/en/product/cell-analysis/real-time-cell-metabolic-analysis/xf-assay-kits-reagents-cell-assay-media/seahorse-xf-cell-mito-stress-test-kit-740885#howitworks

126. Wong SH, Knight JA, Hopfer SM, Zaharia O, Leach CN, Sunderman FW. Lipoperoxides in plasma as measured by liquid-chromatographic separation of malondialdehyde-thiobarbituric acid adduct. Clin Chem. febrero de 1987;33(2 Pt 1):214-20.

127. Bewick V, Cheek L, Ball J. Statistics review 12: Survival analysis. Crit Care. 2004;8(5):389-94.

128. Gimeno-Mallench L, Mas-Bargues C, Inglés M, Olaso G, Borras C, Gambini J, et al. Resveratrol shifts energy metabolism to increase lipid oxidation in healthy old mice. Biomed Pharmacother Biomedecine Pharmacother. octubre de 2019;118:109130.

129. Santos MA, Franco FN, Caldeira CA, de Araújo GR, Vieira A, Chaves MM, et al. Antioxidant effect of Resveratrol: Change in MAPK cell signaling pathway during the aging process. Arch Gerontol Geriatr. 2021;92:104266.

130. Eriksson SE, Ceder S, Bykov VJN, Wiman KG. p53 as a hub in cellular redox regulation and therapeutic target in cancer. J Mol Cell Biol. 1 de abril de 2019;11(4):330-41.

131. Ledesma A, de Lacoba MG, Rial E. The mitochondrial uncoupling proteins. Genome Biol. 2002;3(12):reviews3015.1-reviews3015.9.

132. Alberdi G, Rodríguez VM, Miranda J, Macarulla MT, Churruca I, Portillo MP. Thermogenesis is involved in the body-fat lowering effects of resveratrol in rats. Food Chem. 15 de noviembre de 2013;141(2):1530-5.

133. Walle T, Hsieh F, DeLegge MH, Oatis JE, Walle UK. High Absorption but Very Low Bioavailability of Oral Resveratrol in Humans. Drug Metab Dispos. 1 de diciembre de 2004;32(12):1377-82.

134. Ehala S, Vaher M, Kaljurand M. Characterization of Phenolic Profiles of Northern European Berries by Capillary Electrophoresis and Determination of their Antioxidant Activity. J Agric Food Chem. 1 de agosto de 2005;53(16):6484-90.

135. Burns J, Gardner PT, O'Neil J, Crawford S, Morecroft I, McPhail DB, et al. Relationship among Antioxidant Activity, Vasodilation Capacity, and Phenolic Content of Red Wines. J Agric Food Chem. 1 de febrero de 2000;48(2):220-30.

136. Chukwumah YC, Walker LT, Verghese M, Bokanga M, Ogutu S, Alphonse K. Comparison of Extraction Methods for the Quantification of Selected Phytochemicals in Peanuts (Arachis hypogaea). J Agric Food Chem. 1 de enero de 2007;55(2):285-90.

137. Park SJ, Ahmad F, Philp A, Baar K, Williams T, Luo H, et al. Resveratrol Ameliorates Aging-Related Metabolic Phenotypes by Inhibiting cAMP Phosphodiesterases. Cell. 3 de febrero de 2012;148(3):421-33.

138. Moral-Sanz J, Lewis SA, MacMillan S, Ross FA, Thomson A, Viollet B, et al. The LKB1-AMPK-α1 signaling pathway triggers

hypoxic pulmonary vasoconstriction downstream of mitochondria. Sci Signal. 02 de 2018;11(550).

139. Karacosta LG, Foster BA, Azabdaftari G, Feliciano DM, Edelman AM. A Regulatory Feedback Loop Between Ca2+/Calmodulin-dependent Protein Kinase Kinase 2 (CaMKK2) and the Androgen Receptor in Prostate Cancer Progression. J Biol Chem. 13 de julio de 2012;287(29):24832-43.

140. Navarro G, Allard C, Xu W, Mauvais-Jarvis F. The role of androgens in metabolism, obesity and diabetes in males and females. Obes Silver Spring Md. abril de 2015;23(4):713-9.

141. Wang Y, Romigh T, He X, Orloff MS, Silverman RH, Heston WD, et al. Resveratrol regulates the PTEN/AKT pathway through androgen receptor-dependent and -independent mechanisms in prostate cancer cell lines. Hum Mol Genet. 15 de noviembre de 2010;19(22):4319-29.

142. Gao S, Liu G, Wang Z. Modulation of androgen receptor-dependent transcription by resveratrol and genistein in prostate cancer cells. The Prostate. mayo de 2004;59(2):214-25.

143. Dong YZ, Li L, Espe M, Lu KL, Rahimnejad S. Hydroxytyrosol Attenuates Hepatic Fat Accumulation via Activating Mitochondrial Biogenesis and Autophagy through the AMPK Pathway. J Agric Food Chem. 2 de septiembre de 2020;68(35):9377-86.

144. Hao J, Shen W, Yu G, Jia H, Li X, Feng Z, et al. Hydroxytyrosol promotes mitochondrial biogenesis and mitochondrial function in 3T3-L1 adipocytes. J Nutr Biochem. julio de 2010;21(7):634-44.

145. Peng Y, Hou C, Yang Z, Li C, Jia L, Liu J, et al. Hydroxytyrosol mildly improve cognitive function independent of APP processing in APP/PS1 mice. Mol Nutr Food Res. noviembre de 2016;60(11):2331-42.

146. Cabrerizo S, De La Cruz JP, López-Villodres JA, Muñoz-Marín J, Guerrero A, Reyes JJ, et al. Role of the inhibition of oxidative

stress and inflammatory mediators in the neuroprotective effects of hydroxytyrosol in rat brain slices subjected to hypoxia reoxygenation. J Nutr Biochem. diciembre de 2013;24(12):2152-7.

147. Alemán-Jiménez C, Domínguez-Perles R, Medina S, Prgomet I, López-González I, Simonelli-Muñoz A, et al. Pharmacokinetics and bioavailability of hydroxytyrosol are dependent on the food matrix in humans. Eur J Nutr. 1 de marzo de 2021;60(2):905-15.

148. Romani A, Mulinacci N, Pinelli P, Vincieri FF, Cimato A. Polyphenolic Content in Five Tuscany Cultivars of Olea europaea L. J Agric Food Chem. 1 de marzo de 1999;47(3):964-7.

149. Lavelli V, Fregapane G, Salvador MD. Effect of Storage on Secoiridoid and Tocopherol Contents and Antioxidant Activity of Monovarietal Extra Virgin Olive Oils. J Agric Food Chem. 1 de abril de 2006;54(8):3002-7.

150. Francesca N, Romano R, Sannino C, Le Grottaglie L, Settanni L, Moschetti G. Evolution of microbiological and chemical parameters during red wine making with extended post-fermentation maceration. Int J Food Microbiol. 3 de febrero de 2014;171:84-93.

151. Pastor A, Rodríguez-Morató J, Olesti E, Pujadas M, Pérez-Mañá C, Khymenets O, et al. Analysis of free hydroxytyrosol in human plasma following the administration of olive oil. J Chromatogr A. 11 de marzo de 2016;1437:183-90.

152. De Bock M, Thorstensen EB, Derraik JGB, Henderson HV, Hofman PL, Cutfield WS. Human absorption and metabolism of oleuropein and hydroxytyrosol ingested as olive (*Olea europaea* L.) leaf extract. Mol Nutr Food Res. noviembre de 2013;57(11):2079-85.

153. Domínguez-Perles R, Auñón D, Ferreres F, Gil-Izquierdo A. Gender differences in plasma and urine metabolites from Sprague–Dawley rats after oral administration of normal and high

doses of hydroxytyrosol, hydroxytyrosol acetate, and DOPAC. Eur J Nutr. 1 de febrero de 2017;56(1):215-24.

154. Gambini J, Gimeno-Mallench L, Olaso-Gonzalez G, Mastaloudis A, Traber MG, Monleón D, et al. Moderate Red Wine Consumption Increases the Expression of Longevity-Associated Genes in Controlled Human Populations and Extends Lifespan in Drosophila melanogaster. Antioxid Basel Switz. 16 de febrero de 2021;10(2):301.

155. Troen BR. The biology of aging. Mt Sinai J Med N Y. enero de 2003;70(1):3-22.

156. Naqvi U, Sherman A l. Muscle Strength Grading. En: StatPearls [Internet]. Treasure Island (FL): StatPearls Publishing; 2024 [citado 29 de febrero de 2024]. Disponible en: http://www.ncbi.nlm.nih.gov/books/NBK436008/

157. Jin K. Modern Biological Theories of Aging. Aging Dis. 1 de agosto de 2010;1(2):72-4.

158. Franceschi C, Salvioli S, Garagnani P, de Eguileor M, Monti D, Capri M. Immunobiography and the Heterogeneity of Immune Responses in the Elderly: A Focus on Inflammaging and Trained Immunity. Front Immunol. 2017;8:982.

159. Franceschi C, Bonafè M, Valensin S, Olivieri F, De Luca M, Ottaviani E, et al. Inflamm-aging. An evolutionary perspective on immunosenescence. Ann N Y Acad Sci. junio de 2000;908:244-54.

160. Harman D. Aging: A Theory Based on Free Radical and Radiation Chemistry. J Gerontol. 1 de julio de 1956;11(3):298-300.

161. Viña J. The free radical theory of frailty: Mechanisms and opportunities for interventions to promote successful aging. Free Radic Biol Med. 1 de abril de 2019;134:690-4.

162. Chen Y, Zhang D qi, Liao Z, Wang B, Gong S, Wang C, et al. Anti-oxidant polydatin (piceid) protects against substantia nigral

motor degeneration in multiple rodent models of Parkinson's disease. Mol Neurodegener. 2 de marzo de 2015;10:4.

163. Park B, Jo K, Lee TG, Hyun SW, Kim JS, Kim CS. Polydatin Inhibits NLRP3 Inflammasome in Dry Eye Disease by Attenuating Oxidative Stress and Inhibiting the NF-κB Pathway. Nutrients. 15 de noviembre de 2019;11(11):2792.

164. Calleja M, Peña P, Ugalde C, Ferreiro C, Marco R, Garesse R. Mitochondrial DNA remains intact during Drosophila aging, but the levels of mitochondrial transcripts are significantly reduced. J Biol Chem. 5 de septiembre de 1993;268(25):18891-7.