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EFFECT OF CURCUMIN ON AGING-RELATED GENE EXPRESSION IN FIBROBLAST CELLS

Sabina NEZIRI

MASTER OF SCIENCE THESIS

Department of Molecular Biology and Genetics

Molecular Biology and Genetics Program

Supervisor

Prof. Dr. Nehir ÖZDEMİR ÖZGENTÜRK

Co-Supervisor

Assist. Prof. Dr. Emine KANDEMİŞ

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A thesis submitted by SABINA NEZIRI in partial fulfillment of the requirements for the degree of MASTER OF SCIENCE is approved by the committee on in Department of Molecular Biology and Genetics, Molecular Biology and Genetics Program.

Prof. Dr. Nehir ÖZDEMİR ÖZGENTÜRK	Assist. Prof. Dr. Emine KANDEMİŞ
Yıldız Technical University	Yıldız Technical University
Supervisor	Co-Supervisor

Approved By the Examining Committee

Prof. Dr. Nehir ÖZDEMİR ÖZGENTÜRK, Supervisor
Yildiz Technical University _____

Assoc. Prof. Dr. ŞENAY VURAL KORKUT

Yildiz Technical University _____

PROF. DR. CEMAL ÜN
Ege University _____

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Sabina NEZIRI

Signature

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Dedicated to my parents,

Elmira and Sadi Neziri

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LIST OF ABBREVIATIONS

ADP	Adenosin Diphosphate
AMPK	Adenosine Monophosphate Activated Protein Kinase
ANLN	Anilin Actin Binding Protein
ATP	Adenosine Triphosphate
CAT	Catalase
CCNB1	Cyclin B1
CoQ	Ubiquinone or Coenzyme Q
CoQ	Ubiquinone
CoQH	Ubisemiquinone
CoQH	Ubisemiquinone
$CoQH_2$	Ubiquinol
$CoQH_2$	Ubiquinol
DDR	DNA Damage Repair
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
DNA-SCARS	DNA Segments with Chromatin Alterations Reinforcing
DNMTs	DNA Methyltransferases
ETC	Electron Transport Chain
FOXO	Forkhead Box Transcription Factors
G0	Quiescence
GADPH	Glyceraldehyde-3-Phosphate Dehydrogenase
GH	Growth Hormones
GPx	Glutathione Peroxidase

GR	Glutathione Reductase
GSH-Px	Glutathione Peroxidase
HDAC	Histone Deacetylases
HP1α	Heterochromatin Protein 1α
HSR	The Heat Shock Response
IGF-1	Insulin-Like Growth Factor
IGFBP2	Insulin-Like Growth Factor Binding Protein 2
IIS	Insulin-Like Growth Factor (IGF-1) Signaling Pathway
MAPKs	Mitogen Activated Protein Kinases
MMPs	Matrix Metalloproteinases
MnSOD	Manganese Superoxide Dismutase
MOS	Mild Oxidative Stress
MPO	Myeloperoxidase
mtDNA	Mitochondrial DNA
mTOR	Mechanistic Target of Rapamycin
NADPH	Nicotinamide Adenine Dinucleotide Phosphate+Hydrogen
NTH1	Endonuclease III-Like Protein
OXPHOS	Oxidative Phosphorylation
PBS	Phosphate-Buffered Saline
Pi	Inorganic Phosphate
PI3K	Phosphatidylinositol 3-Kinase
PML NBs	Promyelocytic Leukemia Protein Nuclear Bodies
qPCR	Quantitative Real-Time PCR
RNA	Ribonucleic Acid
RNS	Reactive Nitrogen Species

RONS	Reactive Oxygen and Nitrogen Species
ROS	Reactive Oxygen Species
SAHF	Senescence Associated Heterochromatin Foci
SASP	Senescence-Associated Secretory Phenotype
SA-β-GAL	Senescence-Associated β-Galactosidase
	Senescence
SIPS	Stress-Induced Premature Senescence
SOD	Superoxide Dismutase
SOS	Severe Oxidative Stress
SPT	Skin Phototype
T1D	Type 1 Diabetes
TOS	Temperate Oxidative Stress
UN	United Nations
UPR	Unfolded Protein Response
UPS	Ubiquitin-Proteasome System
WNT16	Wnt Family Member 16

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ABSTRACT

Effect of Curcumin on Aging-Related Gene Expression in Fibroblast Cells

Sabina NEZIRI

Department of Molecular Biology and Genetics

Master of Science Thesis

Supervisor: Prof. Dr. Nehir ÖZDEMİR ÖZGENTÜRK

Co-Supervisor: Assist. Prof. Dr. Emine KANDEMİŞ

The aging process affects almost all species on earth. Intrinsic and extrinsic factors affect aging at different scales. Longitudinal family studies suggest that only 25% of aging is genetic, and the rest is affected by the environment and lifestyle. In recent years food compounds and their influence in the genome and epigenome have gained a lot of attention. Nutraceuticals, or foods with medical benefits are thought to affect lifespan and healthspan. In this research the effect of the well-known compound, curcumin was studied. For centuries, the turmeric plant has been used as a remedy, and curcumin is the most bioactive compound of it. The aim of this thesis is to study the hormetic effect of curcumin, and its effect on senescence and HAS2, IGFBP2, WNT16, GAPDH, ANLN, and CCNB1 gene expressions in late passage foreskin fibroblast. The control group was not treated, while the experimental groups were treated with 1 micromolar curcumin and the other with 0.074% DMSO. All groups were passaged until entering the senescence state. Afterwards, RNA isolation and Real-Time PCR test were done, and the cell viability test was used to find the right curcumin concentration and measure its hormetic effect. As a result, it was found that in our cell line the curcumin retains

its hormetic effect. We also show that curcumin affects the expression of CCNB1 and ANLN genes as expected.

Keywords: Aging, fibroblast, curcumin, gene expression, senescence

ÖZET

Kurkuminin Fibroblast Hücrelerinde Yaşlanmaya Bağlı Gen Ekspresyonu Üzerindeki Etkisi

Sabina NEZIRI

Moleküler Biyoloji ve Genetik Bölümü

Yüksek Lisans Tezi

Danışman: Prof. Dr. Nehir ÖZDEMİR ÖZGENTÜRK

Eş-danışman: Doç. Dr. Emine KANDEMİŞ

Yaşlanma süreci dünyadaki hemen hemen tüm türleri etkiler. İçsel ve dışsal faktörler yaşlanmayı farklı ölçeklerde etkiler. Aile çalışmalarından alınan sonuçlar, yaşlanmanın sadece %25'inin genetik olduğunu ve geri kalanının çevre ve yaşam tarzından etkilendiğini göstermektedir. Son yıllarda gıda bileşikleri ve bunların genom ve epigenom üzerindeki etkileri çok fazla ilgi görmüştür. Nutrasötiklerin veya tıbbi faydaları olan gıdaların yaşam süresini ve sağlıklı yaşam süresini etkilediği düşünülmektedir. Bu araştırmada iyi bilinen bir bileşik olan kurkumin'in etkisi araştırıldı. Yüzyıllardır zerdeçal bitkisi farklı hastalıklar için tedavi olarak kullanılmıştır ve kurkumin bu bitkinin en biyoaktif bileşiğidir. Bu tezin amacı, kurkuminin hormetik etkisini, ve kurkuminin senesans ve HAS2, IGFBP2, WNT16, GAPDH, ANLN ve CCNB1 gen ekspresyonları üzerindeki etkisini geç pasaj sünnet derisi fibroblastında incelemektir. Kontrol grubuna tedavi uygulanmazken, deney gruplarına 1 mikromolar kurkumin ve diğer gruba %0.074 DMSO uygulandı. Tüm gruplar, senesan'a girene kadar pasajlandı. Sonra RNA izolasyonu ve Real-Time PCR testi yapıldı ve ayrıca doğru kurkumin

konsantrasyonunu bulmak ve hormetik etkisini ölçmek için hücre canlılık testi kullanıldı. Sonuç olarak, kurkuminin hücre hattımızda hormetik etkisini koruduğu bulundu. Ayrıca CCNB1 ve ANLN genlerinin ekspresyonu beklendiği şekilde kurkuminden etkilediğini gösterdik.

Anahtar Kelimeler: Yaşlanma, fibroblast, kurkumin, gen ekspresyonu, senesans

1
INTRODUCTION

1.1 Literature Review

Gerontology is the field that studies aging and changes it causes to the organism [1]. Aging or getting older is a process that affects almost all species. According to Otin et. al. aging can be defined as the time-related progressive loss of physiological integrity, resulting in damaged function and increased susceptibility to death [2]. Currently only a few species are known to be biologically "immortal" (they don't die of aging), such as the Turritopsis jellyfish, Hydra, lobsters, and bristlecone pine [3], [4], but the rest are all affected by this process. Otin et. al grouped the hallmarks of aging in three categories: the primary hallmarks, the antagonistic hallmarks and integrative hallmarks [2]. The primary hallmarks damage the cellular functions and include the genome instability, telomere attrition and epigenetic alterations; the antagonistic hallmarks response to this damage and include the deregulated nutrient-sensing, mitochondrial dysfunction and cellular senescence; integrative hallmarks include the culprits of the phenotype such as stem cell exhaustion and altered intercellular communication [2].

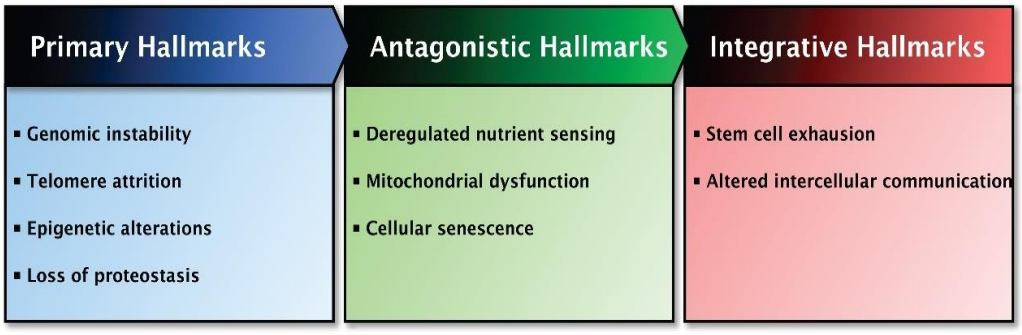


Figure 1.1 Hallmarks of aging [5]

Life expectancy has seriously increased in the last decades. In the beginning of the 19th century the life expectancy was only 40 years in the world, but after the healthcare and lifestyle improvements in 1950 and after it started increasing at almost 60 years in particular states [6]. By 2050 16% of the human population will be over the age of 65 (1 in 6 people), while in 2019 it was only 9% (1 in 11 people) [7]. The increase in population aging indicates the advancements in medicine, public health, social and economic development, contributing in the disease control, injury prevention and decrease in the risk of premature death [8].

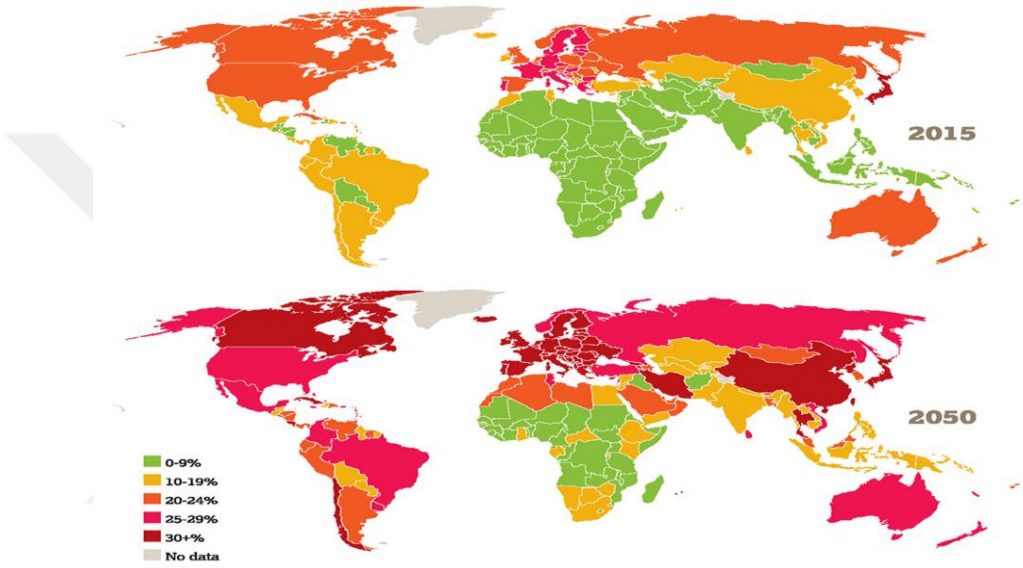


Figure 1.2 Aging speed in the world [9]

Today, scientists suggest that humans will be able to live more than 100 years [10], even reach 150 in a healthy condition [11]. In today's world the top most long-lived countries include: Hong Kong, Japan, Macao, Switzerland, Singapore, Italy, Spain and Australia with life expectancy around 85-84 years [12]. What is clearly seen is that female life expectancy is longer than the one of male's [12]. So far, the oldest female to have ever lived is Jeanne Louise Calment from France who died when she was 122 years old, while the oldest male to have ever lived is Jiroemon Kimura from Japan who died at the age of 116 [13].

We are clearly experiencing an increase in global lifespan and along with this the cases of age-related diseases are also increasing. The improvement of human healthspan is not moving in parallel. With normal aging sensory changes (hearing

loss, visual acuity and vestibular function), muscle strength and fat changes, immunoscenescence and urologic changes take place [14]. Age-related somatic diseases and multiple chronic conditions include: cardiovascular disease, hypertension, cancer, osteoarthritis, osteoporosis, diabetes mellitus [14], neurodegenerative diseases such as Alzheimer's and Parkinson's diseases [15]. an important study case for aging are the premature aging syndromes, also called progeroid syndromes. Segmental progeroid syndromes include more than 100 syndromes with premature aging in more than one type of tissue or organ, genetically and clinically heterogenous [16]. The reason these diseases are called segmental is because they don't affect all the organs [16]. Additionally, centenarians (people living 100 or more years) are important study cases too. Geographically speaking these people are mostly found in Okinawa (Japan), Bulgaria and Sardinia [17]. Scientist have been studying Okinawans for a long time and they suggest that the low caloric intake and the good nutrition are the main reasons for their longevity [17].

For anti-aging related research Greg Bailey says: "I don't think they quite grasp how fast this is going to happen, and how big it's going to be." for Forbes magazine [18]. Currently the company has 12 science-based programs such as senolytics, stem cell research etc. to prevent or modify Parkinson's and Alzheimer's diseases [18]. The focus will also be in addressing fibrosis, inflammation, and tissue regeneration to slow aging [19]. MIT includes anti-aging drugs among the top 10 breakthrough technologies in 2020 [20]. Called senolytics these drugs can remove senescent cells and as a result slow or reverse aging, and first human testings have begun [20].

Several aging theories exist, each of them trying to figure out why we age and what are the underlying mechanisms of it but is the combination among these theories that makes it possible for the aging process to be better understood. Longevity is affected by the genetic and environmental factors. Data from family studies suggest that only 25% of aging is actually determined by the genetic factors [21]. Thomas Johnson was the researcher who initiated the genetics of aging studies [21]. He studied C. elegans mutants, and found out that individuals with age1 gene mutations live longer [22]. Specific gene variants partly determine

longevity, e.g. gene that take part in processes such as: the cell's free radical 'defense' processes; DNA (deoxyribonucleic acid) repair and insulin signaling [23]. As organisms age, the DNA sequence doesn't change, but genes get turn on and off by the epigenetic changes, which play a big role in the process. In a study where white blood cells from elderly man and baby umbilical cord were taken, big methylation pattern changes were found among them [24]. Cytosine methylation significantly was higher in newborns than in centenarians [24].

Otin et. al. categorize the aging hallmarks in: genomic instability, epigenetic alteration, loss of proteostasis, telomere attrition, cellular senescence, altered intercellular communication, deregulated nutrient-sensing, stem cell exhaustion and mitochondrial dysfunction [2]. The genetic damage that occurs includes telomere shortening, chromosomal losses and gains, point mutations, translocations and gene disruption (as a result of transposons or virus integration), thus nuclear architecture defects, laminopathies can trigger the damage and lead to premature aging diseases [2]. Also, the correlation of telomere length, tissue age, and several age-related diseases have been associated with the telomere shortening [25]. Epigenetic changes include DNA methylation, histone modifications, chromatin remodeling, and non-coding RNAs (ribonucleic acid). Epigenetic changes affect processes like gene silencing, transcription, DNA repair, replication, cell cycle progression, telomeres and centromeres [26]. DNA methylations for example are used in the biological age prediction [27]. With age the proteostasis system deteriorates because of the accumulation of damaged and misfolded proteins in cells [28]. Metabolic signaling pathways intermediated by reduced mitochondrial function, dietary restriction, insulin/IGF-1 (insulin-like growth factor 1) signaling can modulate the proteostasis network leading to a maintenance of youthful proteome [28]. The nutrient sensing pathways keep the right nutrition amount by controlling the intake [29]. 4 pathways that are mostly related with aging include: insulin and IGF-1 signaling pathway (IIS), sirtuins, adenosine monophosphate kinase (AMPK) pathway and mechanistic target of rapamycin (mTOR) pathway [29]. For example, when there is abundance in food anabolism and storage are engaged by these pathways [30]. Insulin and insulin-like growth factor (IGF) signaling (IIS) pathway mutations in C. elegans, D.

melanogaster and several mouse models can expend lifespan [31], AMPK plays an really important role in the regulation of some of the essential lifespan and aging determinants [32], while sirtuins are one of the most critical targets when it comes to anti-aging advance [33]. The dysfunction of mitochondria is is linked with aging with the mitochondrial quality control failure, oxidative phosphorylation (OXPHOS) activity failure, enhanced oxidative damage, weakened metabolic enzyme activity, and biogenesis changes, dynamics, and morphology [34], [35]. Senescence is the long-term loss of the metabolically active cells' capacity to proliferate [36]. Senescent cells accumulate in aged organisms [37]. The elimination of these cells is thought to be the cure of numerous age-related diseases and extend the healthspan. With aging even stem cells lose their regenerative functions, and also modifications in the intercellular communication appear [2].

Skin is the largest body organ, where aging gives the clearest signs. Fibroblasts are found in the dermis layer the skin. They are a crucial component in the skin, producing the extracellular matrix and regulating the skin physiology [38]. When it comes to aging, dermal fibroblasts are a strong indicator of it [39]. CCNB1, IGFBP2 (insulin like growth factor binding protein 2 [40]), and WNT16 genes' role in senescence and cell cycle has been proven [41]. CCNB1 gene was downregulated in human senescent fibroblasts, while IGFBP2 and WNT16 gene were upregulated [41]. ANLN gene is another repressed gene during senescence [42]. In fibrotic fibroblasts HAS2 gene (which is responsible for the hyaluronan synthase 2 protein (UniProtKB identifiers: Q92819)), expression was downregulated [43]. It is a well-known fact that hyaluronic acid levels in skin decrease with age and finding nutraceuticals that balance its levels is important.

Curcumin is one of the most bioactive components of the Turmeric plant (Curcuma longa) [44], which is known for the anti-aging, antibiotic, anti-inflammatory, anticancer effects [45]. Curcumin affects diverse biological processes, such as proliferation, apoptosis, inflammation, and redox state, and also affect aging and age-related diseases [46].

Curcumin interacts with receptors, kinases, adhesion molecules, growth factors, tumor necrosis factor, transcription factors, enzymes, proinflammatory cytokines, apoptotic regulators and other proteins [47], [48].

Curcumin has a hormetic effect, which means that its impact depends on the concentration [49], inhibitor in high concentration (genotoxic and cytotoxic agent) and stimulant in low ones (protective agent) [46]. During our cell viability test we also found that curcumin concentrations above 20 μM decrease the absorption, meaning that cell viability or proliferation are negatively affected by it. Meanwhile, lower concentrations had the opposite effect (10-1 μM).

1.2 Objective of the Thesis

The aim of the thesis was to study the effect of curcumin on the expression level of HAS2, IGFBP2, WNT16, GAPDH, ANLN, and CCNB1 genes in dermal fibroblast cells, when added in late passages. For this purpose, the cells were passaged in order to observe the aging process in dermal fibroblasts. The control group was aged naturally, while the experimental groups were treated with 1μM curcumin solution (dissolved in DMSO) and 0.074% DMSO (negative control). Then late passage cells were incubated under appropriate conditions with curcumin, which is known as an anti-aging agent. Within the scope of the thesis, expression levels of HAS2, IGFBP2, WNT16, GAPDH, ANLN, and CCNB1 genes were analyzed with primers designed specifically for the genes. Furthermore, the cell viability was done to measure the hormetic effect of curcumin and to decide the concentration used for cell culture treatment.

1.3 Hypothesis

Our hypothesis is that curcumin, even when added in late passage affects of HAS2, IGFBP2, WNT16, GAPDH, ANLN, and CCNB1 genes, altogether with several pathways in the cell. The effects of curcumin on the expression levels of of HAS2, IGFBP2, WNT16, GAPDH, ANLN, and CCNB1 genes (which are known to be affected in the aging fibroblast cells) have not been studied within the scope of current studies. The data to be obtained will be studied for the first time in the literature and it is thought that it will contribute to the literature and provide new data for other projects.

2
AGING

2.1 Theories of Aging

Why and how do we age? When does aging start in our bodies? Do our genes control our lifespan? How can we increase our lifespan? Is there a clear limit to it? These are some of the questions scientists have been trying to answer for a long time, and still the aging phenomena holds many unknowns.
Several theories of aging exist, exist and many of them interact in complicated ways, but they can be grouped in two categories: programmed and error or damage theories [50]. The programmed theories suggest that there is a biological timetable for aging, while the damage/error ones suggest that the environment induces cumulative damage at different levels leading to aging [50].
The programmed theory is further grouped in three sub-categories:

- Programmed Longevity- according to this theory senescence can be defined as the moment when the age-related characteristics start appearing, and aging itself occurs from chronological turn off and on of particular genes [50].
- Endocrine theory- Hormones define the biological clock and control the aging speed [50]. For example, the insulin/IGF-1 signaling (IIS) pathway has an essential role in the hormone mediated aging regulation [31].
- Immunological Theory- The immune system deteriorates with time, causing enhancement in the vulnerability towards the infectious diseases, and as a result leading to aging and death [50].
- The damage/error theories consist of:
- Wear and tear theory- Vital parts of cells and tissues wear out leading in aging [50].
- Rate of living theory- The greater the rate of oxygen basal metabolism in an organism, the shorter the lifespan [51]. Anyways, this theory is not sufficient in explaining the maximum lifespan [52].
- Cross-linking theory- Proposed by Johan Bjorkseten in 1942, this theory suggests that the accumulation of cross-linked proteins causes damage to cells and tissues,

leading to slowed down processes, leading to aging [53]. There is evidence that cross-linking reactions take part in age-related changes in particular proteins that have been studied [54].

- Free radicals theory- the accumulation of oxidative damage caused by free radicals leads to aging [55].
- Somatic DNA damage theory- Accumulated and not repaired DNA damage has been found in non-dividing mammal cells, leading to cell deterioration (or mitochondrial dysfunction in the case of mitochondrial DNA) [50].
- Telomere theory- Progressive telomere loss leads to genomic instability and boosts aging [56]
- Information Theory of Aging- suggests that epigenetic changes lead to the aging hallmarks, but the genetic code remains almost the same [11].

2.2 Hallmarks of Aging

Lopez Otin et.al. classify the aging hallmarks as [2]:

- Genomic instability
- Epigenetic alteration
- Loss of proteostasis
- Telomere attrition
- Cellular senescence
- Altered intercellular communication
- Deregulated nutrient-sensing
- Stem cell exhaustion and mitochondrial dysfunction

Each of the mentioned hallmarks: must be manifested during normal aging; accelerated aging when aggravated experimentally; slow the normal aging when experimentally ameliorated [2].

2.2.1 Genomic Instability

The increase in the accumulation of DNA damage is experienced in aging and also in premature aging diseases, such as Werner and Bloom syndrome (although these diseases experience only some of the aging hallmarks) [2]. Exogenous chemical, biological and physical agents and endogenous risks, such as reactive oxygen species (ROS), DNA replication errors and spontaneous hydrolytic reactions constantly challenge the DNA stability and integrity [2]. Genome instability includes all the permanent and transmittable DNA sequence alterations (including both nuclear and mitochondrial DNA), as a result of replication or repair errors [57].

2.2.1.1 Nuclear DNA

In the aged cells of humans and other organisms alterations such as: somatic mutations accumulate; chromosomal aneuploidies; clonal mosaicism for chromosomal anomalies have been described [2]. DNA damage consists of spontaneous hydrolysis and deamination, and additional chemical alterations, such as gaps, nicks, breaks, adducts, abasic sites, intrastrand and interstrand DNA-protein crosslinks etc. [57].

Somatic mutations accumulate in our cells with age. While some of these mutations are harmless, others cause problems in the organism. In 1990s, studies reported the existence of mutations in TP_{53} in sun-exposed skin [58]. In 2015, small epidermis (sun-exposed) biopsies from 55-73 aged donors were sequenced to detect mutations [59]. Over 10,000 somatic mutations were found, mostly on important cancer genes leading to accelerated proliferation of the cells and to clonal enlargements [59]. An analogous study was made using esophageal epithelium from individuals aged 20 to 75 [60]. The mutation rate in the these cells was ten times lower compared with the sun-exposed skin, but positive selection was greater, resulting in clones that carry mutations in cancer genes inhabiting most of the esophagus [60]. Mutations in in NOTCH1 and TP_{53} were present in middle-aged and elderly patients in respectively 5-20% and 30% of the cells [60]. The somatic mutations accumulation is strongly related with age [60], [61], with further effect of alcohol consumption and intense smoking [61].

Aneuploidy is an unusual copy number of at chromosomes (one or more), which occurs from abnormalities in cell division [62]. Correct chromosome segregation is depended on the microtubule organization in the bipolar mitotic structure, the right attachment of chromosomes at kinetochores (specific proteinaceous structures in all chromosomes) in spindle microtubules, and in the proper mitosis length of time (for the chromosomes to attach in the right way in the spindle microtubules) [63]. The causes of whole chromosomal aneuploidy include: the spindle assembly checkpoint defects; errors in kinetochore-microtubule attachment; cohesion defects; supernumerary centrosomes; tetra ploidy and telomere dysfunction [63]. On the other hand, structural aneuploidy is a result of DNA breakage and/or damage causing deletions, non-balanced translocations and/or duplications of significant large chromosome regions [63]. Structural aneuploidy can be caused by: unequal crossing over among non-sister chromatids and misalignment of homologous chromosomes during meiosis; incorrect non-allelic homologous recombination leads to variations of DNA copy number because of duplications and deletions; unsolved DNA replication errors in mitosis; partial DNA decatenation in mitosis; chromothripsis (crushing one or more chromosomes because of their highly error-prone re-stitching leading to massive chromosome rearrangements, such as deletions, non-balanced translocations, inversions and duplications) [63].

Genetic mosaicism is the presence of cellular populations with two or more different karyotypes in one individual, resulting from the post-zygotic event in the period of development in germline and somatic cells [64]. At first clonal mosaicism was observed on genetic disorders, but actual data reveals that it can be found in seemingly healthy individuals [65]. Clonal mosaicism rises in frequency with age and mostly occurs in males [65].

2.2.1.2 Mitochondrial DNA

Because of the mitochondrial oxidative environment, absence of mitochondrial DNA (mtDNA) protective histones, and limited mtDNA repair mechanisms, mtDNA is also prone to mutations and deletions associated with aging [2].

Mitochondria are organelles that contain their own DNA. They have an oval shape and differ from 0.5 to 10 μm, and are found in the cytoplasm of nearly all eukaryotic cells (with exception of red blood cells for example, or Monocercomonoides species) [66].

Mitochondria can be observed with a light microscope and it was discovered in 1800s, with its name coming from the Greek words meaning “thread” and “granule” [67]. Its structure includes the two specialized membranes, the intermembrane space, and matrix, each of which has its own specialized proteins [67]. The inner membrane has a high number of foldings, called cristae which increase its complexity [67].

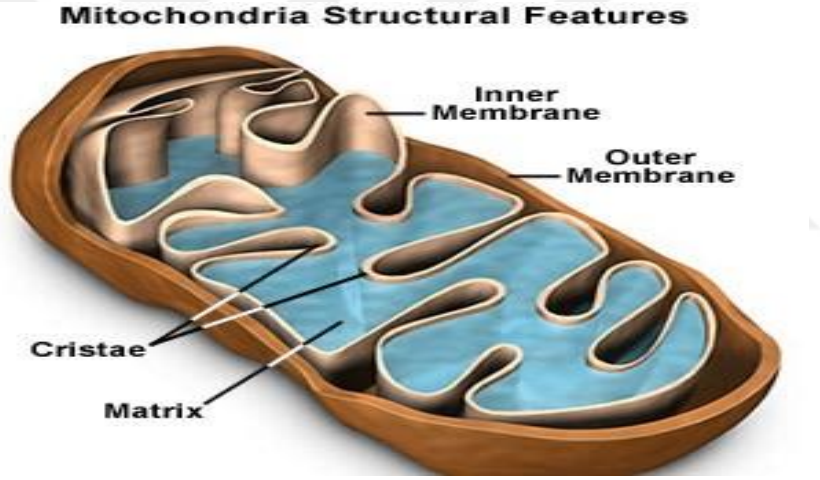


Figure 2.1 Mitochondria structure [67]

Their most important function is the energy production for the cell through the oxidative phosphorylation that occurs in the Electron Transport Chain (ETC). This process is associated with ROS production, which can lead to macromolecules’ damage. mtDNA is not as protected as nuclear DNA is and has less reparation mechanisms to prevent its damage. The other functions of mitochondria also include: cellular homeostasis, apoptosis, intracellular signaling, metabolism (intermediary, amino acids, cholesterol, steroids, lipids and nucleotides) [68]. They store calcium used for cell signaling activities, produce heat and mediate the growth and death of cells [66].

ETC is found in the inner membrane and includes five protein complexes, from I to V [69].

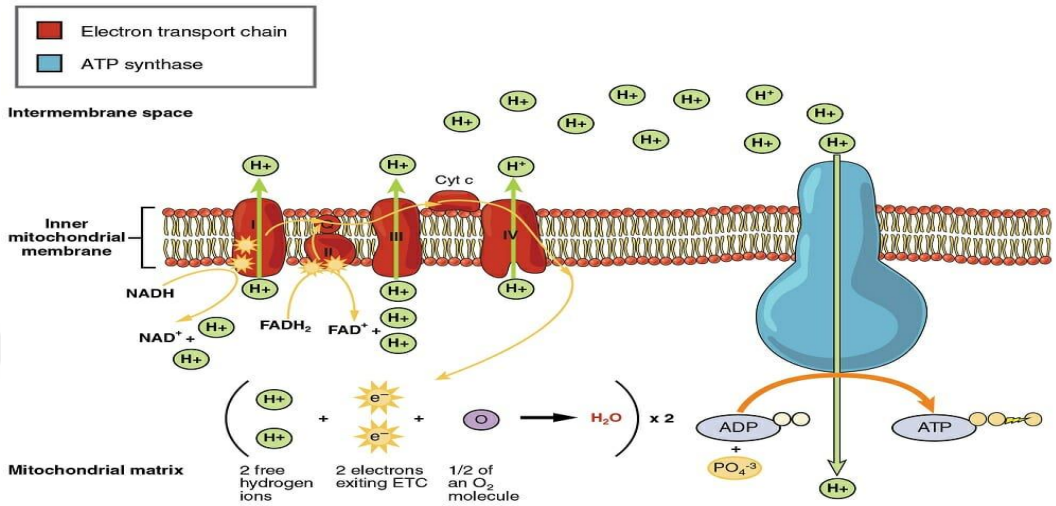


Figure 2.2 The electron transport chain [70]

The reduced cofactors NADH (nicotinamide adenine dinucleotide+hydrogen) and FADH2 (flavine adenine dinucleotide+hydrogen), which are produced during carbohydrates metabolism, fats (β-oxidation), and proteins donate the electrons to the complexes (I and II) [68]. The electrons pass to ubiquinone (coenzyme Q or CoQ) forming ubisemiquinone (CoQH) and later ubiquinol ($CoQH_2$), which transfers the electrons to the complex III, which transmits them to the cytochrome c [68]. From there, electrons flow to complex IV, and this complex is responsible for donating an electron to oxygen leading to water produce [68]. The electron flow generates energy which is used by complex I, III and IV to pump the H+ protons out into the intermembrane space, and the proton gradient that is created has an essential role in the adenosine triphosphate (ATP) synthesis from adenosine diphosphate (ADP) and inorganic phosphate (Pi) by the complex V [68].

Mitochondria have their own circular DNA, with no introns inherited from the maternal line and containing 37 genes, 12S and 16S rRNAs, 13 proteins and 22 tRNAs [68]. mtDNA it is more exposed to damage than the nuclear one, but has

its repair mechanisms [68]. The most important repair mechanism is the base exicision repair (BER), which is encoded by the nuclear DNA [69], [71]. In mitochondria, the enzymes that enable repair are: damage-specific DNA glycosylases, endonuclease III-like protein (NTH1), DNA ligase IIIβ and apyrimidinic/apurinic endonuclease (APE) [68].

In studies with rodents, the mtDNA mutation and base oxidation levels increased with age, while dietary restriction reduced the age-related accumulation of it [72], [73]. ROS toxicity's effect on lifespan is also seen in transgenic Drosophila melanogaster that express enhanced levels of catalase and Cu/Zn SOD (superoxide dismutase) live longer [74]. Furthermore it was seen that mice with inactivated MnSOD died at eight days old [75], [76]. Also, when the human catalase was targeted in mouse mitochondria (transgenic mice) an increase in lifespan has been observed [77]. It is still not known if age-accumulated mtDNA mutations are a cause or consequence of the process of aging [78].

MtDNA somatic mutations are associated with mtDNA heteroplasmy, meaning that there is a mixture of mtDNA molecules in just one cell (normal and mutant) [79]. Mitochondrial DNA mutations include: rearrangement mutations, point mutations affecting rRNA or tRNA, and point mutations affecting protein-coding genes [68]. Since nuclear DNA encodes for 1500 mitochondrial proteins, defects in it affect the oxidative phosphorylation process and the mitochondrial metabolism [80], [81].

2.2.1.3 Nuclear Architecture

Genome instability is also caused by the defects in nuclear lamina [2]. Progerin, the aberrant prelamin A isoform has been found in normal human aging cells, such as production in fibroblasts culture promoted by telomere dysfunction, suggesting the link among them [2]. Lamin B levels also decrease during senescence, making them a biomarker of the process [2].

2.3.2 Telomere Attrition

Telomeres are repetitive non-coding DNA sequences found in the end of each chromosome [82]. Everytime a cell divides they become shorter, till they reach a minimum length were cells can not divide any longer [82]. In humans and other

vertebrates the sequence of telomeres mostly consists of GT-rich repeats (TTAGGG), and has a single-stranded 3'-end overhang [83]. Telomeres have several functions such as: protecting chromosomes from recombination, recognition as damaged DNA, and end to end fusion; providing means for complete chromosome replication; contributing in the gene expression regulation; playing a role in the functional chromosome organization in the nucleus; leading the human cell replicative capacity and their pass to senescence [83].

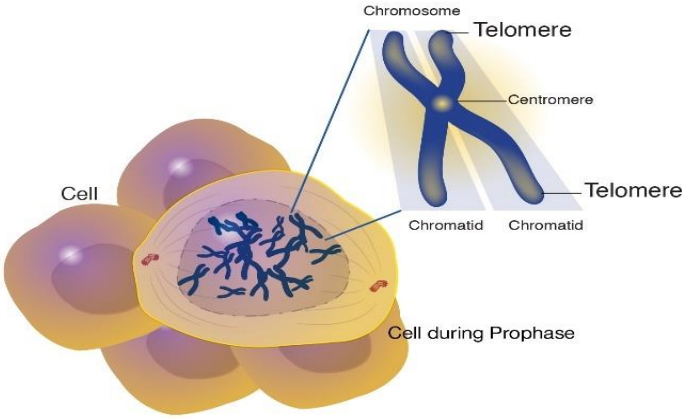


Figure 2.3 Telomeres [82]

It has been shown that the length of telomeres in human fibroblasts decreases with serial passage when in vitro [84]. Several factors such as oxidative damage can accelerate the telomere shortening, and at times even a single dysfunctional telomere can be enough to establish senescence [25]. Several studies give the evidence of inverse correlation of telomere length and particular tissue age, and also various age-related diseases (diabetes, Alezheimer's, cardiovascular diseases) have been linked with the telomere shortening [25].

Telomerase is an enzyme (RNA-depended DNA polymerase) that is responsible for synthethizing telomeric DNA sequences [83]. Telomerase has two important components: the functional RNA component (hTR or HTERC in humans) that is the template for the DNA synthesis, and the catalytic protein (hTERT) that has the

reverse transcriptase activity [83]. The normal chromosome end can look like a double-strand break and can trigger the DNA damage response, that if not fixed by telomerase the cell will enter senescence or die (when too many repeats are lost) [85]. The reasons why the telomere checkpoint fails at times are: selection of cells with defects in the DNA damage response, and the chromosome end fusion and chromatid breaks [85]. The first telomerase disorder that was recognized was the Dyskeratosis congenita (nail dystrophy), which includes mutations in telomerase (the hTERT gene) leading to short telomeres and bone marrow/stem cell failure [85]. There are gender references in leucocyte telomere length, since females show longer telomeres since birth [85]. It is interesting to note that mouse do not exhibit telomere length loss with age [85].

2.3.3 Epigenetic Alterations

Epigenetic traits are heritable phenotypes following chromosome changes without DNA sequence alterations [86]. DNA methylation, histone modifications, non-coding RNAs and chromatin remodeling are the core of the age-related epigenetic changes. Epigenetic alterations considerably affect nuclear processes such as gene silencing and transcription, DNA repair and replication, telomere and centromere function and structure, and the progression of the cell cycle [26].

Epigenetic marks that are associated with age include: enhanced H4K20 trimethylation/H3K4 trimethylation and H4K106 acetylation, and reduced H3K9 methylation/H3K27 trimethylation [2]. The enzymes responsible for the maintenance and production of the epigenetic patterns are DNA methyltransferases, deacetylases, histone acetylases, methylases, demethylases, thus there are other protein complexes that are included in the chromatin remodeling [2].

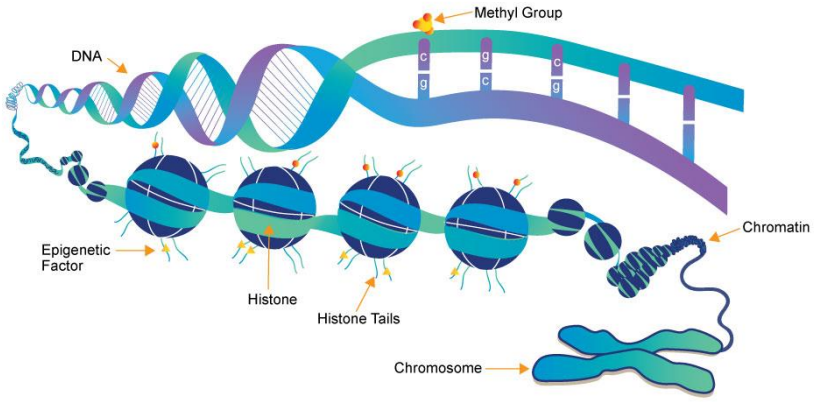


Figure 2.4 Epigenetic modifications [87]

2.3.3.1 Histone Modifications

Chromosomal DNA is well folded by chromatin proteins in eukaryotes, and these proteins are mostly composed of the four core histones H2A, H2B, H3 and H4, but also other histone variants like H3.3 and macroH2A have an essential role in the dynamics of chromatin [88]. Chromatin structure can be remodeled by becoming less condensed and transcriptionally active or highly condensed, and it can change because of the histone modifications, such as methylation and acetylation [88]. Even the histone abundance can change with time [89]. The addition of chemical groups, such as methyl, acetyl, ubiquityl and sumoyl groups to histones modifies the structure of chromatin, and leads to the DNA being less accessible to proteins engaged in DNA repair, transcription, DNA replication and additional processes [88]. The enhance in histone acetylation marks active transcription and has been associated with aging [90]. Lifespan was increased in several model organisms by the histone acetylation inhibition [88]. In contrast to this, extra copies of histone deacetylases (HDACs) such as HST3 or HST4 was found to extend lifespan [91], [92]. In mice for example, the restoration of the H4K12 acetylation was found to delay the age-related memory damage, leading to the conclusion thathistone acetylation can also be beneficial when it comes to aging [93]. The link among histone methylation and aging is more complicated. 2 methylation sites that are related with aging include H3K4me2 (transcription activator), and H3K27me3 (transcription repressor), which have different effect according to the species [88]. The senescence-associated secretory phenotype (SASP) is associated with the

repressive mark H3K27me3 loss, which leads to the upregulation of specific inflammation-related genes associated with the senescent cells' secretory molecules [94], [95, p. 1]. Histones and chromatin remodelers (e.g. SIRT1,SIRT2, SIRT6 and SIRT7), histone acetyltransferases and deacetylases have been linked with longevity and also healthspan [96, p. 6].

2.3.3.2 DNA Methylation

DNA methylation occurs in cytosines with the attachment of a methyl group in the nitrogenous base (C5 position), leading to the formation of the 5-methylcytosine (5mC) [97]. CpG dinucleotides are the palindromic regions that receive the DNA methylation, making possible the passage of it to the offspring [98]. Nevertheless, several studies revealed that even non-CG methylations have biological significance in various cellular processes [98]. The enzymes that are responsible for the cytosine methylation are the DNA methyltransferases (DNMTs) [88]. Methylation patterns change with aging. Generally, with aging CpG islands of particular genes get hyper-methylated, while repetitive elements of the genome and non CpG regions tend to be hypomethylated [88]. Within particular CpG island the age-related methylation or global methylation patterns reveal tissue specificity [99], [100]. There are two kinds of mechanisms that cause the methylome alteration with aging: programmed changes conducted by the environmental exposure and regulated by specific cell mechanisms, and unpredicted changes that occur with/without the environmental stress [88].

DNA methylations are used to predict the biological age, and is related with the phenotypes of some age-related diseases and all-cause mortality rate [27]. The epigenetic clock that is used for the prediction of the biological age can also be used to see how various interventions accelerate or slow the aging process [101]. DNA methylation can be used in predicting the risk of mortality from cardiovascular disease, Alzheimer's disease, and cancer [88]. It has been shown that interventions that prolong lifespan (such as caloric restriction, loss of insulin/IGF-1 signaling, polyphenols, vitamins and exercise) delay the age-related DNA methylation patterns [88].

2.3.3.3 Chromatin Remodeling

Chromosomal proteins (e.g. heterochromatin protein 1α (HP1α)) and chromatin remodeling factors (e.g. Polycomb group proteins; the NuRD complex) demonstrate decreased levels with aging, and participate with histone- and DNA-modifying enzymes, and together with other epigenetic actors determine the chromatin architecture [2]. Loss of heterochromatin has been used for a long time to explain aging [102]. Heterochromatin is really important in repressing several mobile endogenous transposons (e.g. retrotransposons), that can cause mutation in the genome and insert DNA in random regions in the genome [103]. On the other hand, there is a specific heterochromatin state which increases with age, and is called the senescence associated heterochromatin foci (SAHF) [104]. SAHF appears in old but non-senescent cells [105], but also it occurs after the chromatin recondensation in a non-stochastic way [106], [107]. Chromatin and histone modifications are affected by the longevity interventions. CR can prevent the heterochromatin loss [108], modify the histone marks [109], prevent the expression of retrotransposons [110], induce sirtuins [111] etc. Furthermore, rapamycin treatment (mTORC1 inhibition) that leads to extended lifespan in yeast may act partly through the chromatin remodeling [88].

1.3.3.4 Transcriptional Alterations

Transcriptional changes that happen with age, are found to be related with crucial components of mitochondrial, lysosomal, and inflammatory pathways; non-coding RNAs [2]. It has been showed in studies that small RNAs are obligatory for the interventions in order to promote the longevity, and for the healthspan and lifespan regulation [88].

2.3.4 Loss of Proteostasis

Protein homeostasis or proteostasis system is responsible for the generation, the maintenance of the protein fold and the remove of proteins [112]. The proteostasis system includes more than 2000 chaperones and degradative components as well as their specific signaling pathways [112]. Signaling pathways which modulate the proteostasis network consist of: unfolded protein response (UPR), ubiquitin-proteasome system (UPS), the heat shock response (HSR),

inflammatory pathways and Ca^{2+} sensing [112]. Protective degrative pathways consist of the autophagic-lysosomal-endosomal pathways and UPS [112].

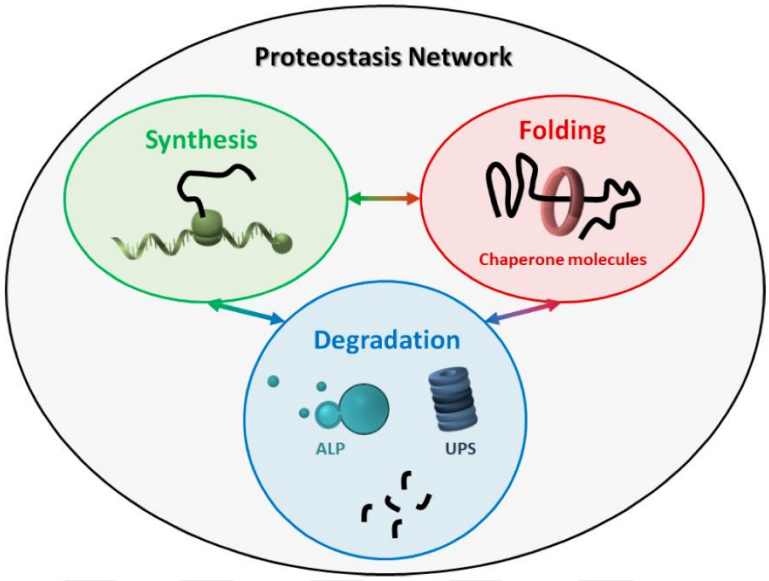


Figure 2.5 Proteostasis network [113]

As proteostasis system declines with aging damaged and misfolded proteins accumulate in cells, leading to diminished cellular viability and the development of diseases that are related with the protein misfolding (e.g. Huntington's and Alzheimer's) [28]. Proteostasis network can be modulated by metabolic signaling pathways mediated by dietaryrestriction, insulin/IGF-1 signaling, reduced mitochondrial function, leading to a longer maintenance of youthful proteome and inhibition of the age-related diseases [28].

2.3.4.1 Chaperone-Mediated Protein Folding and Stability

The production of cytosolic or organelle chaperones is decreased with aging [2]. Chaperones can operate alone or in different combinations with cochaperones in order to regulate the interactions, disaggregation, folding, degradations and trafficking of the proteins within the cell [114].

2.3.4.2 Proteolytic Systems

In the cases when the proteins cannot be aggregated or misfolded, chaperones redirect them to the degradation pathways, in proteosomes or lysosomes where they can be degraded individually or in masses, respectively [114]. Proteasomes

are complexes consisting of 20S proteolytic core and 19S regulatory cap [115]. The 19S regulatory subunit identifies the ubiquitylated substrates, removes the ubiquitin chains, unfolds the protein to permit the 20S core entry where it will degrade into peptides [114]. Proteosome is the main protein degradation source in the cell, but cannot degrade very large or unfolded protein complexes [116]. Substrates such as big inclusions are directed to lysosomes for degradation, organelles that contain a variety of nonspecific proteases [117]. Lysosomes are the last step of autophagy [114], which itself is controlled by mTORC1 and mTORC2, integrating the proteostasis network with the cell's metabolic state, organisms' nutritional status and the protein synthesis rates [117].

2.3.5 Deregulated Nutrient-Sensing

2.3.5.1 The Insulin and IGDF-1 Signaling Pathway

Insulin-like growth factor I (IGF-1) is a polypeptide hormone that is produced in liver as a response to the stimulus of endocrine growth hormones (GH), and also secreted by various tissues with the paracrine/autocrine effect [118]. IGF-1 is responsible for the GH activities, but also it has anabolic, anti-inflammatory, cytoprotective and antioxidant actions [118]. Some of the IGF-1 deficiency conditions include: Lason Syndrome (in children), intrauterine growth restriction, liver cirrhosis, and aging and age related-neurological and cardiovascular diseases [118]. Insulin-like growth factor 1 and 2 are two active substances in human serum and were named like this because of their resemblance to the proinsulin [119]. GH's mitogenic and anabolic activities are mediated by the IGF-1 [119]. 99% of plasma IGFs are complexed to binding proteins [119]. 80% of the circulating IGF-1 is bonded to IGFBP-3, but there are 5 other binding proteins [119]. IGFs stimulate growth, decrease glucose levels in blood, and IGF-1 and 2 differ in the receptors they bind to and activate [120].

It has been showed that insulin and insulin-like growth factor (IGF) signaling (IIS) pathway has an essential role in longevity. In C. elegans, even single mutations that reduce insulin/IGF-1 signaling can expend lifespan more than twofold, and the similar result are also seen in the case of D. melanogaster [31]. Even in various mouse models, the decrease in insulin or GH/IGF-1 signaling leads to prolonged

lifespan [31]. In mammals, insulin binds to IGF-1 and IGF-2 receptors with various affinity [31]. The findings have been contradictory when it comes to humans. In humans the defects in GH/IGF-1 networking have been related with growth defects and enhanced cardiovascular diseases' risk [121]. On the other hand, the Italian centenarians are found to be rich in the PI3KCB and IGF-IR gene combination, which leads to a low IGF-1 plasma level [122].

2.3.5.2 Other Nutrient-Sensing Sytems: mTOR, AMPK and Sirtuins

The proteins that belong to the sirtuin family are one of the most important targets when it comes to anti-aging approaches [33]. mTOR pathway is a component of the phosphatidylinositol 3-kinase (PI3K) pathway, a lipid kinases family that is implicated in cell proliferation, growth and survival [123]. mTOR, being a serine-threonine kinase controls several cell functions, such as transcription and translation, cell growth, and autophagy (the degradation of the cell's components in lysosomes) [123]. mTOR pathway is activated by the signals of growth factor and nutrients [123]. mTOR has two multiprotein complexes: mTOR complex 1 and 2 (mTORC1 and mTORC2 respectively) [123]. The mechanistic target of rapamycin (mTOR) synchronizes the metabolism and growth of eukaryotic cells with growth factors and nutrients [124]. It takes the name from the fact that is the target of rapamycin-FKBP12 complex [124]. The production of nucleotides, proteins and lipids must be increased for a cell to normally grow and divide, and meanwhile the catabolic pathways (e.g. autophagy) must be suppressed, and mTORC1 has an essential role in these processes by controlling the anabolism and catabolism balance towards the environmental circumstances [124]. mTORC1 promotes the protein synthesis, promotes nucleotide and de novo lipid synthesis, and promotes a shift in the glucose mechanism, regulates the protein turnover (suppresses protein catabolism) [124]. mTORC2 regulates the cell survival and proliferation, by phosphorylating the AGC members of the protein kinases family [124]. The increase of lifespan firstly was made possible by the inactivation of protein kinase, target of rapamycin (mTOR) in yeast[125]. mTOR can be activated by particular growth stress and growth factors (e.g. oxidative stress, temperature etc.), insulin and leucine amino acid, and inhibited by DNA damage, caffeine and hypoxia[125].

AMP activated protein kinase (AMPK) senses the energy status of the cell, and is expressed in nearly all eukaryotic cells [126]. When the energy state is decreased in a cell, AMPK activation restores its balance by accelerating the catabolic processes that produce ATP, and blocking the anabolic processes that consume it [127]. AMPK plays an really important role in the regulation of the cell's metabolism, homeostasis, cell growth, survival and death, stress resistance, and autophagy- some of the crucial lifespan and aging determinants [32]. Several studies suggest that AMPK activation and responsiveness declines with age, explaining the modified metabolic regulation leading to oxidative stress and reduced autophagy of unnecessary or harmful products [32]. Caloric restriction effects senescence by decreasing the oxidative damage and enhancing autophagy [128]. AMPK activation in D. melanogaster's gastrointestinal tract leads to a 30% increased lifespan [129]. In rats the AMPK activation (induced by the caloric restriction) protects from senescence via enhancing the autophagy and decreasing the oxidative damage [128], process which is partly performed by AMPK-FOXO signaling pathway as seen in C. elegans studies [130]. The transcription factors family FoxO regulate a variety of crucial biological processes, like cell cycle progression, apoptosis, oxidative stress resistance, senescence, differentiation and metabolism [131], [132], [133]. Today, there are more than 100 natural products that modulate the AMPK activity, such as metformin, salicylate, berberine etc. [127].

Sirtuins are members of class III histone deacetylases (HDAC), and this class' enzyme activity is depended on NAD+ and controlled by the NAD+/NADH ratio and NAD+ levels [33]. Sirtuins also deacetylate other cytoplasmic proteins and transcription factors [33]. The proteins that belong to the sirtuin family are one of the most important targets when it comes to anti-aging approaches [33]. The human sirtuin family includes seven members, SIRT1-7, which have mono-ADP ribosyl transferase or deacetylase activities [33]. Nearly 40% if the cells of mice that lack both copies of SIRT1 gene were found to have damaged chromosome structure, such as disorganized chromatin and breaks, compared with 5% in normal mice [134]. SIRT1 is also found in peri centromere regions and telomeres, and oxidative stress can block this interaction leading to modified gene expression

[135], [136]. SIRT1 also regulated the expression of histones, and regulates the activity and level of various histone modifying enzymes [137]. On the other side, SIRT2 takes part in the metaphase chromosome formation through the H4K16 deacetylation [138]. Its levels, reach the at M phase and the G2/M transition during the cell cycle [138]. Mitotic exit can be delayed because of SIRT2 overexpression [139]. SIRT3 is the most important mitochondrial deacetylase, and has a crucial part in these organelles' homeostasis, and in the anti-oxidative defense [33]. SIRT3 deacetylates H4K16 and H3K9, this way regulating the expression of genes that are engaged in the mitochondrial metabolism and biogenesis [140]. It deacetylated the mitochondrial complex I and III, leading to an increase in electron transport efficiency which is associated with the prevention of ROS production [141]. SIRT6 deacetylates H3K9 in several promotor regions, among which are the genes that take part in metabolism [142]. SIRT6 also protects from telomere dysfunction [143], and the dynamic of telomere binding is highest in the S phase (in cell cycle) [144]. H2BK12 and H3K56 are also substrates of SIRT6, whose enhanced acetylation level is related with genome instability [145], [146]. SIRT7 cooperates with the transcribed regions and promoter of the rDNA genes, and is related with the oppression of tumor suppressor genes [33]. Sirtuins also modulate the antioxidant enzymes, for example SIRT1 deacetylates FOXO3a leading to an enhancement in the catalase and manganese superoxide dismutase (MnSOD) levels [147]. SIRT5 can increase the activity of SOD1 [148]. When it comes to proteins involved in senescence, sirtuins interact with: p53 (inhibits p53 activity to promote senescence and cell cycle arrest), FOXO family (deacetylation of which increases particular proteins expression that protect against oxidative stress, take part in DNA repair and cell cycle checkpoints; NFκB (inhibits it leading to decreased inflammation); AMPK (increase its activity); mTOR (inhibits it); IGF-1/insulin signaling pathway [33].

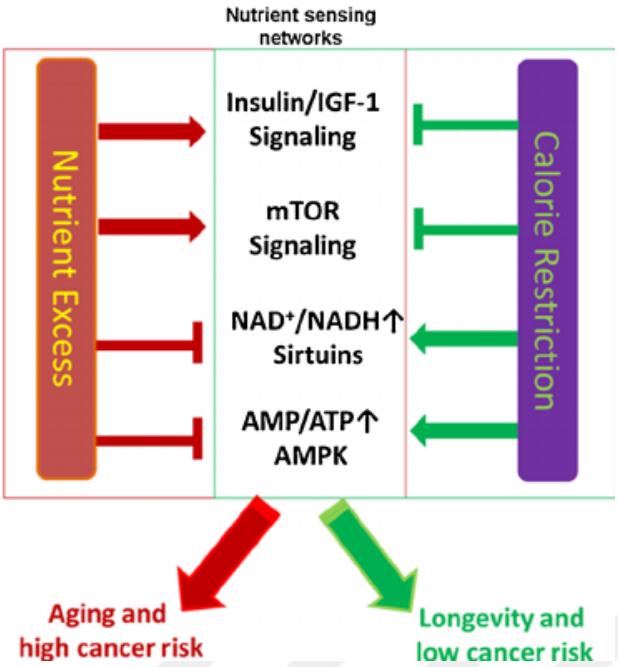


Figure 2.6 Nutrient sensing networks [149]

2.3.6 Mitochondrial Dysfunction

Mitochondria are the key components in the regulation of homeostasis and cellular metabolism, because of their roles in anabolism, catabolism, bioenergetics, calcium and iron homeostasis, generation of ROS, heme and iron-sulfur cluster biosynthesis, signal transduction and apoptosis [150]. The dysfunction of mitochondria is related with aging in these aspects: damaged activity of oxidative phosphorylation (OXPHOS), failure in mitochondrial quality control, enhanced oxidative damage, diminished metabolic enzyme activity, and changes in biogenesis, morphology and dynamics [34], [35]. Mitochondrial dysfunction is also related with several age-related diseases, such as obesity, cancer, and cardiovascular and neurodegenerative disorders [150].

2.3.6.1 ROS

In chemistry a free radical is a molecule that consists of at least one unpaired electron and has negative, positive, or no electrical charge[151]. These molecules are usually extremely reactive [151]. In biology, free radicals are types of unstable molecules that are produced during the normal cell metabolism, which can cause damage to various molecules, e.g. DNA, carbohydrates, lipids, and proteins [152]. “Free radicals” and “reactive oxygen species” terms are usually used

interchangeably, but at times this in incorrect [153]. The cytochrome oxidase in the electron transport chain consumes 90% of the oxygen in aerobic conditions, without ROS release [153]. Less than 10% of the oxygen has its reduction by the one-electron continuous pathways, leading to the formation of the anion radical O2, and followed with the formation of the hydrogen peroxide H2O2, which is not a free radical, but is included in ROS because of its reactivity [153]. At the moment free radicals are commonly named reactive oxygen species (ROS) and reactive nitrogen species (RNS) [154]. ROS includes the superoxide, peroxyl, and hydroxyl radicals, and hydrogen peroxide, while RNS includes the peroxynitrite and nitric oxide [154]. Even though some of these molecules may be harmless at times (e.g. superoxide and nitric oxide) they may produce damaging radicals leading to aging and other pathological disorders, thus at particular conditions the trouble in the ROS and RNS signaling regulation may cause dangerous enzymatic cascades' activation [154]. These free radicals are produces by enzymes, such as xanthine oxidase, nitric oxide synthase and NADPH oxidase, mitochondria, and display signaling functions by activating/inhibiting various enzymes (e.g. MAPK kinases, gene-dependent cascades etc.) [154]. In the time Dr. Hartman developed the free radical theory of aging, free radicals were considered to have a negative impact in the organism, and only formed because of different environmental factors, but never to be metabolites of physiological processes [154]. The free radical theory of aging, or the oxidative stress theory of aging (as later named) claims that the accumulation of the oxidative damage to the cell macromolecules leads to age-related functional loses [155]. In normal physiological conditions ROS signaling effects several enzymes and gene catalyzed processes, but with aging these processes may become damaging, usually because of ROS overproduction, and this may be more harmful than the direct harm of these free radicals [154]. The only real signaling species are: hydrogen peroxide, nitric oxide and superoxide [154]. Several protein kinases, especially the Akt/B protein kinase and the mitogen activated protein kinases (MAPKs), and aging-regulating genes, such as Sirtuin, p66shc, Klotho and FOXO3a play a really important role in the enzyme/gene cascades in which ROS signaling occurs [154]. We can classify the oxidative stress as: mild oxidative stress (MOS), temperate oxidative stress (TOS),

and severe oxidative stress (SOS) [153]. Various endogenous and exogenous processes produce ROS and RNSs, that get neutralized by the antioxidant defenses. It is the imbalance between them that causes the oxidative stress. The exogenous sources of RONS (reactive oxygen and nitrogen species) include alcohol, tobacco, water and air pollution, radiation, industrial solvents, food, drugs and heavy or transition metals, while the endogenous sources include the mitochondria, phagocytic cells, and peroxisomes [156]. Endogenous sources also include angiotensin II, NADPH oxidase, lipoxygenase and myeloperoxidase (MPO) [157]. It has been reported that the oxidative stress is involved in various diseases such as: cardiovascular diseases, diabetes mellitus, respiratory diseases, neurodegenerative disorders, cancer, cataract development and rheumatoid arthritis [156]. It is thought that oxidative stress leads to cellular senescence. RONS induce senescence by affecting different SASP components such as control of mTOR complexes' function, IL-1α production (proinflammatory state), generation of MMPs expression (linked with the chronic and age-related diseases), FOXO (forkhead box transcription factors) proteins' activity inhibition (part of the insulin or insulin-like growth factor-1-mediated oxidative stress protection), p16INK4a/pRB and p53/p21 pathways' regulation (causing senescence) [158], sarco/endoplasmic reticulum Ca^{2+}-ATPase activity's reduction (causing cardiac senescence) [159], sirtuins activity inhibition causing SOD inhibition which leads to enhanced RONS production [160].

2.3.6.2 Mitochondrial Integrity and Biogenesis

Mitochondria produce energy or adenosine triphosphate (ATP) via breakdown of sources (e.g. glucose, fatty acids) during the redox reactions that happen in the electron transport chain (ETC), assisted by five mammalian OXPHOS system's enzyme complexes [161]. During the mtDNA replication or the damage repair, the most common mutations in genome include the deletions and point mutations [150]. Enhanced frequency of mtDNA insertions/deletions have been seen in human and animal models with increasing age [162], [163].

The maintenance of homeostasis and mitochondria necessitates a strong regulation and coordination among new generated and damaged mitochondria

[150]. New mitochondria is synthesized via the mitochondrial biogenesis, while the damaged one is degraded via mitophagy (mitochondria-specific autophagy) [150]. The balance of these processes is crucial for aging and longevity [164], [165], [166]. The rates of mitophagy differ among tissues [167], and as a reaction to intracellular and environmental factors [150]. On the other side, mitochondrial biogenesis is a process that includes the coordination of the transcriptional regulation of mitochondrial and nuclear genomes in order to generate a new mitochondria [166], [168]. Deterioration in autophagy and mitophagy pathways are related with age and age-related diseases, such as cancer, kidney failure; cardiac, immune system and neurodegenerative disorders; hepatic dysfunction [34], [169], [170], [171], [172].

2.3.7 Cellular Senescence

The word senescence derives from the Latin word “senex”, meaning old age or old man and is used interchangeably with aging [173]. Senescence is also called replicative senescence or cellular aging, while cells that are not senescent are called ‘young’, pre-senescence, proliferating or early passage [173]. Based on proliferation, we can classify cells as mortal (with limited replication number) and immortal (with unlimited replication lifespan) [173]. The primary cells that are explanted from human tissues have a limit of proliferation capacity, which exhibits three phases: I- the little proliferation while the culture establishes (before the 1st passage); II- the fast proliferation; III- proliferation decreases till a stop [174]. In 1965, Hayflick came with the hypothesis that “The finite lifetime of diploid cell strains in vitro may be an expression of aging or senescence at the cellular level.” [175]. So senescence means that there is a long-term loss of the cells capacity to proliferate, even though metabolic activity and viability continues [36].

Cells continually experience endogenous and exogenous stresses. The response to these stresses differs from recovery to cell death. One of the theories of aging is the antagonistic pleiotropy, which defines the processes that have both beneficial and harmful effect on the organism, beneficial for young and harmful for the old ages [173]. The types of cells in complex organisms include the mitotic and post-mitotic cells [173]. Mitotic cells proliferate, or spend particular intervals in the

quiescence (G0) state, in which a growth arrest is experienced till particular physiological signals stimulate the proliferation [173]. On the other hand, the post-mitotic cells have no dividing ability, and arrest is irreversible leading to apoptosis resistance and altered gene expression, both features of the senescent phenotype [173]. The senescent phenotype includes the patterns of changes in cell structure, behavior and function that associate cellular senescence [173].

Proliferating cells respond to cellular stress (mostly DNA damage) can start a program that results in senescence, which means permanent cell cycle arrest. Triggers such as telomere dysfunction, activated oncogenes, DNA-replication stress, cell-cell fusion and oxidative stress lead to short-term or log-term senescence [176]. Short-term senescence includes tumor suppression, embryonic development and limits tissue damage, while long-term senescence includes tissue aging and tumorigenesis [176]. Senescence takes part in four opposing biological processes: tissue repair and aging; tumor suppression and tumor promotion [177]. The mechanisms and biomarkers of cellular senescence include cycle cell arrest, morphological transformation, ROS, DNA damage-induced senescence, senescence-associated heterochromatic foci (SAHF), activation of tumor suppressor networks, induction of senescence-associated β-galactosidase (SA-β-GAL) activity, autophagy, and secreted factors [36]. Senescent cells with constant DNA damage response signaling harbor DNA segments with chromatin alterations reinforcing senescence (DNA-SCARS), and secrete cytokines, growth factors, proteases, and various factors with effective paracrine and autocrine activities [177].

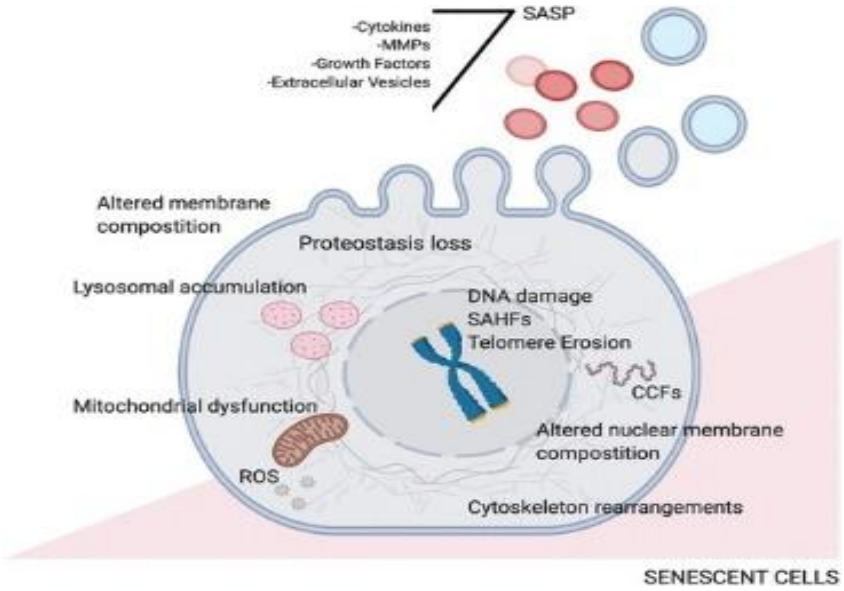


Figure 2.7 Senescent cell hallmarks [178]

The size of senescent cells increases, at times twofold compared to the non-senescent ones [175]. Physiological stimuli cannot reverse senescence [177]. Senescence is not static, but it has phenotypically different cellular states following the first growth arrest [179]. Senescence include changes in chromatin and secretome, and activation of tumor-suppressor [179]. Replicative senescence is related with telomere attrition, and it was shown that the expression of telomerase extends the cell doubling (lifespan) [180].

The growth arrest takes place in G1 phase, and even though individual cells experience arrest quickly, cell cultures are usually asynchronous and experience arrest some weeks later [25]. Two categories of the replicative cellular senescence include the response mediated by p53 tumor suppressor pathway, and the CDKN2A upregulation [25]. Senescent cells have the ability to change the tissue microenvironment, such example are the skin fibroblasts which during senescence secrete degradative enzymes, growth factors, and inflammatory cytokines, leading to altered skin morphology [25]. p16 expression is low in young organisms, but enhances with age, for example p16 upregulation happens in aging human skin [25]. p16 inactivates CDK4 and CDK6, leading to the arrest of cell cycle in the G1 phase [25].

Premature senescence in vitro can be grouped in oncogene-induced senescence (OIS), stress-induced senescence and tumor suppressor loss-induced senescence [36]. Stress-induced premature senescence (SIPS) can be activated by chemotherapeutic drugs, UV light, radiation and ROS (intracellular and extracellular), which is not depended in telomere length (just like oncogene-induced senescence) but in DNA damage repair (DDR) [176].

Two are the key approaches to target senescent cells: the SASP inhibition and the senescent cells specific elimination [37]. Strategies we can use to eliminate or prevent senescent cells include the enhanced immune response, increased replicative potential, cellular reprogramming/reversion and pharmacological cell death induction [176]. The age-associated deficiency in the antioxidant and mitochondrial enzyme systems can result in the enhancement of the ROS-related damage [181]. Anyway, it is still not clear if SIPS has a role in the physiological aging, as seen in cases when increased particular antioxidant enzymes production does not prolong lifespan in mice [182]. The elimination of the senescent cells can be the cure of various age-related diseases and prolong the healthspan. It was shown that the removal of $p16^{Ink4a}$- positive senescent cells delays the onset of the age-related disorders or reduce their progression (when removed late in life) [183]. Also, the elimination of senescent beta cells was shown to prevent the type 1 diabetes (T1D) [184].

Senescent cells are also detected in vivo, and in rodents, primates and humans these cells are found in various renewable tissues, such as the vasculature, hematopoietic system, stroma and several epithelial organs [173]. One of the things that aged organisms have in common is the accumulation of the senescent cells, which make the onset and the progression of the age-related diseases more favorable [37].

Correlations exist among senescence (limited proliferative capacity) of somatic cells and the physiology of an entire organisms, which makes it a suitable system for studying human aging. The in vitro indicators of aging include the cellular biomarkers and the morphological changes while cells age. The most common morphological changes associated with replicative senescence include the increase

in the cell, nuclear and nucleolar size; increased number of multinucleated cells, vacuoles in cytoplasm and endoplasmic reticulum, cytoplasmic microfilaments, prominent Golgi apparatus and large lysosomal bodies [185]. It is thought that the increase in cell and nuclear size, and numbers of inclusion bodies is caused by the increase of intracellular RNA and protein content of the cells, caused by the decreased protein degradation by the proteosome-mediated pathways, reduced RNA turnover, the disconnection of the cell growth and cell division and the block in the G1 phase of the senescent cells [185]. Furthermore, senescent cells display an enhanced sensitivity to cell contact, probably because of the differences in interaction with the extra-cellular matrix or secreted proteins' expressions, leading to decreased harvesting [185]. Cellular biomarkers includes differences in the expression of IGF-1, EGF, c-fos; enhanced activity of SA-β-Ga, enhanced formation of SAHF and promyelocytic leukemia protein nuclear bodies (PML NBs), inflammatory secretome, chromosomal instability and long-lasting DNA damage, enhanced cell cycle time (since G1 intervals become longer) [185].

2.3.8 Stem Cell Exhaustion

Stem cells' life-long persistence leads in the susceptibility of cellular damage accumulation, which can cause senescence, cell dearth ot regenerative function loss and this leads to decreased effectiveness of tissue regeneration and replacement of cells in an aged organism [186]. For example, the number of stem cells that provide the blood formation decreases with age [85].

2.3.9 Altered Intercellular Communication

Inflammaging results from various causes, e.g. dysfunctional immune system and dysfunctional host cells, the secretion of the pro-inflammatory cytokines from the senescent cells, defective autophagy response, increase of pro-inflammatory tissue damage and the increased stimulation of the NF-κB transcription factor [2]. Inter-organ aging coordination can also be explained with the induce of senescence by the gap junction-mediated cell contact among senescence cells and normal senesce, and ROS involving processes [2]. Nutritional, pharmacological, or genetic interventions can improve the cell to cell communication elements that are affected by aging [2].

2.4 Skin Aging

2.4.1 Skin Anatomy

The skin is the largest organ in our body. It is a sensory organ that also protects our body from various mechanical, chemical, or physical factors; prevents moisture loss; produces the D vitamin; regulates the body temperature; acts as an immune organ to discover infections. The skin consists of three tissue layers: epidermis, dermis, and hypodermis.

Epidermis is the outer layer of the skin, avascular and very cellular. It has four layers itself: stratum corneum, stratum granulosum, stratum spinosum and stratum basale [187]. Also, in the palms of our hands and the heel the layer of stratum lucidum is found between stratum granulosum and corneum [187].

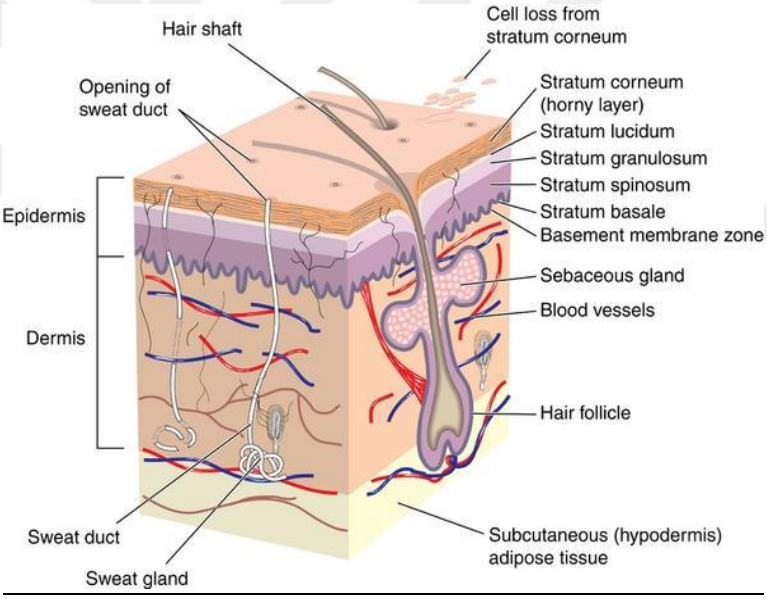


Figure 2.8 Skin layers [188]

-Stratum basale (stratum germinativum)- the lowest layer which via collagen fibers attaches the epidermis to basal lamina (under which lies the dermis) [189]. Basal stem cells of this layer produce the keratinocytes, Merkel cells senses the stimulations that are perceived as touch in the brain, and melanocytes produce the melanin pigment (which gives color to the hair and skin and protects from UV radiation) [189].

-Stratum spinosum- 10 layers of keratinocytes which via the desmosome structure, and among which Langerhans cells are found, which function as macrophages (for foreign particles, damaged cells or bacteria) [189].

-Stratum granulosum- 3-5 layers of keratinocytes, which become flatter with thicker cell membranes and produce large volumes of proteins (keratin-fibrous, keratohyalin-within cells granules) [189].

-Stratum lucidum- found only in the thick skin (palms, soles and digits), composed by flattened and dead keratinocytes which are packed in eleidin (protein rich in lipids) [189].

-Stratum corneum- dead and dry layer exposed to the outer environment with 15-30 layers of cells, which shed regularly (the layer is replaced in about 4 weeks) [189].

Dermis is the second layer composed of a dense extracellular matrix (ECM), which supports the nerve cells, hair follicles and sweat glands and the dermal vasculature [187]. ECM is primarily populated by fibroblasts that synthesize the structural matrix composed of collagens, elastic fibers, hyaluronic acid and proteoglycans [187].

Hypodermis mainly includes fatty acids (responsible for the nutrition storage, thermoregulation, protection and insulation), and white adipose tissue (pre-adipocytes, adipocytes, fibroblasts and macrophages) [187]. Nerve endings and hair terminate in this layer [187].

Skin aging is the most clear sign of the aging process, and furthermore skin can serve as an indicator of the systemic diseases, such as osteoporosis, metabolic diseases, neurodegenerative diseases and the cardiac surgery outcomes [190]. Skin protects the organism from several deleterious environmental agents, and is essential in maintain the electrolyte, fluid and temperature balance [190]. Furthermore, skin is responsible for the biosynthesis, processing and the metabolism of several structural proteins, lipids, and glucans, which makes it a classic endocrine organ [190]. Downregulation of the lipid synthesis processes occurs in both chronological and photoaging [190]. Downregulated genes include those engaged in fatty acid and cholesterol synthesis, and those linked with the

epidermal differentiation, such as envelop components and keratin filaments [190].

Among continents, and even countries and cities skin aging characteristics vary, such as wrinkling, melanin pigment, sebaceous secretion, intensity, sagging and physiology [190]. Melanin and the photoprotection it provides influences the skin aging variation among various racial groups, and usually Caucasians experience earlier onset and larger wrinkling, sagging and pigmentary problems in their skins [191]. The 'ethnic' and 'race' words are interchangeably used literature, though there are differences among them [191]. The 'ethnic' word stands for a broad group of population with common culture and/or language, while 'race' stands for a specific population according to the genetic similarities [191]. Furthermore, another classification system includes the one of the skin phototype (SPT), which classifies all people, involving those with pigmented skin according their skin responsiveness to UV radiation [191].

2.4.2 Dermal Fibroblasts

Fibroblasts, the most important cells of the connective tissue play a very important role in the stem cells maintenance, fibrosis, repair, healing and tissue remodeling [192]. Furthermore fibroblasts can be group in subtypes according to the regions of the body where they are found, and this heterogeneity (genetic/epigenetic) makes them really important in aging studies [192]. In a study, naturally aged human foreskin cell and children foreskin cell gene expression were compared [193]. It was found that 105 genes' expression changes, 62 upregulated and 43 downregulated genes, related with cycle control, signaling, cytoskeletal changes, metabolism, and inflammatory response [193]. The genes we chose to study are: HAS2, IGFBP2, WNT16, GAPDH, ANLN, and CCNB1.

The HAS2 gene synthetizes the hyaluronan synthase 2 protein, which is involved in the hyaluronan biosynthesis (part of Glycan biosynthesis) and it is expressed in fibroblasts. The protein catalyzes the GlcNAc or GlcUA monosaccharides addition to the developing hyaluronan polymer, playing an essential role in extracellular matrices (regulating this way the cell adhesion, differentiation and migration) (UniProtKB identifiers: Q92819). 2 isoenzymes (HA synthases) are coded by 3

different genes, but HAS2 gene is the most important one [194, p. 2]. HA synthase 2 is a multipass transmembrane protein located in the plasma membrane and is responsible for hyaluronan deposition in the extracellular matrix [194, p. 2]. As a member of glycosyl transferase 2 protein family it catalyzes the UDP-esterified residues' addition, forming the disaccharides (glucuronic acid and N-acetylglucosamine) that are present n the HA molecule [194, p. 2]. Hyaluronan in the extracellular matrix is related with really important cellular processes, such as morphogenesis, cell division, and motility [194, p. 2]. Mutations disrupting HAS2 during development resulted embryonic lethal [194, p. 2].

IGFBP2 gene is responsible for the insulin like growth factor binding protein 2. IGFBPs modulate the IGFs ability to trigger the IGF-I receptors, but some also are IGF-independent and translocate into the cell [40]. IGFBP2 is the second among most abundant proteins that bind IGF [40]. In the 20-year longitudinal study of van den Beld et. al. was found a link between mortality and IGFBP-2 levels; serum levels which enhance after the age of 50 and parallelly progress with the insulin sensibility. IGFBP-2 serum levels can be used in the mortality prediction in an aging population. A variety of studies suggest that concentrations of the insulin-like growth factor 1 (IGF-I) and the growth hormone (GH) decline with age.[40]

Wnt family member 16 (WNT16) is responsible for Wnt-16 protein, a probable developmental protein [195, p. 16], [196, p. 16]. Wnt family's genes encode secreted signaling proteins, and play an important role in various developmental processes, such as embryogenesis, cell fate regulation and also oncogenesis [197].

Cyclin B1 (CCNB1) gene encodes the G2/mitotic-specific cyclin-B1 protein, which is crucial for the control of G2/M transition (mitosis) [198, p. 1], [199, p. 1].

ANLN gene (anillin actin binding protein) encodes anillin, which is needed for cytokinesis, and also plays a role in metaphase and anaphase of mitosis [200], [201].

GAPDH encodes the glyceraldehyde-3-phosphate dehydrogenase, which plays a role in glycolysis and nuclear functions (DNA replication, transcription, RNA transport and apoptosis) [202], [203].

3
ANTIOXIDANTS AND CURCUMIN

3.1 Antioxidants

Aerobic organisms have an antioxidant defense system, which contain series of enzymes, such as catalase (CAT), superoxide dismutase (SOD), glutathione reductase (GR) and glutathione peroxidase (GPx) [204]. Thus, there are the dietary antioxidants, such as: ascorbic acid, vitamin A and C, flavonoids etc., which are seen to extend the lifespan in fruit flies [204]. The free radical reaction have 3 major stages: initiation, propagation and termination [204]. ROS play a really important role in differentiation, proliferation and the host defense response, but also can cause DNA damage and protein/lipid oxidation [204]. The antioxidant systems are enzymatic and nonenzymatic [204]. The enzymatic ones contain the SOD, CAT, GPx and GR enzymes, while the nonenzymatic antioxidants serve as second defense against free radicals [204].

Antioxidant defense also includes endogenous and exogenous molecules. The endogenous ones consist of both enzymatic and non-enzymatic pathways [159]. The most important enzymes are SOD, glutathione peroxidase (GSH-Px) and catalase (CAT) [159]. For example, SOD coverts O2 to H2O2, which is later turned to oxygen and water by CAT inhibiting the production of the hydroxyl radicals; GSH-Px turn hydroxyl radicals and peroxides in nontoxic forms [159]. The nonenzymatic antioxidants include β-carotene, bilirubin and vitamin E (α-tocopherol) in blood, and uric acid and albumin with 85% of antioxidant function in plasma [205]. Exogenous antioxidants include the vitamin E (preventing the lipid peroxidation of the cell membrane), vitamin C (ascorbic acid- scavenges the superoxide and hydroxyl radicals), zinc, selenium, several drugs, oil lectins and phenolic antioxidants (phenolic acids, flavonoids and resveratrol) [206].

One of the aging theories is the oxidative stress theory, which supports the idea that the ROS overproduction and lack of antioxidants lead to DNA and cell membrane damage [125]. Data show that diets rich in antioxidants and anti-inflammatory agents, such as Mediterranean and Okinawa-type diets positively impact the longevity by decreasing the oxidative stress and influencing the epigenetic factors [125]. Dietary and endogenous antioxidants are part of the antioxidant defense by reducing free radicals, DNA damage and inhibiting the lipid peroxidation in cells [125]. Diets rich in flavonoids and antioxidants can modulate gene and protein expression, modifying homeostasis and the endogenous metabolic pathways, and inducing epigenetic changes[125]. Telomeres are also effected by antioxidants and supplements such as curcumin, resveratrol, vitamin B, D, E, and C [125].

Furthermore aging and diet influence the inflammatory status of an organism by the processes of inflammaging and meta inflammation, thus aging is associated with oxidative stress and changes in inflammatory processes [207]. Inflammaging is the cause of a chronic and systemic inflammation associated with age-related pathologies, such as diabetes, cancer etc., pathologies which share excessive levels of inflammatory cytokines and mediators [207]. Macrophages and adipocytes get stimulated by the surfeit of the circulating fatty acids and obesity, triggers differences in the signal transduction, effecting the pro-inflammatory status [207]. This leads to increases in ROS, pro-inflammatory cytokines and decrease in adiponectin and endothelial nitric oxide levels, leading to the chronic inflammatory status known as meta inflammation, which depends not only in obesity or overweight, but in aging also [207]. Meta inflammation and inflammaging induce the proinflammatory status, which causes senescence and cellular damage [207]. Senescence itself has a worsening impact on the chronic inflammatory status since the senescence cells produce various proteases, growth factors, pro-inflammatory cytokines, and chemokines[207]. “Dietary inflammatory index” was developed in order to measure the influence of specific food regimes on the inflammatory profile of individuals

[207]. Foods can alter the cellular homeostasis and cellular processes, such as proliferation, senescence and apoptosis by their inflammatory capacity [207]. For example, high inflammatory index foods are associated with senescence and oxidative stress, while low inflammatory index foods decrease the oxidative stress due to the antioxidants they contain [207].

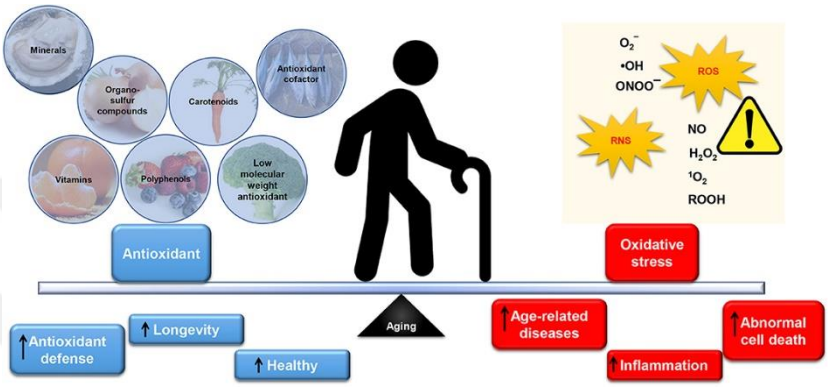


Figure 3.1 Oxidative stress and antioxidant balance effect on aging [208]

There is no evidence of a “death gene”, but 25% of lifespan variations can be related with heredity, and these genetic factors may influence aging via their impacts on somatic maintenance [209]. The other 75% of variation is thought to be because of the stochastic events and environmental responses [209]. The aging phenotype is the product of the damage accumulation in cells’ macromolecules, and this leads to the idea that nutrients decrease aging must reduce this amount of damage by increasing the cell, tissue or organism ability to deal with this damage [209]. The plasticity of the aging process is supported by date from the experiments with pharmaceutical agents (e.g. rapamycin), thus is obvious that modulating main pathways to nutrient sensing, cellular defense and energy is essential to the aging process [209]. Furthermore, there is data showing that nutritional intake impacts the DNA repair, which may explain the inter-individual variety in DNA repair ability in humans [209]. Such example of the diet-aging link is the Mediterranean diet, which is associated with increased risk of various age-related disease [209].

ROS generation, when in specific boundaries is crucial to maintain the homeostasis, but an increase in the intracellular antioxidant levels can activate various signaling pathways and damage the cell components, gaining the ability to influence aging and the age-related diseases [210]. ROS species are produced exogenously or generated intracellularly from various sources [210]. Oxidative stress is influenced by the systems of cytosolic enzymes, such as NADPH oxidases (a system that produces superoxide's) [210]. NADPH oxidases either trigger senescence or the transformation of cellular components, because different NADPH oxidase family members can trigger widely different biological results which emphasizes the complication in regulating the cellular oxidant response [210]. Influencing factors may be: cell type; the level and duration of the production of oxidants; ROS species that are produced and the particular intracellular ROS production [210]. Most of intracellular ROS generation comes from mitochondria [210]. The mitochondrial radicals of superoxide get produced firstly at two points of the electron transport chain: at complex I (NADH dehydrogenase) and complex III (ubiquinone-cytochrome c reductase), which is the main site for ROS generation in normal metabolic circumstances [210].

Diseases that can be prevented/modified by nutrition are some of the biggest causes of deaths in developing and developed countries, diseases such as cancer, heart-related problems, stroke, hypertension, Alzheimer's, type 2 diabetes, depression, osteoporosis, arthritis, macular degeneration, cataracts, infectious diseases and pulmonary complications [211]. Longevity-related genes have been identified, genes that can alter lifespan and health in several organisms (such as yeast, worms, flies, rodents), and some of these genes are part of the nutrient sensing pathways (sirtuin, target of rapamycin, and insulin/insulin-like growth factor-1-like signaling pathways) [211]. Cellular amino-acid, NAD+/NADH and glucose levels are sensed by these pathways [211]. Nutraceuticals include dietary supplements, medical food and functional foods, which provide health/medical benefits [211]. According to the chemical composition they can be classified as: isoprenoid derivatives, phenolic compounds, carbohydrate derivates, structural lipids and fatty acid, amino acid derivatives, microbes and minerals [211]. Foods rich in polyphenol can relieve the

aging symptoms with their anti-oxidant and anti-inflammatory properties [46]. Curcumin prolonged the lifespan of nematodes, fruit flies and mice when taken as a diet supplementation [212], [213], [214], [215].

3.2 Curcumin

Turmeric (Curcuma longa) is a plant in the Zingiberaceae family, with the origin in India, though at the moment being grown in Latin America, China, and Southeast Asia [45]. Turmeric or as called 'Indian saffron' has been used since year 4000 bc, and its name is found in the old Indian system of medicine (Ayurveda), and its benefits were recognized by the Greco-Roman, Middle East and Egyptian regions [216]. More than 40 species of the Curcuma genus are native in India, while there are 70-110 species of this genus all over tropical Asia, and at times the species show huge diversity according to the places they grow [216]. Turmeric is coded "E 100" when used as food additive, e.g. in beverages, ice cream, dairy or baked products, popcorn, gelatin, mustard, cakes, sweets, and pickles [216]. 80% of the worlds Turmeric is produced in India (which also is the major exporter and consumer), followed by Thailand, Taiwan, China, Pacific islands, Nigeria, Bangladesh and South America etc.[216].



Figure 3.2 Curcuma Longa [217]

In modern medicine nutraceutical characteristics of turmeric are broadly recognized, with its role in arthritis, wound healing, inflammatory bowel disease, respiratory

disorders, gastrointestinal disorders, Alzheimer, cosmetology and antioxidant, antimutagenic, antithrombotic, antimicrobial, chemo preventive, anticarcinogenic, bioprotectant, antidiabetic, antiangiogenic, hepatoprotective and antimicrobial properties [216]. Turmeric (Curcuma longa) includes 3 curcuminoids: demethoxycurcumin, bisdemethoxycurcumin, and curcumin which is the most widespread bioactive ingredient in this plant [44]. Curcumin makes up 60-70% of the turmeric extract, is responsible for the yellow pigment, and is the main curcuminoid with its health-promoting abilities [44].

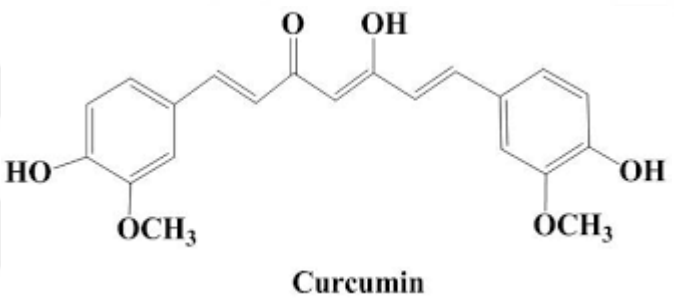


Figure 3.3 Molecular composition of curcumin [218]

Curcumin (1,7-bis-(4-hydroxy-3-methoxyphenyl)-hepta-1,6-diene-3,5-dione) gained a lot of attention in the last decades because of its anticancer, anti-aging, antibiotic and anti-inflammatory effects [45]. In 2019 the value of the global market size of curcumin was 58.4 million $, and is expected to reach the value of 7.9 million $ by 2027, since it is being widely used in skincare applications, medical products, food, tissue engineering, textiles, dye-sensitized PV technology [219].

Several research resulted in the improvement in the bioavailability of curcumin, like inclusion of piperine, encapsulation with turmeric's essential oils (especially turmerone), and the use of various delivery systems, such as micelles, liposomes, microemulsions, lipid particles, nanoparticles and biopolymer particles [44]. The amount of turmeric powder added to foods it is not enough to furnish health benefits for conditions such as, arthritis, metabolic syndrome and type 2 diabetes mellitus, other new formulations and curcuminoid extracts must be used to provide the right dose intake [44].

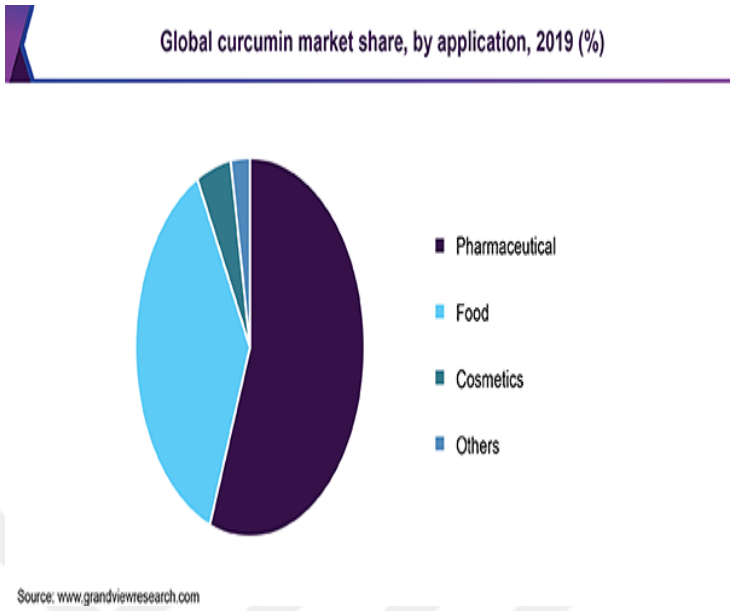


Figure 3.4 The global curcumin market share [219]

Curcumin first was isolated in 1815, by the German scientists, Vogel and Pelletier, though the first study in its bioactivity is registered in Nature, in 1949, while the first clinical trial in 1937, in The Lancet [48]. It has been found that it has anti-inflammatory activity by suppressing several cell signaling pathways, such as ROS, Nrf2, COX-2, STAT3 and NF- κB [48]. There is evidence that curcumin also synergizes with nutraceuticals, such as piperine, resveratrol, catechins, genistein and quercetin [48]. Unlike 'smart drugs' that affect only a particular target, drugs that affect several signaling pathways are required in complex diseases, like cancer and cardiovascular diseases, and this brings the need to develop multi-targeted agents [48]. Curcumin has: antioxidant, anti-aging, anti-inflammatory, antigrowth, antiarthritic, antidepressant, memory-enhancing, anti-atherosclerotic, antidiabetic, wound healing antimicrobial activities, and further chemo preventive, radio sensitization and chemo sensitization effects [48].

Curcumin has low solubility in water, MeOH and high solubility in chloroform and DMSO[220]. Curcumin has a low stability in physiological conditions, short time in the gastrointestinal tract, low bioavailability, and weak pharmacokinetic profile (absorption, distribution, metabolism, excretion)[45]. To increase curcumin

bioavailability several methods are used, e.g. adjuvants, curcumin nanoparticles, liposomal curcumin, curcumin that is reformulated with different oils and metabolism inhibitors, phospholipid complexes of curcumin, curcumin linked with polyethylene glycol and conjugation of curcumin prodrugs [48]. Curcumin was shown to be not toxic in humans when orally taken up to 8000 mg/day for three months [221].After taking 4000, 6000 and 8000 mg of curcumin, the serum concentrations peaked in 1 to 2 hours with 0.51 +/- 0.11 microM, 0.63 +/- 0.06 microM and 1.77 +/- 1.87 microM correspondingly [221].

Curcumin can modify various biological targets, such as protein kinases, apoptotic proteins, cell cycle proteins, enzymes, growth factors, transcription factors, cytokines and inflammatory pathways [45]. Curcumin has a crucial role in regulating kinases, cytokines, receptors, enzymes, growth factors, apoptotic and metastatic molecules, and transcription factors [222]. Curcumin has anti-photoaging effects, which is caused by the sunlight and especially the ultraviolet radiation, which is associated with an increased production of matrix metalloproteinases (MMPs)[223]. MMPs expression is induced by the suppression of NF-κB and activation of AP-1 transcription factors via MAPK signaling pathway [223]. MMPs cause the collagenous extracellular matrix (ECM) degradation in the connective tissue [223]. Curcumin has pro-antioxidant and antioxidant activities, and is a hydrogen donor and scavenges free radicals, is an iron chelator and connects metals (especially copper and iron) [217]. It has low bioavailability, but is non-toxic [217]. Curcumin, currently in various clinical trials can be a very good therapeutic agent for several conditions, such as pancreatic and colon cancer, multiple myeloma, myelodysplastic syndromes, Alzheimer's disease [217]. Curcumin affects Nrf2 dependent cytoprotective pathways; NF-κB, Akt, and growth factors regulated survival pathways; angiogenic pathways and metastatic pathways [217]. In human skin or in in vitro dermal fibroblast culture ultraviolet irradiation also increases ROS production [223]. Bo-Mi Hwang at al. made their research in foreskin human fibroblasts, and it was concluded that in photoaged cells curcumin reduced the ROS levels; inhibited of MMPs (MMP-1 and MMP-3) [223]. Moreover, curcumin regulates various miRNAs, and plays a role

in the epigenetic changes, since it inhibits DNA methyltransferases and effects the histone acetyltransferases and deacetylases [46]. As seen, curcumin affects different biological processes, for instance proliferation, apoptosis, migration, inflammation, redox state, wound healing, and as a result has a positive effect on aging, age-related diseases, and memory [46]. Some studies suggest that curcumin can postpone senescence, while others think it just effects the nutrient-sensing signaling pathways (e.g. sirtuins; AMPK) [46].

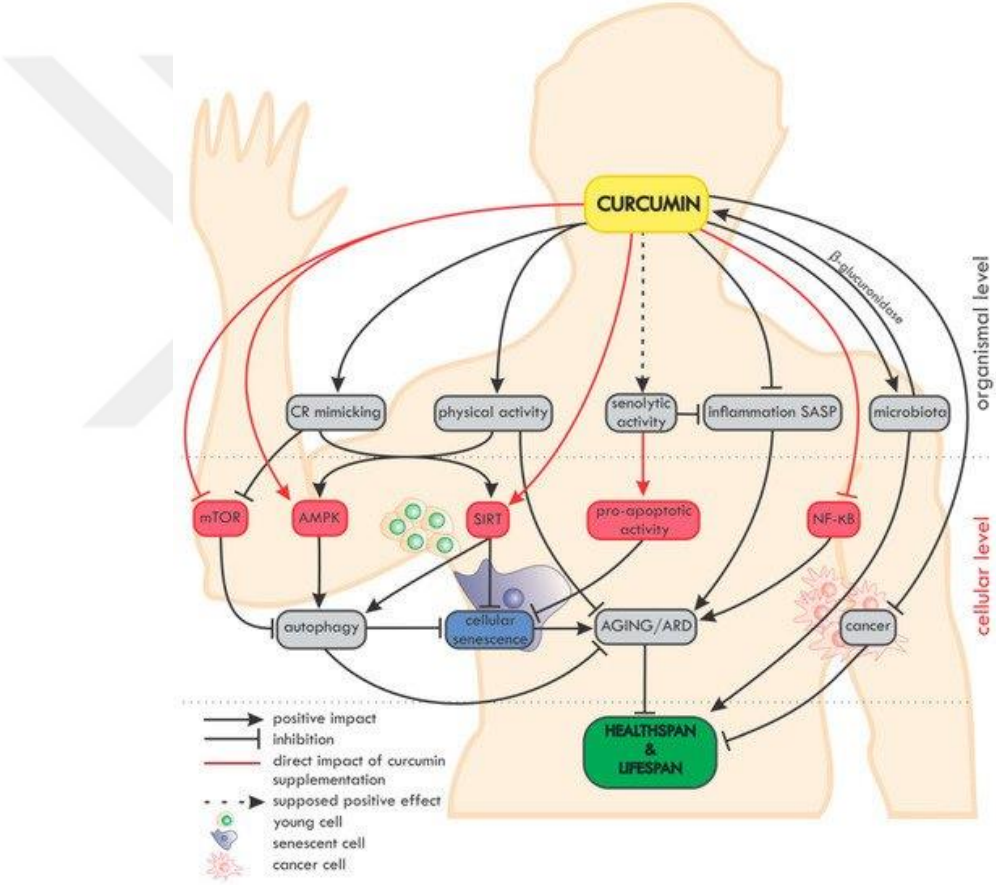


Figure 3.5 Curcumin effect on human body [46]

Curcumin known for its antiaging, antioxidant, anti-inflammatory, anticarcinogenic, and wound healing properties [49]. Curcumin is a hormetin (hormetic agent) which means that its effect changes with dosage [49]. At low doses curcumin has stimulatory effect and in high ones it has inhibitory effects, also can have specific

functions when in low doses, such as antioxidant agent and other in high doses such as induction of cell death[49].

Various studies demonstrated the hormetic effect of curcumin in human skin cells [49]. In low doses curcumin stimulates the proteasome, increases stress tolerance and enhances the keratinocyte's differentiation [49]. Data suggests that when at low concentrations (0.25-5 μM) curcumin has the potential to improve wound healing (in late passaged cells), while the concentrations above 5 μM did not have a statistical importance [224]. Other dietary hormetins that affect aging, wound healing and differentiation include vitamins, resveratrol, minerals (zinc, iodine, iron, copper, selenium and fluorine), trace elements, antioxidants (coenzyme Q10, alpha lipoic acid), kinetin, thymidine-dimer, and other medicinal plants[224]. In another study also it was found that in normal human skin fibroblasts (ASF-2) curcumin has a stimulatory effect in the doses from 1 to 5 μM, and inhibitory effect in greater doses, and this was also occurring in the stimulation of the stress response pathways, such as HO-1 and Nrf2 [225]. Curcumin prolonged the lifespan of nematodes, fruit flies, mice, and lessened the symptoms of some age-related diseases [212]. Furthermore, it enhanced the differentiative ability of cells during senescence [226]. Various studies show that curcumin increases the SIRT1 activation and decreases the oxidative stress [227], [228]. Curcumin can interact with several receptors (e.g. CXCR4, EGFR), kinases (e.g. FAK, MAPK), adhesion molecules, transcription factors, growth factors, enzymes, proinflammatory cytokines, tumor necrosis factor, apoptotic regulators and other proteins (e.g. cyclin B1,p53) [47], [48]. Moreover, curcumin can regulate several miRNAs [229] and histone modifications, and can inhibit DNA methyltransferases [230], [231], [232]. Furthermore, curcumin acts in the cellular level. It protected the HUVEC cells against the peroxide-induced senescence [233], enhanced the human epidermal keratinocytes' ability to differentiate during senescence [226]. In endothelial cells (EC) and vascular smooth muscle cells (VSMC) curcumin could not postpone or protect cells against doxorubicin-induced senescence, even in low concentrations [234]. Anyway, it increased the AMPK and sirtuin levels in VSMC, leading to the conclusion that its effect can be attributed to

AMPK and sirtuin induction, but not senescence inhibition [234]. In rats and mice, curcumin enhanced the exercise effect, prevented fatigue, and increased the activity and level of AMPK and SIRT1 in muscles [235], [236].

Diseases prevented by curcumin include diabetes, oral cancer, stomach cancer, colorectal cancer, liver cancer, lung cancer, bladder cancer, pancreas cancer, breast cancer, arthritis, cataract and cardiovascular diseases [237]. In a phase I clinical trial with 25 patients with high-risk or premalignant lesions was indicated that curcumin may affect the cancer chemoprevention [221]. Curcumin treatment also was able to inhibit the IKKβ kinase activity and decrease the number of inflammatory cytokines in head and neck squamous cell carcinoma [238]. In another clinical trial, the curcumin and gemcitabine-based chemotherapy combination resulted safe in patients with pancreatic cancer [239]. Curcumin was also pharmacologically active in colorectum, in patients with colorectal cancer, which a significant distribution outside the gut [240]. Curcumin's bioavailability is low because of the fast metabolism, low absorption, and quick systemic elimination [241]. It was found that when combined with ascorbic acid, the in vitro antioxidant and antifungal effects of curcumin improved [242].

In clinical trials it was found that curcumin can lessen particular age-related diseases symptoms (e.g. cancer, atherosclerosis and diabetes) [243], [244].

To sum up, curcumin is engaged in the nutrient-signaling pathways regulation (AMPK, sirtuins), enhances mild physical activity's benefits, and can mimic the caloric restriction [33]. Curcumin can also reduce the number of cancer cells (because it induces apoptosis in cancer cells), inhibit metastasis (it contains anti-angiogenic properties), and protects against tumorigenesis (protects against toxicity of particular environmental factors and during therapy) [245].

4
MATERIALS AND METHODS

4.1 Cell Culture

We used the CCD-1072Sk (ATCC CRL-2088) cell line. The foreskin fibroblasts were taken from normal foreskin from caucasian infants. Cells were negative for HIV-1, Hepatitis-B, Hepatitis-C, mycoplasma, bacteria, yeast and fungi.

Cells were cultured in DMEM/F-12, HEPES (Biological Industries), 10% fetal bovine serum (FBS) (HyClone), 1% L-glutamine and 1% 1X non-essential amino acids (NEAA), at 37°C in 5% CO_2. Cells reaching 70%-90% density were passaged. Cells reaching the appropriate density were washed with 1X phosphate-buffered saline (PBS) solution. After the solution were removed, the cells were passaged using Trypsin/EDTA (Biological Industries) solution which detaches the adherent cells from plate's surface. The detachment was checked with microscope. After cell detachment medium was added to neutralize the tripsin's effect. The cell suspension was transferred in a sterile centrifuge tube and centrifuged in 1500 rpm for 5 minutes, and then the supernatant is discarded. Then, the medium was added, and the cell pellet was resuspended by pipetting.

The experiment plan is showed in Figure 4.1.

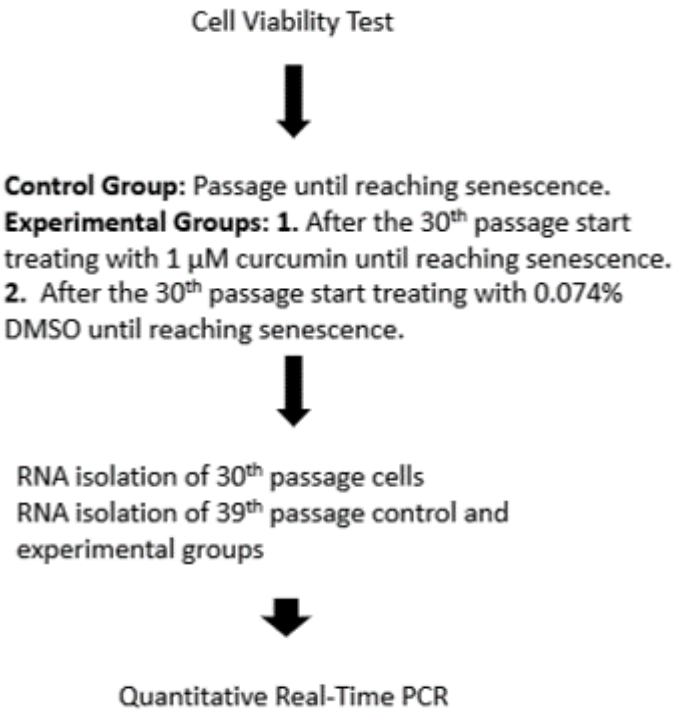


Figure 4.1 Experiment plan

4.2 Cell Viability Test

Cell viability test will be performed to make appropriate concentration determinations for the active substance (curcumin, Sigma, Catalog no: C1386) for cells. For this purpose, cell viability was analyzed using the MTT kit after curcumin has been dissolved in dimethyl sulfoxide (DMSO) (25mg/ml). 50, 30, 20, 10, 5, 2.5, 1 and 0.5 curcumin concentrations were tested. In all concentrations the DMSO concentration was maintained in 0.074%.

Fibroblast cells (35th passage) were seeded in appropriate numbers in 96-well cell culture dishes and incubated overnight at 37°C in the presence of 5% CO2. After incubation, the cell culture medium was removed. Cells were washed with 1X PBS, and later the curcumin concentrations were added for 24, 48 and 72 hours. The

negative control (cells cultivated in medium) and blank (only medium, no cells) was used for the calculations. After the given times (24, 48 and 72h), the medium was discarded and the MTT solution (MTT and medium mix in 1/10 ratio) was added and the plate put back in incubator for 4 hours. Afterwards, MTT solution was removed and 100 µl DMSO was added to dissolve the formazan crystals, products of alive cells (which gives the solution a purple color). After this the reading was made at 650 nm with the help of the Eliza reader and based on the absorption of each well cell viability was calculated.

4.3 RNA Isolation

The RNA isolation was performed with GeneMark Total RNA Purification Kit. Firstly, the medium was removed, and the cells were washed with PBS. The lysis of the attached cells was performed directly in the culture dish by adding 350 µl of Lysis/2-Me Solution and collecting the lysate with a rubber policeman. The lysate was pipetted until the clump was gone, and later homogenized with a 20G needle. After this, 350 µl 70% ethanol was added in the lysate. 700 µl of the lysate/ethanol mixture was transferred in the RNA Spin Column, which was inserted in a 2 ml Collection Tube, and spined at top speed (12000-14000 xg) for 1 minute. The flow-through was then discarded and 500 µl of RNA Wash Solution I was added and spined in top speed for one minute. The flow-through was discarded and in each column 82 µl solution (80 µl DNase I Incubation Buffer+ 2 µl DNase I was added in the center of the RNA Spin Column. After 15-minute incubation in the room temperature, 500 µl of RNA wash solution was added followed by a spin at top speed for 1 minute, after which the flow-through was removed. 600 µl of RNA Wash Solution 2 was added and the RNA Spin Column and Collection tube were put to spin at top speed for 1 minute. This step was repeated one more time, and after this the RNA Spin Column and Collection tube were put in centrifuge, in top speed for 3 minutes, to remove the residual ethanol. In the end, the RNA Spin Column was put into a 1.5 ml microcentrifuge tube. 50 µl of Nuclease-free water were added, and after 1 minute of

incubation in room temperature it was centrifuged for 1 minute at top speed. After this, the collected RNA was stored at -80ºC.

4.4 Quantitative Polymerase Chain Reaction (qPCR)

We performed one step quantitative real-time PCR (qPCR), using the BIO-RAD iTAQ Universal SYBR Green One-Step Kit and Argilent's AriaMx Real-Time PCR instrument. For a 10 μl reaction we mixed: 5 μl iTaq universal SYBR Green reaction mix (2x), 0.125 μl iScript reverse transcriptase, 1 μl forward and 1 μl reverse primers, 2 μl RNA and 0.875 μl nuclease-free water. GAPDH was used as a housekeeping gene.

Firstly, the RNA was turned into cDNA, and then the quantitative reactions followed. PCR steps are showed in the table below:

Table 4.1 qPCR- thermal cycling

Setting /Scan Mode	Reverse Transcri-ption Reaction	Polymerase Activation and DNA Denaturation	Amplification			Melt-Curve Analysis
			Denaturation	Annealing/Extension + Plate Read at 60ºC	Cycles	
SYBR	10 min at 50 ºC	1 min at 95 ºC	10 sec	10-30 sec	35-40	65-95 ºC 0.5ºC increment 2-5 sec/step

5
RESULTS AND DISCUSSION

5.1 Cell Viability Test Results

We studied the hormetic effect on foreskin fibroblasts, the effect of 1 μM curcumin on senescence and on late passaged fibroblasts' gene expression. We used late passaged fibroblasts to study whether late intake of curcumin treatment affects senescence and aging-related gene expression.

The hormetic effects of curcumin have been studied before [49]. We conclude that also in foreskin fibroblasts curcumin can act as an inhibitor in high concentrations and a stimulant in low ones. In our cell viability test, cells at the 35th passage number were used. We used normal medium for cells as negative control, and 0.074% DMSO medium as positive control, while curcumin concentrations varied from 50 to 0.5 μM. It was seen that the absorption of the cells in high concentrations (50, 30 and 20 μM) decreased compared to the control group, suggesting that curcumin kills the cells or stops their proliferation.

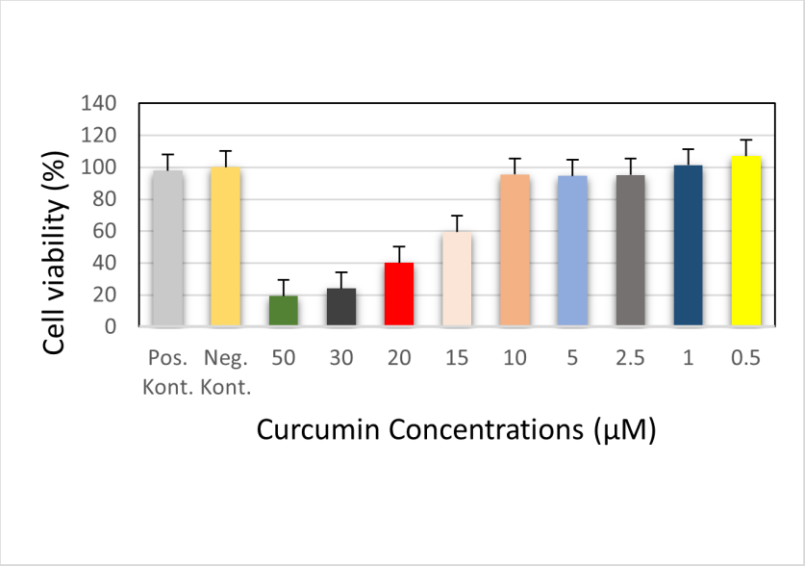


Figure 5.1 Cell viability (after curcumin treatment) in 24 hours

As showed in Fig 5.1, after 24 hours the 50, 30, 20 and 15 μM curcumin concentrations decreased the cell viability. Meanwhile 10, 5, 2.5, 1 and 0.5 μM concentrations didn't cause a significant change in the cell viability.

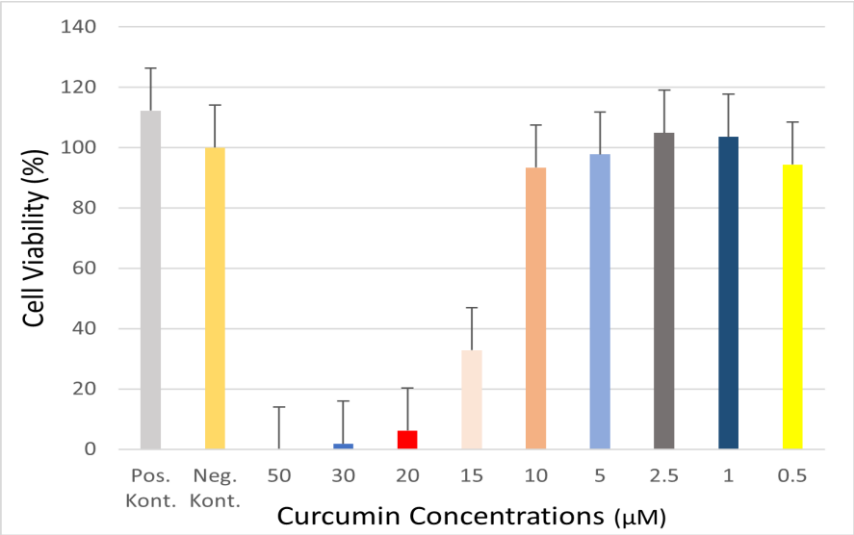


Figure 5.2 Cell viability (after curcumin treatment) in 48 hours

In the Fig. 5.2 we can clearly see that after 48 hours treatment the 50, 30 and 20 μM concentrations drastically decrease the cell viability. 15 μM concentration also as a stronger effect compared to the 24-hour treatment. In addition, cell viability when treated with 10, 5, 2.5, 1 and 0.5 concentrations remains almost same as in the case of the 24-hour treatment.

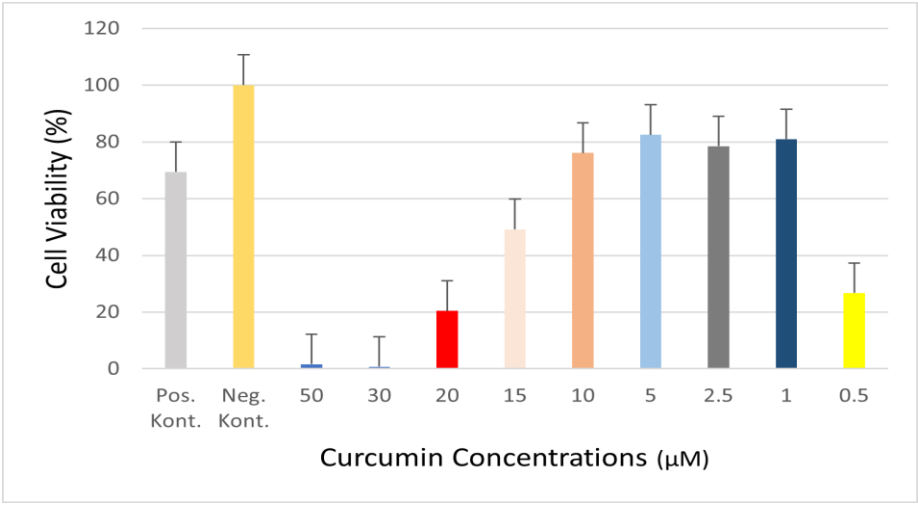


Figure 5.3 Cell viability (after curcumin treatment) in 72 hours

In the 72-hour test the values change even more. As we can see in the Fig. 5.3, the effect of 50, 30, 20 and 15 μM concentrations on decreasing the cell viability is clear. Furthermore, 10, 5, 2.5 and 1 μM concentrations started affecting the cell viability after this period. The 0.5 μM concentration displayed a decreasing affect.

5.2 Cell Culture and Senescence

During cellular senescence human diploid fibroblasts express altered morphology, changing their shape from spindle to irregular, flattened and expanded [246]. We studied 3 groups of cells. The control group was passaged with normal medium; the experimental group was passaged with 1 μM curcumin dissolved in DMSO (total DMSO concentration was 0.074%), and the negative control was treated with medium containing 0.074% DMSO. We used the last group to differ the 0,074% DMSO effect from curcumin's effect. Curcumin was added at the 30th passage in cells that start experiencing senescence after at least 35 passages (according to the product characteristics).

We concluded that 1 μM curcumin does not prevent senescence and does not delay the process in late passage foreskin fibroblast cells. All 3 study groups entered senescence at almost the same time.

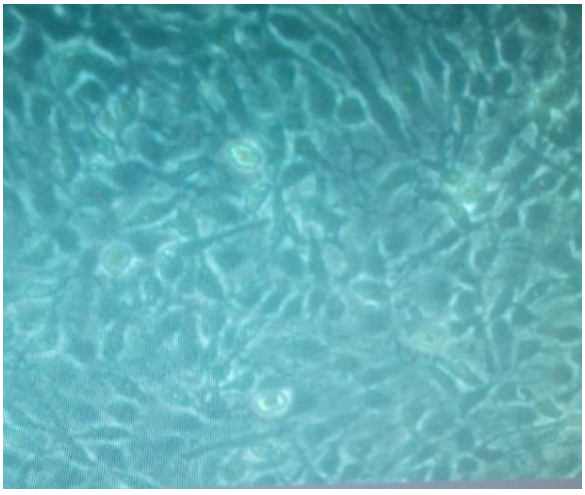


Figure 5.4 Cells at 29th passage (20x)

It is clear to see that the morphology in the 29th passage is normal (Fig. 5.4 and 5.5), the fibroblasts maintain their spindle shape.

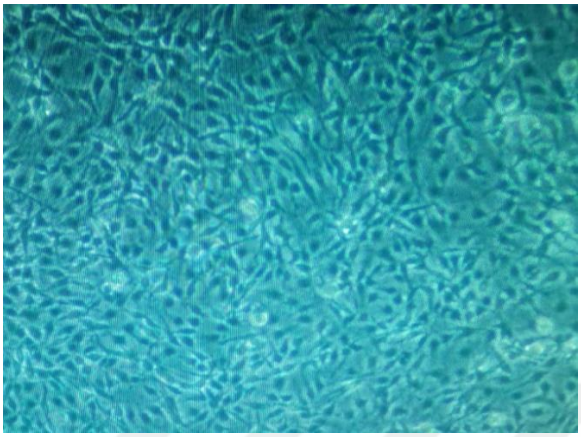


Figure 5.5 Cells at 29th passage (10x)

The CCD-1072Sk cell culture is expected to enter senescence state after the 35th passage. Anyhow, this can happen at a different scale for cells in a dish (not all the cultured cells experience senescence at the same time).

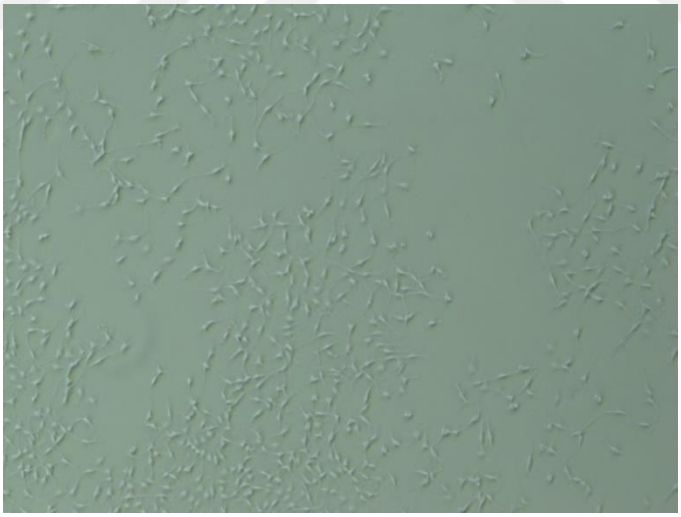


Figure 5.6 Cells at 35th passage (5x)

In our case, at 35th passage (Fig. 5.6) the general morphology of the plate was still normal and only some cells individually started entering senescence.

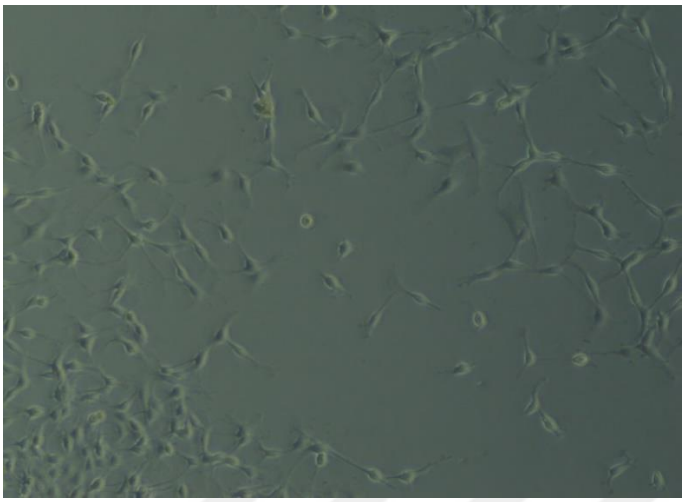


Figure 5.7 Cells at 35th passage (curcumin treated)

The same condition was present in the curcumin-treated culture (Fig. 5.7) and 0.074% DMSO-treated culture (Fig. 5.8). Cell morphology mostly remained the same.

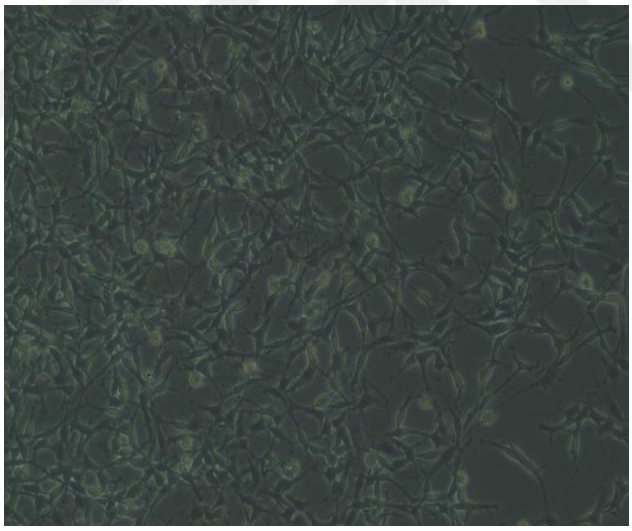


Figure 5.8 Cells at 35th passage (0.074% DMSO treated)

Changes in morphology slightly started becoming clearer after the 37th passage (Fig. 5.9). The cells have a round and altered shape. As mentioned above, there are no significant differences among the control and experimental groups when it comes to the time they enter senescence.

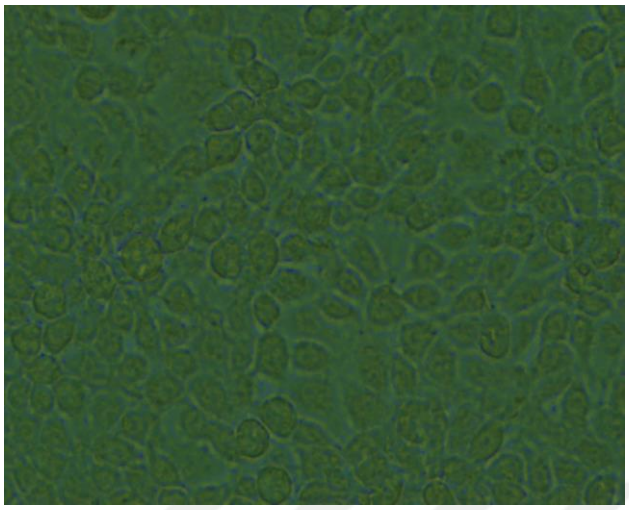


Figure 5.9 Cells at 39th passage (control group-40x)

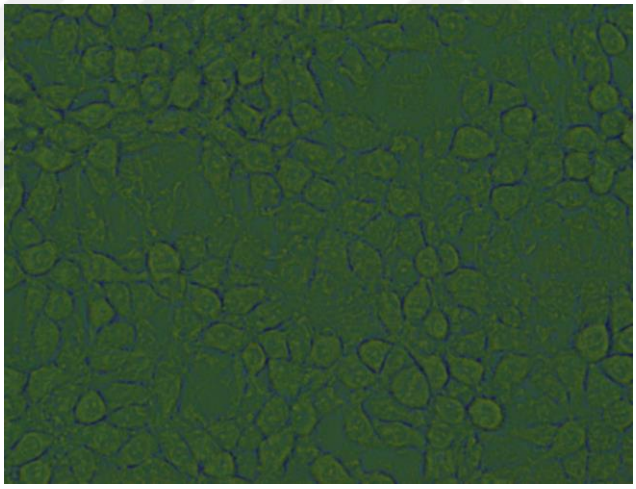


Figure 5.10 Cells at 39th passage (0.074% DMSO treated-40x)

In 0.074%DMSO and curcumin-treated groups (Fig. 5.10 and 5.11) same morphological changes were observed.

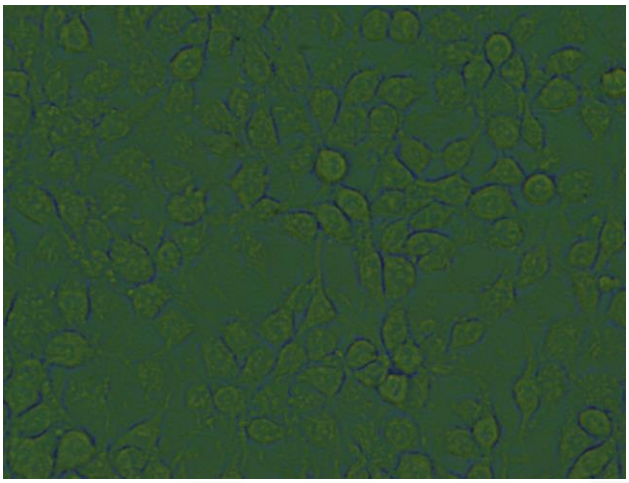


Figure 5.11 Cells at 39th passage (curcumin treated-40x)

5.3 Real-Time PCR Results

The HAS2, IGFBP2, WNT16, GAPDH, ANLN, and CCNB1 gene expressions were measured. The comparison was made among the 31st passage, 39th (non-treated) passage, 39th 0.074% DMSO-treated and 39th 1 μM curcumin-treated passages. 31st passage was a late passage, but cell culture didn't enter senescence yet. Our aim was to understand whether there would be differences among the selected gene expressions between late passage and senescence state, and whether 'late' usage of curcumin still affects the cells. T test was used to make the calculations and compare the groups.

5.3.1 HAS2 Gene-qPCR Results

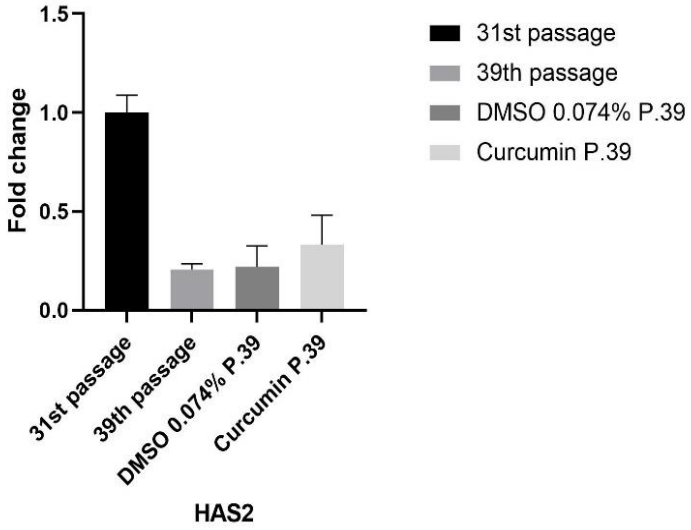


Figure 5.12 HAS2 gene

HAS2 expression decreases with senescence (Fig. 5.12). The first column shows the gene expression in the 31st passage and the second the decrease it has when cells enter senescence. In the third (0.074% DMSO-treated cells) and the last column (curcumin-treated cells) we see that there was a slight change, but it was not significant. We concluded that curcumin cannot affect the hyaluronan (hyaluronic acid) production when added in late passage foreskin fibroblast cells.

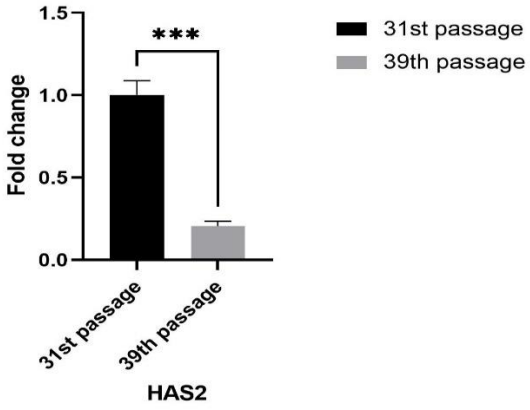


Figure 5.13 HAS2 gene- significant change among young and senescent cells

In Fig. 5.13 we can clearly see the significance of the difference among late passage and senescent cells in HAS2 expression.

5.3.2 IGFBP2 Gene-qPCR Results

In previous studies IGFBP2 gene expression which plays a role in senescence and cell cycle, was upregulated with aging of foreskin fibroblasts [41]. On another study in which keratinocytes from psoriatic plaques were used, during senescence intracellular IGFBP2 was highly expressed, suggesting its effect on senescence and apoptosis inhibition [247]. Our results were contradictory when it comes to IGFBP2 gene expression in senescence.

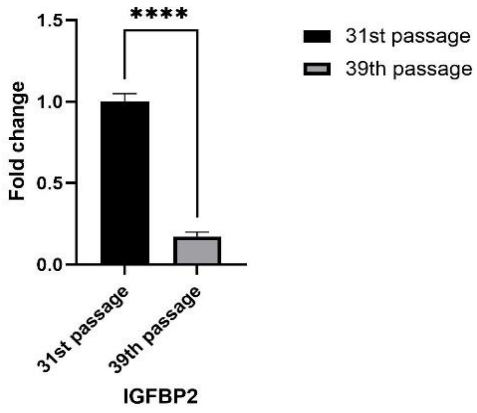


Figure 5.14 IGFBP2 gene- significant change among young and senescent cells

In Fig. 5.14 the significance between the 31st and 39th passage is shown. In our case IGFBP2 is downregulated with senescence, and the change is considerable.

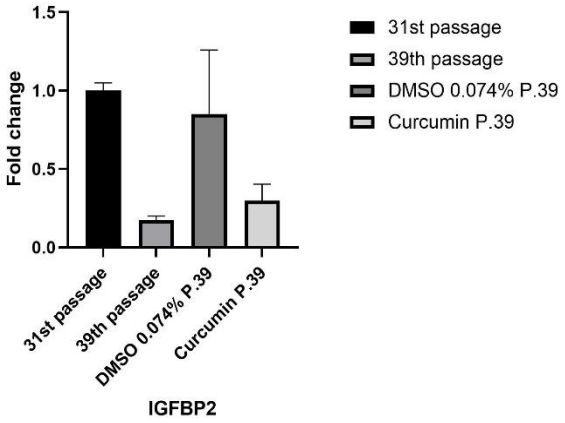


Figure 5.15 IGFBP2 gene

In Fig. 5.15 is interesting to observe that the 0.074% DMSO treatment had a significant effect on increasing the gene expression (3rd column in the graph), while curcumin (last column in the graph) had no significant effect in this gene. These results must further be studied.

5.3.3 WNT16 Gene-qPCR Results

WNT16B gene (one of the WNT16 isoforms [248]) was found to be overexpressed in lung fibroblasts in the case of stress-induced senescence [249]. In our case also, WNT16 gene was upregulated with aging. In both experimental groups a high increase of this gene expression was seen. These results also require further research.

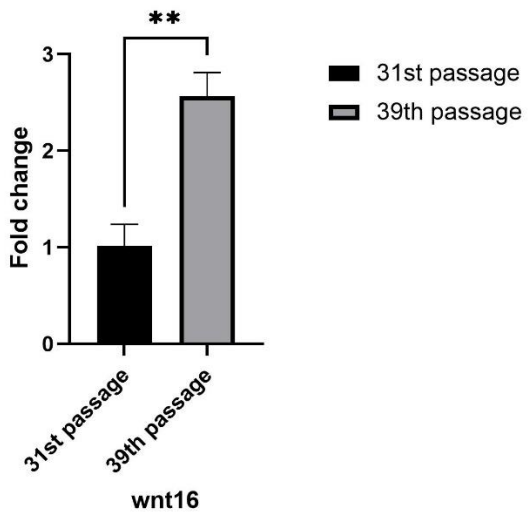


Figure 5.16 WNT16 gene- significant change among young and senescent cells

In Fig. 5.17 the significant difference among young and senescence cells is shown. The second column shows the upregulation of this gene expression in senescence cells.

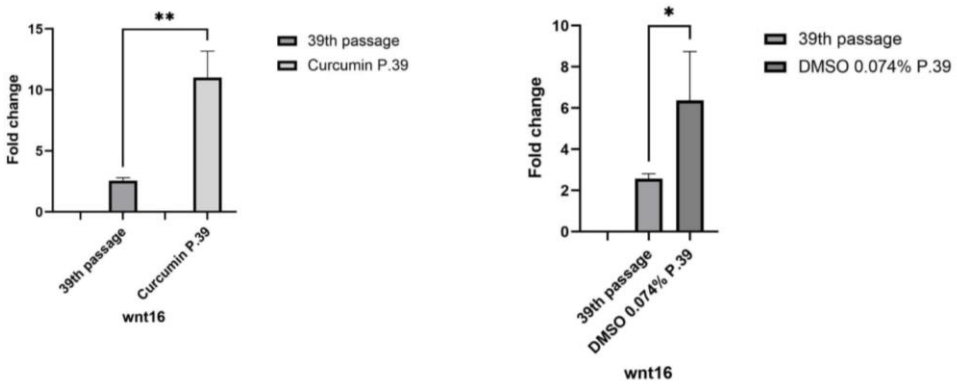


Figure 5.17 WNT16 gene- experimental groups

It was surprising for us to see that curcumin and DMSO furtherly upregulated the gene expression, suggesting that they may have caused stress to the senescent cells. These conclusions also must be studied with further experiments.

5.3.4 CCNB1 Gene-qPCR Results

Curcumin upregulated the CCNB1 expression (Fig.5.20). As mentioned before, this gene is essential for mitosis control and cell viability.

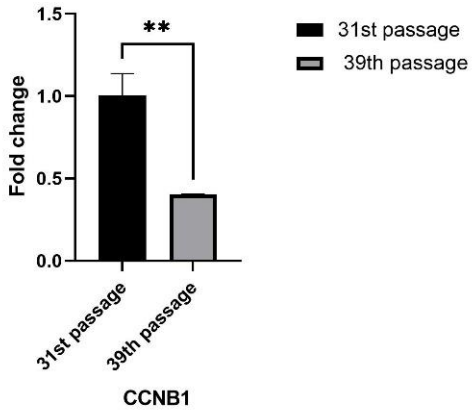


Figure 5.18 CCNB1 gene- significant change among young and senescent cells

In Fig. 5.17 we show the CCNB1 gene downregulation in senescence, compared with the non-senescent cells (first column).

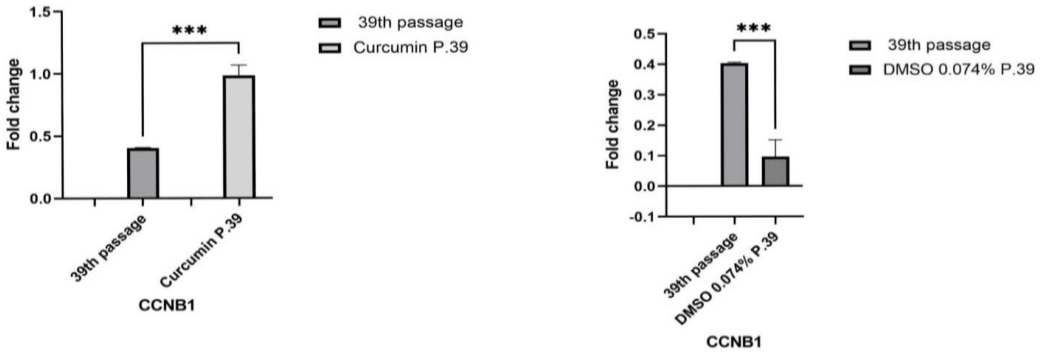


Figure 5.19 CCNB1 gene- experimental groups

In the first graph in Fig.5.20 we see that curcumin was effective on increasing the gene expression. Senescent cells that were treated with 1 μM curcumin showed significant upregulation in CCNB1 expression. Contrary to this, 0.074% DMSO treatment (second graph, Fig.5.20) downregulated the CCNB1 expression even more, compared with the 39th passage (senescent cells).

5.3.5 ANLN Gene-qPCR Results

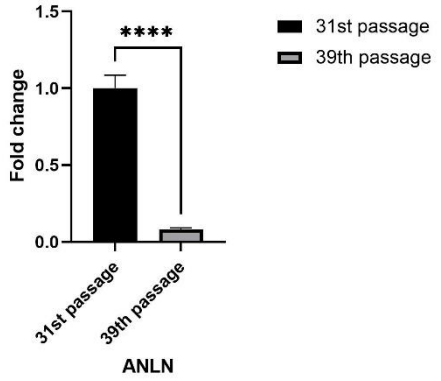


Figure 5.20 ANLN gene- significant change among young and senescent cells

ANLN gene was significantly decreased with senescence as seen in Fig. 5.21, where the first column shows the gene expression in young cells and the second column shows the gene expression in the senescent cells.

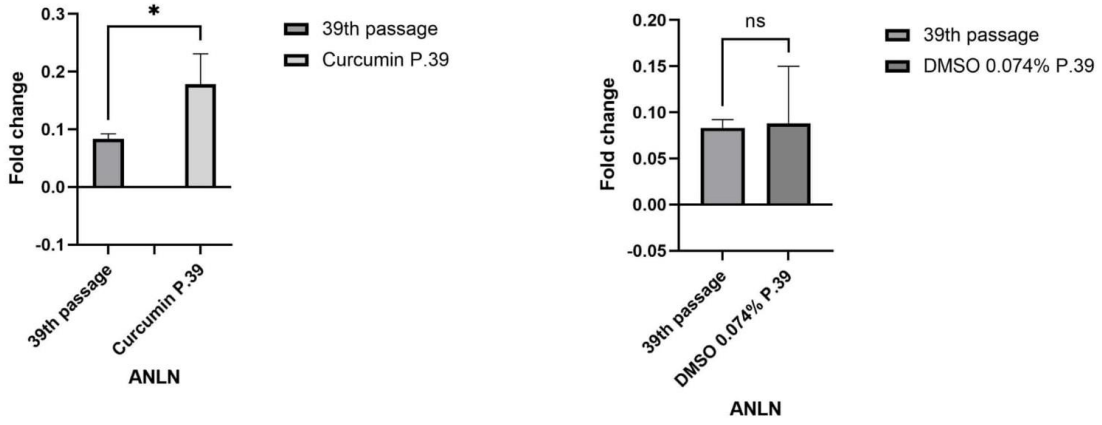


Figure 5.21 ANLN gene- experimental groups

0.074% DMSO treatment had no significant effect, as seen in the second graph, Fig. 5.22. On the other hand, curcumin treatment increased the ANLN expression, as seen in the first graph in Fig. 5.22.

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PUBLICATIONS FROM THE THESIS

Conference Papers

1. Curcumin effect on HAS2 gene expression on senescent fibroblasts [250]

Projects

1. Effect of curcumin on aging-related gene expression in fibroblast cells
BAP Projesi (Project ID-3701)