

# INTERNATIONAL ACADEMIC STUDIES IN THE FIELD OF BIOLOGY

*June 2024*

EDITOR

PROF. DR. HASAN AKGÜL

**Genel Yayın Yönetmeni / Editor in Chief • C. Cansın Selin Temana**

**Kapak & İç Tasarım / Cover & Interior Design • Serüven Yayınevi**

**Birinci Basım / First Edition • © Haziran 2024**

**ISBN • 978-625-6319-35-6**

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**Serüven Yayınevi / Serüven Publishing**

**Türkiye Adres / Turkey Address:** Kızılay Mah. Fevzi Çakmak 1. Sokak

Ümit Apt No: 22/A Çankaya/ANKARA

**Telefon / Phone:** 05437675765

**web:** www.seruenyayinevi.com

**e-mail:** seruenyayinevi@gmail.com

**Baskı & Cilt / Printing & Volume**

Sertifika / Certificate No: 47083

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# *Chapter 1*

## **THE EVALUATION OF VARIATIONS WITHIN *Q. COCCIFERA* L. USING TWO BARCODING REGIONS BELONGING TO NUCLEAR DNA**

*Aykut YILMAZ*<sup>1</sup>

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## INTRODUCTION

The genus *Quercus* L. belonging to the family Fagaceae have over 500 woody plant species with high economic value and diversity distributed across the wide area in Northern Hemisphere (Hubert et al., 2014; Backs and Ashley, 2021; Ozturk and Altay, 2021; Ngoc et al., 2022).

Interspecific gene flow among especially close related species is frequently observed in the genus *Quercus* (Muir et al., 2000; de Casas et al., 2007; Ortego and Bonal, 2010). Frequent interspecific gene exchange in the genus leads some problems in aspect of biological species concept and make it debatable (Van Valen, 1976; Borazan and Babaç, 2003; Yılmaz et al., 2011). Species diversity, wide distribution areas that have variable ecologic and climatic conditions for many species, hybridization between oak species caused by weak reproductive barriers, mixed populations and gene flow bring about variations in morphological characters and increase taxonomic problems in the genus *Quercus* (Yılmaz, 2018). *Q. coccifera* L. is evergreen oaks and has wide distribution area in forest habitats of Mediterranean Region. It can be stated that environmental stress is effective factor in the reduction of mate recognition and later in the hybridization in the oaks (Williams et al., 2001). In this concept, the Mediterranean climate zone has high potential of hybridization. Two evergreen oaks, *Q. coccifera* and *Q. ilex* L. generate mixed populations in this region where hybridisation may take place (de Casas et al., 2007). Furthermore, wide distribution of the *Q. coccifera* across Mediterranean leads to variations under influence of different ecologic and climatic conditions.

There are confused and unclear states in the taxonomic status of some *Q. coccifera* populations. It is stated by many researchers that the populations of *Q. coccifera* belonging to East Mediterranean region exhibit differences in the comparison to others (Toumi and Lumaret, 2001; Yılmaz et al., 2013; Yılmaz et al., 2017). In other words, the region called as Levant containing from Eastern Mediterranean region of Türkiye to Egypt are the distribution areas where exhibit the highest variation for *Q. coccifera*. Toumi and Lumaret (2001) states that East Mediterranean region is the living regions for *Q. calliprinos* (Webb) Holmboe. Similarly, Zohary (1966) mentioned the two subspecies of *Q. calliprinos* as *Q. calliprinos* subsp. *coccifera* and *Q. calliprinos* subsp. *calliprinos*. Unlike, it was described the two subspecies of *Q. coccifera* as *Q. coccifera* subsp. *coccifera* and subsp. *calliprinos* (Schwarz, 1936; Tutin et al., 1993). Recently, Yılmaz et al. (2013 and 2017) states in the both of molecular and morphological based studies that the populations of *Q. coccifera* belonging to Eastern Mediterranean region in Turkey exhibited variations and finally this supported the presence of second group within *Q. coccifera*. However, it is taxonomically controversial and unclear whether *Q. coccifera* and *Q. calliprinos* are separate species or subspecies (Schwarz, 1936; Camus, 1938; Zohary, 1961; Salvatore and Paola, 1976; Tutin et al., 1993; Toumi and Lumaret, 2001; Toumi



and Lumaret, 2010; Yilmaz et al. 2013; Yilmaz et al. 2017; Ozturk and Altay, 2021).

DNA barcoding studies have been commonly using for many plant groups that are especially taxonomically problematic in the determination of variations, taxonomic problems and phylogenetic relationships, recently. For this aim, sequences information containing sufficient variations belonging to chloroplast DNA (cpDNA) and nuclear DNA as a barcoding regions are applied by researchers. However, there is not universal barcoding region for plants and the same barcoding region may show differences based on its species identification and separation ability, in addition to the grouping of species in aspect of common characteristics and the solving of taxonomic problem. Therefore, it is necessary to has knowledge about suitable barcoding sequences for each plant groups and afterwards, to use correct combinations of barcoding regions to get more informative results.

In this study, the sequence data for all populations belonging to *Q. coccifera* were separately acquired from National Center for Biotechnology Information (NCBI) for the barcoding regions covering 5.8S rRNA gene-ITS2 and 5S rRNA gene- intergenic spacer (IGS). Afterwards, they were examined for the determination of variations within *Q. coccifera* based on populations distributed in different habitats. Furthermore, it was aimed to make suggestion about taxonomic status of the populations distributed East Mediterranean region.

#### **MATERIAL AND METHODS FOR THE ANALYSIS OF VARIATIONS WITHIN *Q. COCCIFERA***

The populations belonging to *Q. coccifera* used in the study consist of the samples from 3 continents containing Mediterranean coastal countries. All sequence data for 5.8S rRNA gene-ITS2 and 5S rRNA gene-IGS regions were acquired from the NCBI database. The sequences which is compatible for both barcoding regions were determined and then examined in further analysis.

In this study, with the selection of sequences belonging to different localities and so the use of as many populations of *Q. coccifera* as possible, it is aimed:

- to determine the effects of different localities on the relationships among *Q. coccifera* populations

- to reveal the populations of *Q. coccifera* with the highest variation. For this purpose, firstly, the sequence data for the regions containing 18S rRNA gene-ITS1-5.8S rRNA gene- ITS2- 25S rRNA gene, ITS1-5.8S rRNA gene- ITS2 and 5.8S rRNA gene- ITS2 were provided and then the sequences belonging to 5.8S rRNA gene-ITS2 were extracted for effective analysis. Similarly, in addition to the use as many populations of *Q. coccifera* as possible, by the aim

of determination of the accuracy and compatibility of the sequences shared in NCBI by different researchers at different periods, some localities were represented by a few populations.

15 populations from 8 countries for 5.8S rRNA gene-ITS2 region and 15 populations from 7 countries for 5S rRNA gene-IGS region were examined based on sequence data. GenBank codes containing all populations examined were given in Supplementary Material Table S1. After the determination of compatible sequences for both regions of interest, sequence alignments were performed by using Molecular Evolutionary Genetics Analysis (MEGA 11) (Tamura et al., 2021). Nucleotide sequences for variable and parsimony informative sites (parsim-info) were determined separately for both barcoding regions to evaluate the populations phylogenetically and variable sites were shown in Supplementary Material Table S2 and S3. Furthermore, base substitution probabilities were computed for 5.8S rRNA gene-ITS2 and 5S rRNA gene-IGS sequences. Thus, transitional and transversional substitutions (%) were computed, in addition to transition/transversion ratios for purines and pyrimidines. Nucleotide frequencies for both barcoding regions were determined as A+T/U % and G+C %.

The Maximum Parsimony (MP) dendrogram was performed to evaluate the genetic distance and phylogenetic relationships of *Q. coccifera* populations. Additionally, it was aimed the determination of populations with highest variations, besides revealing the effect of habitats on the variations of populations.

#### **ANALYSIS RESULTS PROVIDED FROM 5.8S rRNA GENE-ITS2 AND 5S rRNA GENE-IGS SEQUENCES**

The all sequence data for 5.8S rRNA gene-ITS2 and 5S rRNA gene-IGS regions belonging to *Q. coccifera* populations were provided from NCBI database. Many regions that contain gene and spacer sequence information for both barcoding regions of interest were determined and then related sequences based on their compatibilities were extracted to obtain significative and comprehensive results (Supplementary Material Table S1).

Firstly, the sequences defined for each region were aligned to determine the variations and relationships among populations. Alignment lengths for 5.8S rRNA gene-ITS2 and 5S rRNA gene-IGS regions were determined as 379 and 401 bp, respectively. The variable sites and parsim-info sites are very important in the forming of dendrograms giving the information about taxonomic and phylogenetic relationships of the samples examined. These sites were observed in 12 and 4 nucleotides for 5.8S rRNA gene-ITS2 sequences, in 61 and 24 nucleotides for 5S rRNA gene-IGS sequences, respectively. Sequence variations for 5S rRNA gene-IGS and 5.8S rRNA gene-ITS2 based on the variable sites were determined with the rates of 15.21% and 3.16%, respectively. The comparisons

based on alignment lengths and variable sites of the sequences examined show to us that although alignment lengths of both barcoding regions are very close, 5S rRNA gene-IGS region exhibit more sequence variation than other region. Similarly, parsim-info sites which contain the least two types of nucleotides with a minimum frequency of two revealed that 5S rRNA gene-IGS sequences have more variations than 5.8S rRNA gene-ITS2 sequences. Although the substitutions between nucleotides for variable and parsim-info sites in this study represent the intraspecific variations, it can be stated for both barcoding regions that they display distinctive and important data in the evaluation of *Q. coccifera* populations.

The probabilities of base substitutions for each region were created as Table 1 and 2, and after that transitional and transversional substitutions were computed using tables. Transitional and transversional substitutions were determined as 73.5% and 26.5% for 5.8SrDNA-ITS2 sequences, 43.24% and 56.76% for 5S rRNA gene-IGS sequences, respectively. In other words, it was observed that transitional substitutions are higher than the transversional substitutions in 5.8SrDNA-ITS2, contrary to 5S rRNA gene-IGS.

**Table 1.** *The probabilities of substitution (r) from one base (row) to another base (column) for 5.8SrDNA-ITS2 sequences (Transitional substitutions are shown in bold)*

	A	T	C	G
A	-	2.68	4.38	<b>4.06</b>
T	2.31	-	<b>41.55</b>	3.88
C	2.31	<b>25.47</b>	-	3.88
G	<b>2.42</b>	2.68	4.38	-

**Table 2.** *The probabilities of substitution (r) from one base (row) to another base (column) for 5S rDNA-IGS sequences (Transitional substitutions are shown in bold)*

	A	T	C	G
A	-	8.78	8.03	<b>14.77</b>
T	4.65	-	<b>8.85</b>	6.92
C	4.65	<b>9.68</b>	-	6.92
G	<b>9.94</b>	8.78	8.03	-

Furthermore, the highest substitutions between bases were observed as 41.55% from T to C and 25.47% from C to T for the region containing 5.8SrDNA-ITS2 sequences. Finally, transition/transversion ratio of purines (k1), pyrimidines (k2) and overall were determined as 1.04, 9.49 and 2.76, respectively for 5.8SrDNA-ITS2. In the comparison of transition/transversion ratio for the states evaluated, purines for the 5S rRNA gene-IGS sequences show higher value than pyrimidines, contrary to 5.8SrDNA-ITS2 sequences (Table 3).

**Table 3.** The information of the *Q. coccifera* populations examined based on 5.8SrDNA-ITS2 and 5S rDNA-IGS sequences

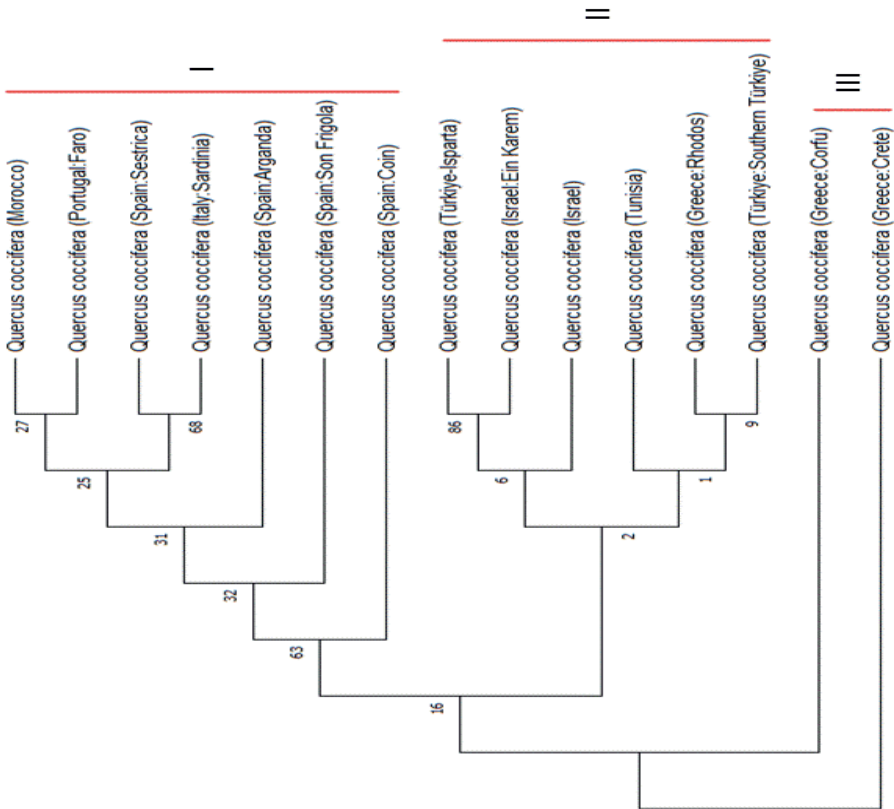
DNA regions	Pop. (Num.)	Align. length (bp)	Variab. site	Parsim-info site	Transitional substitutions (%)	Transversional substitutions (%)	Transition/Transversion Purine Pyrimid. Overall (k <sub>1</sub> ) (k <sub>2</sub> ) (R)	Nucleotide frek. (%) A+T/U G+C
5.8SrDNA-ITS2	15	379	12	4	73.5	26.50	1.04 9.49 2.76	37.68/62.32
5SrDNA-IGS	15	401	61	24	43.24	56.76	2.13 1.10 0.75	47.33/52.67

Nucleotide frequencies (%) for A+T/U bases and G+C bases were computed as 37.68 and 62.32 for 5.8SrDNA-ITS2, 47.33 and 52.67 for 5S rDNA gene-IGS sequences, respectively. In other words, it can be stated that both barcoding regions analysed for *Q. coccifera* populations consist of highly G and C bases (Table 3).

Maximum Parsimony (MP) dendrograms which are useful tool in the evaluation of taxa in terms of their phylogenetic relationships and taxonomic statues and help to overcome the problem related to species identification were drawn separately for each barcoding region (Figure 1, 2). MP dendrogram provided from 5.8SrDNA-ITS2 sequences separated the *Q. coccifera* populations into three main groups. It was observed that the *Q. coccifera* populations were clustered based on their geographical distribution that is under the influence of different ecological and climatic conditions (Figure 1). The dendrogram provided from 5S rDNA-IGS sequences separated the *Q. coccifera* populations into two main groups. The highest variations were observed in the populations belonging to Türkiye and Israel forming outmost clade in MP tree (Figure 2).

There are many factors that make it difficult to define the oak species and lead to the problems in terms of species concept. Although DNA barcoding is especially valuable approach in the solution of taxonomic and phylogenetic problems, each barcoding region has not equal efficiency based on species recognition and identification ability. Furthermore, it can be observed the variations for same barcoding region in different plant groups. In other words, the determination of suitable sequences and thus, the using of correct combinations of barcoding regions have crucial importance to get more comprehensive and informative results in the solution of problems detected. In this study, short DNA sequences that contain 5.8SrDNA-ITS2 and 5S rDNA-IGS regions were analysed for the evaluation phylogenetically of *Q. coccifera* populations and the determination of variations among populations that have different distribution areas.

The Phylogenetic tree provided from 5.8SrDNA-ITS2 sequences separated the populations into three main groups. Group I consists of seven populations collected in Spain, Portugal, Morocco and Italy (Figure 1). The populations belonging to Spain, Portugal and Morocco have close localities and it is observed in the phylogenetic tree that all populations exhibit the closely clustering together. Island population of Italy: Sardinia showed affinity to these clade. Group II in phylogenetic tree is represented by a population belonging to Southern Türkiye together with Isparta population from Southwest Türkiye, two populations from Israel, an island population of Greece (Rhodes) and a population of Tunisia.



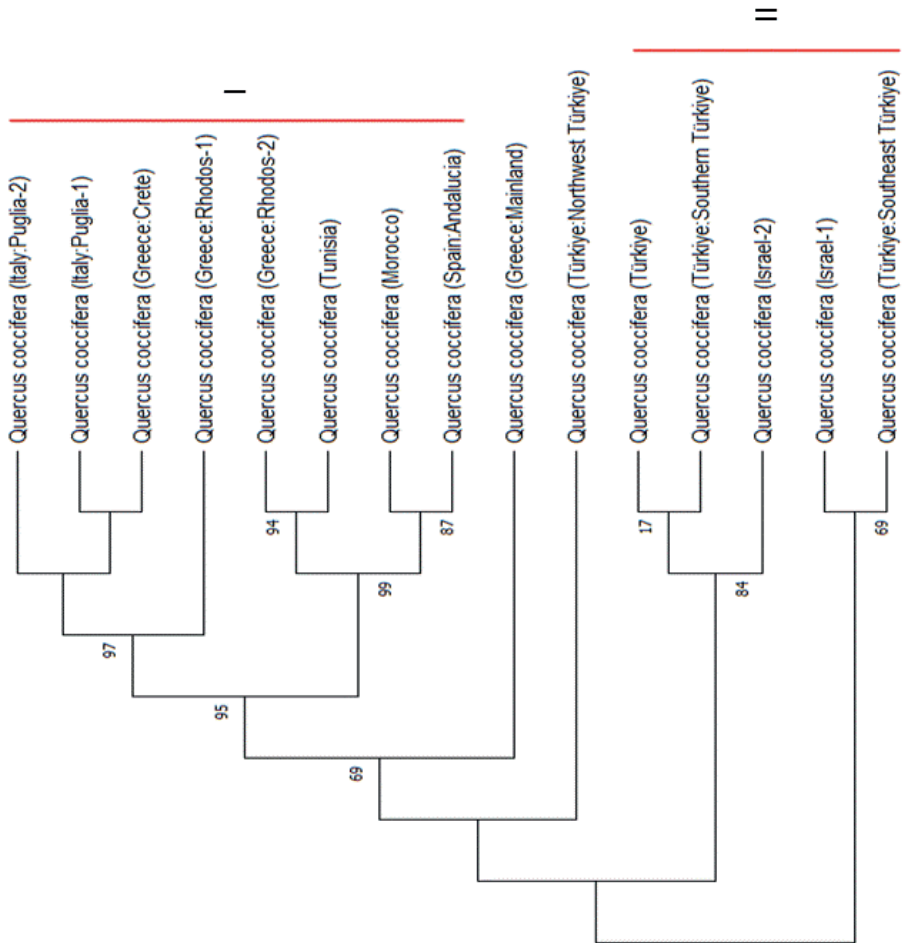
**Figure 1.** MP tree provided from 5.8SrDNA-ITS2 sequences

The region called Levant that is crossroad between continents is very important in terms of diversification (Douaihy et al., 2020). Yılmaz et al. (2017) in the study based on morphological variability of evergreen oaks that consist of *Q. coccifera*, *Q. aucheri* and *Q. ilex* in Türkiye state that the highest variations within 16 *Q. coccifera* populations collected from different localities were observed in East Mediterranean region. Similar segregation in the *Q. coccifera*

populations belonging to the East Mediterranean region were determined by Yılmaz et al. (2013) in the study based on molecular diversity among Turkish oaks using RAPD markers. In other words, two distinct studies based on morphological and molecular data revealed that *Q. coccifera* populations collected from East Mediterranean region in Türkiye show distinction from other. Furthermore, Toumi and Lumaret (2001) states that the living area of the populations that show differences within *Q. coccifera* is East Mediterranean region and they are called as *Q. calliprinos* Webb. Phylogenetic tree provided from 5.8SrDNA-ITS2 sequences show that the populations belonging to the Levant exhibit differences, in addition to cluster together (Figure 1). Two island populations from Greece: Corfu and Crete formed outmost samples in dendrogram.

The Phylogenetic tree provided from 5S rDNA-IGS sequences separated the populations into two main groups. Group I consists of the populations collected in Italy, Greece, Tunisia, Morocco and Spain. It can be stated that the populations that are close in terms of their localities were clustered together. *Q. coccifera* populations collected from Türkiye, along with populations from Israel generate group II that consists of outmost populations in phylogenetic tree and have highest variations (Figure 2). It was observed that the samples of *Q. coccifera* collected from the South of Türkiye, with the samples from Israel showed the highest distinction in dendrogram provided from 5S rDNA-IGS sequences. In other words, the relationships between *Q. coccifera* populations exposed the consistency between both barcoding regions and supported that the populations belonging to the Levant exhibit differences in comparison to other. However, taxonomic status of these samples are still confused. The evaluation taxonomically of the samples belonging to Levant show difference in many studies (Zohary, 1966; Salvatore and Paola, 1976; Blondel and Aronson, 1999; Toumi and Lumaret, 2001; Tutin et al., 2010; Yılmaz et al., 2017; Ozturk and Altay, 2021). It is observed different descriptions for the samples provided from Levant such as *Q. calliprinos* (a separate species) or *Q. coccifera* subsp. *calliprinos* (an intraspecific taxa).

It is clear that the populations collected from Levant and expressed as *Q. coccifera* show differences. However, *Q. coccifera* has wide distribution area along Mediterranean Region containing the samples from three continents.



**Figure 2.** MP tree provided from 5S rDNA-IGS sequences

Besides variable ecological and climatic conditions, interspecific gene flow are states frequently observed and giving rise to variations within the taxon (Denk and Grimm, 2010; Simeone et al., 2013). In other words, this needs to be supported by studies including all *Q. coccifera* and *Q. calliprinos* samples based on the population genetics to provide the most comprehensive and accurate results. Moreover, phylogenetic relationships between oak species with weak reproductive barriers should be examined based on the population genetics.

Both barcoding regions examined in this study exposed the consistent results with each other and supported the presence of a second group with higher variations than others within *Q. coccifera*. However, there are other issues with the sequence data in NCBI such as the absence of habitat information belonging to the samples or the only presence of the country information for

samples that were collected from different locations of same country. All of these make difficult to interpret the *Q. coccifera* based on the populations.

Finally, it can be stated that both barcoding regions that contain 5.8SrDNA-ITS2 and 5S rDNA-IGS sequences have important data for their ability to reveal the phylogenetic relationships among the populations, also recommended for further studies.

### **Acknowledgements**

The author would like to thank to NCBI for sequence information used in this study and the authors sharing the sequence information in NCBI.

### **Supplementary Material**

**Table S1.** GenBank codes for all populations examined.

#### **5.8S rDNA-ITS2**

FM244333, FM244331, FM244324, FM244320, FM244311, FM244310, DQ342352, DQ342348, DQ342347, DQ342346, DQ342345, HE583689, HE583687, HE583685, HE583683

#### **5S rDNA-IGS**

MT219885, MT219884, MT219880, MT219869, FM243558, FM243550, FM243541, FM243537, FM243525, FM243517, FM243501, FM243494, FM243481, FM243475, FM243462



**Table S2.** *Q. coccifera* populations and variable sites belonging to 5.8S rDNA-ITS2 sequences (The numbers show variable nucleotides).

	1	1	1	1	2	3	3	3				
	1	3	9	1	7	9	9	6	2	5	7	
	5	2	4	9	0	9	1	3	0	4	5	0
<i>Quercus coccifera</i> (Israel)	C	C	C	G	C	C	C	G	C	C	G	C
<i>Quercus coccifera</i> (Greece:Rhodos)	C	C	C	G	C	C	C	T	C	C	G	T
<i>Quercus coccifera</i> (Greece:Crete)	T	T	T	G	C	C	C	G	C	C	G	C
<i>Quercus coccifera</i> (Türkiye:Southern Türkiye)	C	C	C	G	C	-	C	G	C	C	G	C
<i>Quercus coccifera</i> (Tunisia)	C	C	C	G	C	A	C	G	C	C	A	C
<i>Quercus coccifera</i> (Morocco)	C	C	C	G	C	C	T	G	A	C	G	C
<i>Quercus coccifera</i> (Greece:Corfu)	C	C	C	G	C	C	C	G	C	C	G	C
<i>Quercus coccifera</i> (Spain:Coin)	C	C	C	G	C	C	T	G	C	C	G	C
<i>Quercus coccifera</i> (Spain:Son Frigola)	C	C	C	G	C	C	T	G	C	C	G	C
<i>Quercus coccifera</i> (Spain:Arganda)	C	C	C	G	C	C	T	G	C	C	G	C
<i>Quercus coccifera</i> (Spain:Sestrica)	C	C	C	G	C	C	T	G	C	T	G	C
<i>Quercus coccifera</i> (Italy:Sardinia)	C	C	C	G	C	C	T	G	C	T	G	C
<i>Quercus coccifera</i> (Portugal:Faro)	C	C	C	G	C	C	T	G	C	C	G	C
<i>Quercus coccifera</i> (Türkiye-Isparta)	C	C	C	T	T	-	C	G	C	C	G	C
<i>Quercus coccifera</i> (Israel:Ein Karem)	C	C	C	T	T	-	C	G	C	C	G	C



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## *Chapter 2*

# **ROLE OF THE CHOLINERGIC SYSTEM IN AGING AND NEUROINFLAMMATION**

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## 1. INTRODUCTION

Aging encompasses all the irreversible, functional, and structural changes that occur over time at an organism's cellular, tissue, and systemic levels, constituting a physiological process (Aslan & Hocoğlu, 2017). Aging is a comprehensive process from birth to death. The onset of old age varies from society to society and even within the same society over the years, depending on gender, education level, economic conditions, and psychological age (Arioğul, 2006; Kutsal, 2007). According to the health report on old age published by the World Health Organization (WHO) in 1998, old age was defined as increased disabilities and dependence on others. The age limit was set at 65 years (Kalinkara, 2011). World Health Organization (WHO) classifies the concept of old age under three headings: Young, old age (65-74 years), middle old age (75-84 years), and advanced old age-elderly (85 and over) (Alterovitz & Mendelsohn, 2013).

The population aged 65 and over, considered the elderly population, has increased by 24% in the last five years in our country, and its proportion in the total population increased to 9.9% in 2022 (TÜİK, 2023). With this demographic change and increase in life expectancy, interest in healthy aging and old age is increasing both in the world and in our country. In addition, the increase in human lifespan has increased the negative impact of aging on cognitive performance. However, it is essential to increase the percentage of healthy older adults in the population since healthy living and aging benefit individuals, their environment, and the society in which they live in many different aspects, such as physical, cognitive, psychological, sociological, cultural, economic, etc. Therefore, there is a growing need to improve knowledge about the mechanisms underlying the aging process.

## 2. AGING AND CHOLINERGIC DYSFUNCTION

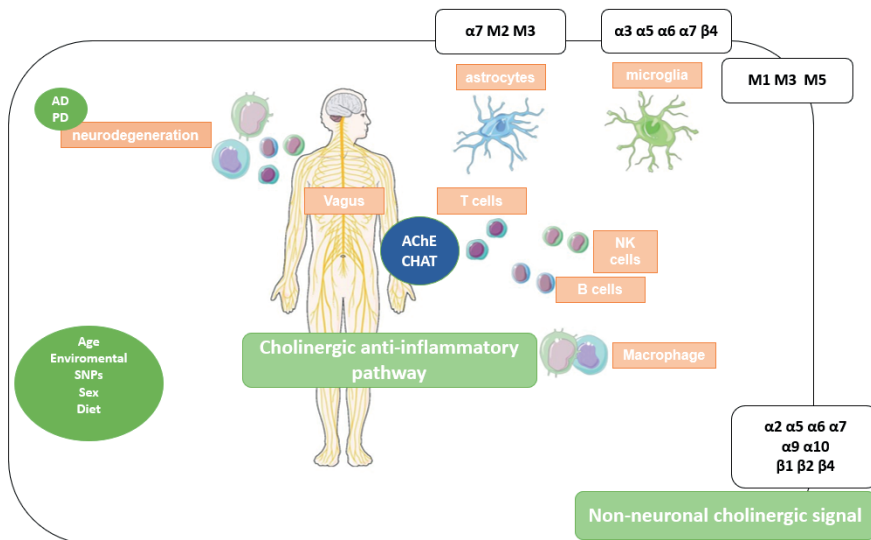
Aging is a physiological phenomenon marked by reduced brain function, diminished synaptic plasticity, and central nervous system (CNS) alterations, potentially impacting neurotransmission and cognitive abilities (S.-C. Li, Lindenberger, & Sikström, 2001; Mahncke, Bronstone, & Merzenich, 2006). Oxidative stress and inflammation occur in brain aging (Kuzumaki et al., 2010; Sierra, Gottfried-Blackmore, McEwen, & Bulloch, 2007). Glial cells (such as microglia, astrocytes, and dendritic cells) in the CNS, which are innate immune system elements, generate an inflammatory response (Benfante, Di Lascio, Cardani, & Fornasari, 2021). Neuroinflammatory responses such as glial activation, over-expression of proinflammatory cytokines, and abnormal neuronal signaling can negatively affect the CNS. These mechanisms may cause an acceleration of cognitive impairment. Neuroinflammation and cholinergic dysfunction cause cognitive impairment underlie **aging and neurodegenerative diseases** (Benfante et al., 2021).

Cognitive impairments are mainly caused by central cholinergic neuron degeneration. In general, decreased synthesis and release of acetylcholine in aging and neuroinflammation, changes in the expression of various pro- and anti-inflammatory genes, and acetylcholine receptors (nicotinic and muscarinic) (Schliebs & Arendt, 2011).

### 3. ROLE OF THE CHOLINERGIC SYSTEM IN NEUROINFLAMMATION

Acetylcholine (ACh), the first neurotransmitter discovered, plays many physiological roles in the central, peripheral, and autonomic nervous systems. The choline acetyltransferase (ChAT) synthesizes acetylcholine (ACh) from acetyl CoA and choline. Postsynaptic acetylcholine receptors involved in ACh-induced nerve conduction are classified according to their agonist ligand binding affinity. The acetylcholine molecule is hydrolyzed by acetylcholinesterase (AChE, E.C. 3.1.1.7) and butyrylcholinesterase (BChE, E.C. 3.1.1.8) enzymes to acetate and choline. Choline transporters take up the released choline. The system composed of all these components is called the cholinergic system. The cholinergic system is found in neuronal and non-neuronal cells (Figure 1) (Halder & Lal, 2021; Jia et al., 2004; Kawashima & Fujii, 2000; Soreq, 2015; Winek, Soreq, & Meisel, 2021).

Based on ligand affinities, ACh receptors divide into muscarinic (mAChR) and nicotinic acetylcholine receptors (nAChR).



**Figure 1.** Cholinergic anti-inflammatory pathway (CAIP) in the human body

AD, Alzheimer's disease; AChE, acetylcholinesterase; ChAT, choline acetyltransferase; PD, Parkinson's disease,  $\alpha$  and  $\beta$ : nicotinic acetylcholine

The cholinergic anti-inflammatory pathway (CAIP) is the efferent arm of the inflammatory reflex. This system consists of the vagus nerve, acetylcholine, cytokines and the  $\alpha 7$  subunit of the nicotinic acetylcholine receptor (Borovikova et al., 2000). Further studies have elucidated the anatomy of this reflex. Pathogen-associated molecular patterns (PAMPs) or pro-inflammatory cytokines mediate signaling to the nucleus tractus solitarius (NTS) through vagal afferents. The nucleus tractus solitarius (NTS) initiates activation of the cholinergic efferent fibers, transmitting output signals through the celiac ganglion and N. splenicus towards the spleen, the main target organ of the inflammatory reflex. Subsequently, the splenic nerve releases norepinephrine, which triggers the stimulation of  $\beta 2$ -adrenergic receptors and prompts the synthesis and secretion of acetylcholine (ACh) (Benfante et al., 2021).

Microglial and astrocytic cells in the CNS express muscarinic and nicotinic ACh receptors. Activation of  $\alpha 7$ nAChR on glial cells has been associated with anti-inflammatory effects (Mizrachi, Vaknin-Dembinsky, Brenner, & Treinin, 2021). Non-neuronal cells such as microglia and astrocytes regulate neuronal activity by mediating the release of inflammatory molecules. Over- and under-expression of pro- and anti-inflammatory molecules can lead to neuroinflammation and thus to the onset/progression of any neurodegenerative disease (DiSabato, Quan, & Godbout, 2016). Microglia and astrocytes contribute to the inflammation process by being in two different phenotypes: neuroprotective and neurotoxic (Kwon & Koh, 2020).

### **3.1.Role of Glial Cells in the Cholinergic System**

#### **3.1.1. Microglia Cells**

Innate immune responses are considered the first defense against invading pathogens (Muzio, Viotti, & Martino, 2021). Microglia are the primary innate immune cells and are critical first responders to pathological processes (Baufeld, O'Loughlin, Calcagno, Madore, & Butovsky, 2018; Heneka, Kummer, & Latz, 2014).

Microglia participate in maintaining homeostasis and executing defense mechanisms through three fundamental functions, with their primary task being the detection of environmental changes (S. E. Hickman et al., 2013). The second is to enable migration to damaged sites, remodeling synapses, and maintaining myelin homeostasis. The final task is to provide cellular protection against pathogens. Microglia express cellular receptors such as Toll-like receptors (TLRs), nuclear oligomerization domain-like receptors (NODs), and viral receptors (Glass, Saijo, Winner, Marchetto, & Gage, 2010; Stephenson, Nutma, van der Valk, & Amor, 2018). As a result, microglia produce proinflammatory cytokines such as IL-1 $\beta$ , TNF- $\alpha$ , and IL-18 in response to removing pathological agents (Glass et al., 2010; S. Hickman, Izzy, Sen, Morsett, & El Khoury, 2018). Microglia play a role in triggering mediators

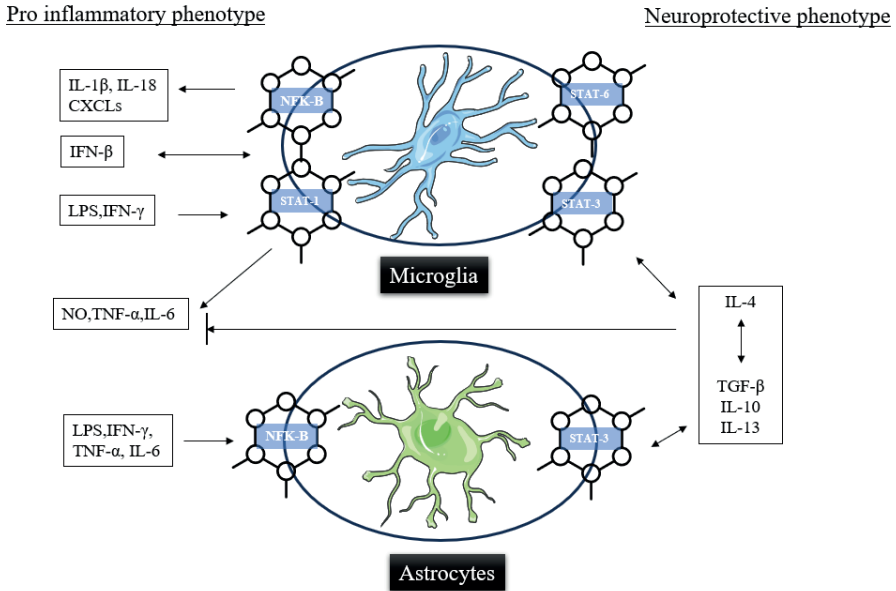


like cytokines and chemokines, leading to inflammation, and their numbers increase in almost all neurodegenerative diseases (Muzio et al., 2021).

### **3.1.2. Astrocyte Cells**

Astrocytes are one of the primary cells in the CNS. They generally function in the blood-brain barrier, regulating blood flow and ion balance and modulating synaptic activity (Colombo & Farina, 2016; Oksanen et al., 2019; Sofroniew, 2009). Activated astrocytes contribute to the pathogenesis of neurodegenerative diseases, inflammatory CNS disorders, and injuries (Ludwin, Rao, Moore, & Antel, 2016; Sadick & Liddelw, 2019; Zhou et al., 2020). They also have essential roles in neuroprotection (B. Liu, Teschemacher, & Kasparov, 2017).

Activated astrocytes induce proinflammatory factors by activating different genes (Glass et al., 2010; Liddelw & Barres, 2017). Signaling pathways such as the Nuclear factor kappa-B (NF- $\kappa$ B) pathway (Brambilla et al., 2005), the JAK/STAT3 pathway (Ceyzériat, Abjean, Carrillo-de Sauvage, Haim, & Escartin, 2016; Herrmann et al., 2008), and the MAPK pathway initiate and modulate astrocyte activity. Cytokines released from activated astrocytes activate glial cells and secreted cytokines, and chemokines facilitate the recruitment of peripheral immune cells (Esen, Tanga, DeLeo, & Kielian, 2004; Rosciszewski et al., 2018). Furthermore, inflammatory mediators secreted by microglia, such as IL-1s and TNF- $\alpha$ , can activate proinflammatory astrocytes and cause a secondary inflammatory response (Liddelw & Barres, 2017). Regulating the polarization of astrocytes into proinflammatory and neuroprotective phenotypes plays a central role in balancing the mechanisms of action of activated astrocytes (L. Li, Acioglu, Heary, & Elkabes, 2021).



**Figure 2.** Signaling associated with microglia and astrocytes.

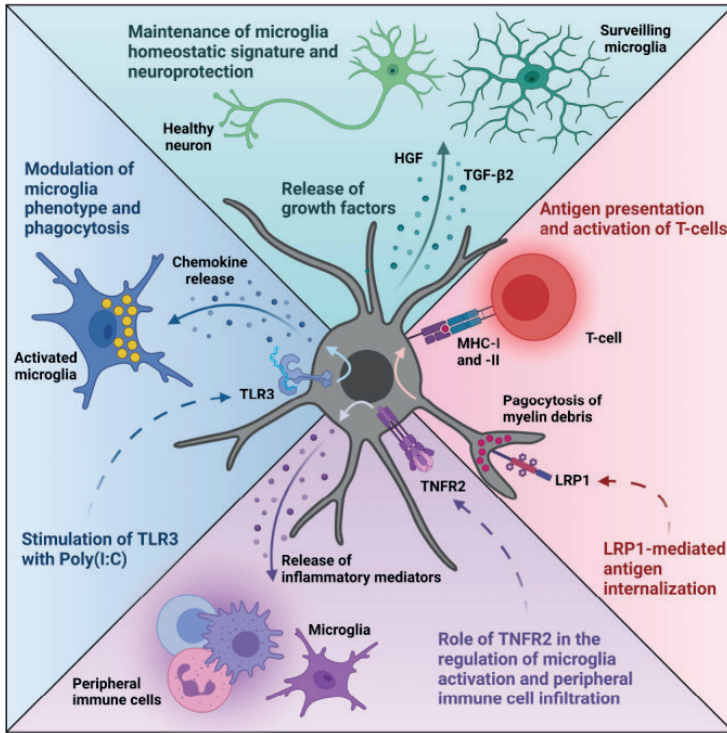
### 3.1.3. Oligodendrocytes

Oligodendrocytes are one of the primary cell types in the CNS, produced from oligodendrocyte progenitor cells (OPCs) (Bradl & Lassmann, 2010). They are required for myelin formation in the developing CNS and are critical for myelin regeneration after injury (Nave, 2010).

Oligodendrocytes communicate with microglia and astrocyte cells (Koning, Swaab, Hoek, & Huitinga, 2009). Under physiological conditions, the release of growth factors by OPCs is essential for the protection of microglia and neurons from degeneration (Nakano et al., 2017; Zhang et al., 2019). In CNS injury, oligodendrocytes help the inflammatory response by expressing inflammatory mediators and various receptors for immune-related molecules. Oligodendroglia tumor necrosis factor receptor 2 (TNFR2) regulates the expression of inflammatory molecules and controls excessive microglia activation (Figure 3) (Desu et al., 2021; Madsen et al., 2020).

Toll-like receptors (TLRs), a member of the pattern recognition receptor (PRR) family, are thought to be necessary for the maturation and survival of oligodendrocytes (Sloane et al., 2010). Stimulation of TLR3 induces homeostasis of the microglia phenotype by affecting the immunomodulatory properties of OLs (Bocczazzi et al., 2021). Researchers believe that oligodendrocyte-microglia and oligodendrocyte-astrocyte cross-talk occur at the level of pattern recognition receptors (PRRs) in neurodegenerative diseases. However, they

consider studies on this pathway insufficient (Sloane et al., 2010). In addition, oligodendrocytes contribute to the inflammatory response by being involved in the release of a wide range of inflammatory cytokines, including IL family (Peferoen, Kipp, van der Valk, van Noort, & Amor, 2014).



**Figure 3.** Functions of oligodendrocytes in neuroinflammation (Boccazzi, Raffaele, & Fumagalli, 2022).

Acetylcholine regulates the inflammatory reflex of innate immune cells such as microglia, astrocytes, and oligodendrocytes by inhibiting the release of pro-inflammatory cytokines. CAIP-mediated signals are involved in releasing and inhibiting inflammatory cytokines secreted from macrophages and peripheral tissue by stimulating acetylcholine release (Borovikova et al., 2000; De Angelis, Bernardo, Magnaghi, Minghetti, & Tata, 2012). Microglia, astrocytes, and oligodendrocyte cells in the CNS express muscarinic and nicotinic AChRs and regulate the cholinergic anti-inflammatory pathway. Activation of  $\alpha 7$ nAChR on glial cells, particularly microglia, has been associated with anti-inflammatory effects, leading to suppression of pro-inflammatory cytokines and reduced inflammation in the CNS (De Angelis et al., 2012; Mizrachi et al., 2021; Ramos-Martínez, Rodríguez, Cerbón, Ramos-Martínez, & Ramos-Martínez, 2021). Specifically, activation of  $\alpha 7$ nAChR in macrophages, monocytes and dendritic cells inhibits the synthesis of pro-

inflammatory, allowing the generation of anti-inflammatory responses in the CAIP pathway (Hoover, 2017).

#### 4. ROLE OF CYTOKINES IN NEUROINFLAMMATION

Cytokines are proteins (15-25 kDa) that are released from immune cells such as monocytes, macrophages, and lymphocytes. Cytokines are activated when inflammation, infection, and immunologic changes occur and are mainly involved in repairing damaged tissues and regulating homeostasis (Nathan, 2002; Woodroffe, 1995). Cytokines facilitate communication between immune cells and can be classified as either pro-inflammatory, promoting inflammation, or anti-inflammatory, suppressing it (Table 1) (Kim, Na, Myint, & Leonard, 2016).

**Table 1.** *Cytokines and their functions*

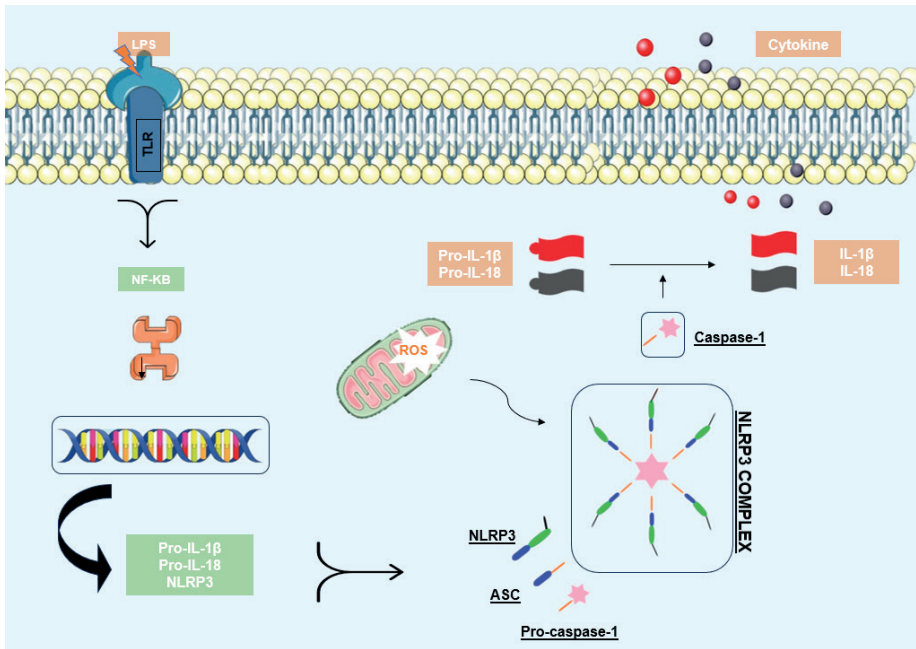
Inflammatory molecule	Family	Source	Function
<b>Pro-inflammatory cytokines</b>			
<b>IL-1<math>\beta</math></b>	IL-1	Macrophage, monocyte	Proliferation, apoptosis, and differentiation
<b>IL-6</b>	IL-6	Macrophages, T cells and adipocytes	Differentiation and cytokine production
<b>IL-8</b>	CXC	Macrophages, epithelial and endothelial cells	Chemotaxis, and angiogenesis
<b>IL-12</b>	IL-12	Dendritic cells, macrophages, and neutrophils	Cell differentiation and activation of NK cells
<b>TNF-<math>\alpha</math></b>	TNF	Macrophages, NK cells, CD4+ lymphocytes and adipocytes	Cytokine production, cell proliferation, apoptosis and anti-infection
<b>IFN-<math>\gamma</math></b>	IFN	T cells, NK cells and NK cells	Innate and acquired immunity
<b>Anti-inflammatory cytokines</b>			
<b>IL-4</b>	IL-4	T cells	T and B cell proliferation and B cell differentiation
<b>IL-10</b>	IL-10	Monocytes, T and B cells	Inhibition of proinflammatory cytokines
<b>IL-11</b>	IL-6	Fibroblasts, neurons, and epithelial cells	Promotes differentiation and induces acute phase protein
<b>TGF-<math>\beta</math></b>	TGF	Macrophages and T cells	Inhibition of pro-inflammatory cytokines

## 5. INFLAMMASOME COMPLEX AND NEUROINFLAMMATION

Microglia and astrocytes are integral components in orchestrating the innate immune response within the CNS, actively engaging in the defense against infections, neurodegenerative pathologies, and traumatic injuries (Farina, Aloisi, & Meinl, 2007; Rivest, 2009). Pattern recognition receptors (PRRs), which play an important role in mediating innate immune responses, are expressed by glial cells (Bsibsi, Ravid, Gveric, & van Noort, 2002; Farina et al., 2005; Takeuchi & Akira, 2010).

Glial PRRs are involved in the neuroinflammation process as key “danger sensors” in CNS (Heneka et al., 2014). PRRs mediate the cross-talk between glial cells and neurons and facilitate interactions between immune cells and cells specific to the central nervous system (CNS). The PRR member with the most knowledge is toll-like receptors (TLRs) (Kawai & Akira, 2011; Takeda & Akira, 2005). In the CNS, TLRs exhibit widespread expression and actively contribute to cell survival, playing a pro-inflammatory role against pathogens. TLRs are involved in AD patients (Y. Liu et al., 2005), and other neurodegenerative diseases such as ALS and PD (Bsibsi et al., 2002; Walter et al., 2006). Plasma membrane TLRs recognize bacterial-associated lipid and protein, including lipopolysaccharide (LPS). Each TLR specifically recognizes bacterial-associated structures. For instance, TLR4 can specifically recognize Gram-negative bacterial LPS (Figuroa-Hall, Paulus, & Savitz, 2020). TLRs mediate the activation of inflammatory regulators through the generation of nucleotide-binding oligomerization (NOD)-like receptors (NLRs), a newly identified type of PRRs (Bourgeois & Kuchler, 2012; Shao, Xu, Han, Su, & Liu, 2015). Structurally, NLRs are usually composed of C-terminal leucine-rich repeats (LRRs) and a central oligomerization domain (NOD/NACHT) flanked by an N-terminal caspase recognition domain (CARD) or pyrin domains (PYDs).

There are four defined types of inflammasomes (NLRP1, NLRP3, NLRC4, and Aim2 inflammasomes) (Lamkanfi & Dixit, 2014; Lu et al., 2020) NLRP3 plays an important role both in shaping immune responses and in many common inflammatory diseases (Pellegrini, Antonioli, Lopez-Castejon, Blandizzi, & Fornai, 2017). NLRP3, a multiprotein complex comprised of ASC (apoptosis-associated speck-like protein), an adaptor protein linked to apoptosis, and the effector procaspase-1, engages in interactions with ASC to facilitate the binding and activation of procaspase-1. This interaction enables the activation of caspase-1. Active caspase-1 catalyzes the conversion of pro-IL-1 $\beta$  and pro-IL-18 to mature and biologically active IL-1 $\beta$  and IL-18. These activated cytokines initiate a cascade of inflammatory responses (Figure 4.) (Liston & Masters, 2017; A. Lu et al., 2014; Mangan et al., 2018; Próchnicki & Latz, 2017).



**Figure 4.** *LPS-induced NLRP3 inflammasome pathway*

Inflammasomes and cholinergic systems are closely related to the aging process and each other. Their activation causes accelerated progression of biological aging and age-related disorders (Figure 5) (Hu et al., 2019; Schliebs & Arendt, 2011). Microglia and macrophages, astrocytes, neurons, and other cells in the brain are the cell types in which inflammasomes and cholinergic pathways are activated during aging processes and are also critical effectors and regulators in various neurodegenerative diseases (Alen, 2022). Several studies are reporting that activating  $\alpha 7nAChR$  can inhibit the NLRP3 inflammasome (Ke et al., 2017; B. Lu et al., 2014). However, the underlying mechanism remains unclear.

The role of inflammasomes in neurodegenerative disease pathways makes them a potential drug target for neuroinflammation and neurodegeneration. NLRP3 inhibitors can be categorized into two main groups: those directly hindering NLRP3 activity and those deactivating NLRP3 by targeting its constituents or impeding its signaling cascades. Some drugs approved by the US Food and Drug Administration (FDA) for clinical use, such as anakinra, canakinumab, and riloncept, inhibit the cytokines released upon NLRP3 inflammasome activation. However, therapeutic approaches targeting the mechanism of inflammasome-mediated inflammation still need to be explored despite representing a promising avenue for future research.

**Table 2.** Association of the inflammasome complex with some neurodegenerative diseases (Brahadeeswaran, Sivagurunathan, & Calivarathan, 2022)

Alzheimer's Disease	Parkinson's Disease	Huntington's Disease
<p style="text-align: center;"><u>Causes</u></p> <p>Genetic mutations Oxidative stress Environmental factors</p> <p style="text-align: center;">↓</p> <p style="text-align: center;"><u>Pathological changes</u></p> <p>Aβ plaque formation Formation of tau proteins and Hyperphosphorylation of tau Neurofibrillary entanglement</p> <p style="text-align: center;">↓</p> <p>Immune system and activation of microglia</p> <p style="text-align: center;">↓</p> <p>Activation of inflammasomes (NLRP3) and production of inflammatory cytokines</p> <p style="text-align: center;">↓</p> <p>Neuroinflammation and degeneration</p> <p style="text-align: center;">↓</p> <p>Synapse loss and neuronal cell death</p> <p style="text-align: center;"><u>Symptoms</u></p> <p>Short-term memory loss Impaired judgment Language dysfunction Behavioral disorders</p>	<p style="text-align: center;"><u>Causes</u></p> <p>Genetic mutations Oxidative stress Environmental factors</p> <p style="text-align: center;">↓</p> <p style="text-align: center;"><u>Pathological changes</u></p> <p>Protein Aggregation, Misfolding, Mitochondrial dysfunction etc. Synuclein accumulation and Lewy body formation</p> <p style="text-align: center;">↓</p> <p>Immune system and activation of microglia</p> <p style="text-align: center;">↓</p> <p>Activation of inflammasomes (NLRP3) and production of inflammatory cytokines</p> <p style="text-align: center;">↓</p> <p>Neuroinflammation and degeneration of dopaminergic neurons</p> <p style="text-align: center;">↓</p> <p>Decreased dopamine levels</p> <p style="text-align: center;"><u>Symptoms</u></p> <p>Bradykinesia Postural instability Stiffness and Tremor</p>	<p style="text-align: center;"><u>Causes</u></p> <p>Genetic mutations in the Huntington's gene</p> <p style="text-align: center;">↓</p> <p style="text-align: center;"><u>Pathological changes</u></p> <p>Mutant Huntingtin protein formation and aggregation Mitochondrial dysfunction, abnormal ROS production and Decreased ATP synthesis</p> <p style="text-align: center;">↓</p> <p>Immune system and activation of microglia</p> <p style="text-align: center;">↓</p> <p>Activation of inflammasomes (NLRP3) and production of inflammatory cytokines</p> <p style="text-align: center;">↓</p> <p>Neuroinflammation and impaired synaptic transmission</p> <p style="text-align: center;">↓</p> <p>Decreased levels of GABA and substance P</p> <p style="text-align: center;"><u>Symptoms</u></p> <p>Dementia Psychiatric Symptoms Abnormal movements</p>

### Acknowledgement

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## *Chapter 3*

### **THE RELATIONSHIP OF MITOCHONDRIA AND AGING IN THE WOUND HEALING PROCESS**

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## 1. INTRODUCTION

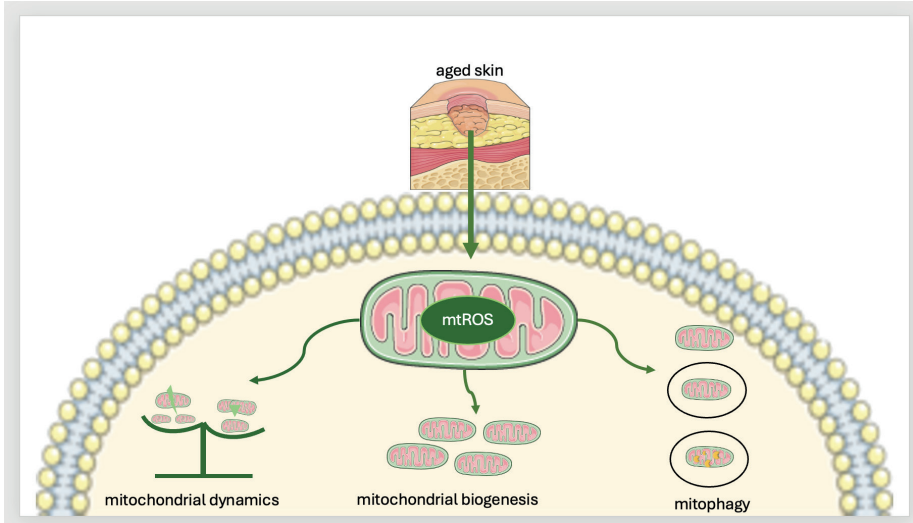
Impairment of wound healing is a clinical problem that negatively affects the quality of life of individuals. The wound healing process is not limited to the wound site but affects the whole system. The wound healing process consists of sequential and intertwined stages: hemostasis, inflammation, proliferation, and remodeling. During a healthy wound-healing period, these stages are completed in full. However, the adverse effects of many systemic and local factors can impair wound healing. Impaired wound healing is characterized by prolonged inflammation, defective extracellular matrix formation, and impaired re-epithelialization. Prolonged wound-healing processes lead to the formation of non-healing chronic wounds (Robson, Steed, & Franz, 2001; Viera, Vivas, & Berman, 2012). Aging is a risk factor for delayed wound healing. Our skin changes as we age, which can cause delays or impairments in the healing process. These changes can affect how our skin looks and functions, making older people more susceptible to wound infection, trauma, and the development of chronic wounds (Gosain & Dipietro, 2004). Venous leg ulcers, pressure ulcers, and diabetic foot ulcers are among the most common types of chronic wounds in the elderly. The risk of chronic wounds increases as older people undergo surgery more frequently, and their physical abilities deteriorate over time (Eming, Martin, & Tomic-Canic, 2014).

Mitochondria are the energy source needed to sustain the life of cells and tissue metabolism. The skin is constantly renewed, and the energy requirement for this is adenosine triphosphate (ATP). The organelle responsible for producing ATP in eukaryotic cells is the mitochondria. ATP is produced due to oxidative phosphorylation in mitochondria (Cooper & Hausman, 2000). As a result of OXPHOS, some harmful by-products are formed in addition to ATP. These are reactive oxygen species (ROS), such as superoxide anion, singlet oxygen, and peroxides that damage cellular structures. Oxidative damage occurs with increased ROS production, and aging constitutes a crucial molecular basis of various pathophysiological conditions such as cancer. In aging cells, the respiratory chain becomes less efficient (Kirkinezos & Moraes, 2001; Sreedhar, Aguilera-Aguirre, & Singh, 2020). Mitochondrial dysfunction, one of the essential indicators of aging, occurs with inadequate mitochondrial dynamics. In addition, dysfunctions occurring in mitochondria affect various pathophysiological processes, including aging and wound healing, as they affect oxidative damage (Sreedhar et al., 2020).

Oxidative stress, exacerbated by increased ROS production, underlies mitochondrial dysfunction in the skin and other organs. Therefore, controlling mitochondrial health is essential for skin aging and wound healing. Control of mitochondrial health is achieved through the regulation of mitochondrial dynamics, mitochondrial biogenesis, and mitophagy (Chang et al., 2022). The balance of mitochondrial dynamics is achieved through fission and



fusion events (Zacharioudakis & Gavathiotis, 2023). The available evidence suggests that various abnormalities in mitochondrial biological processes can be considered alterations in mitochondrial function and have been shown to play a vital role in wound healing (Rizwan, Pal, Sabnam, & Pal, 2020). In this section, we will describe the changes in wound healing with aging and the fundamental role of mitochondria in regulating normal skin physiology, skin aging, and wound healing (Figure 1).



**Figure 1.** Mitochondrial network in wound healing in aged skin. Figures used in the figure are illustrated using Servier Medical Art, licensed under a Creative Commons Attribution 4.0 Unported License (<https://creativecommons.org/licenses/by/4.0/>).

## 2. WOUND HEALING

Disruption of the normal integrity of living tissue due to physical, chemical, or thermal damage triggers wound healing, returning the tissue to its former form (Nagle, Stevens, & Wilbraham, 2022). Wound healing is a well-organized biological process of sequential stages, including hemostasis, inflammatory events, cell proliferation, and tissue remodeling (Oliveira, Simões, Ascenso, & Reis, 2022). Hemostasis, the first stage of wound healing, is achieved by constricting blood vessels and preventing bleeding. The hemostatic process begins with forming a fibrin clot by platelet cells. The blood clot also serves as a temporary matrix to defend against microbial invasion and recruit inflammatory cells (Sen and Roy, 2012). Growth factors (such as growth factor-beta (TGF-beta), platelet-derived growth factor (PDGF), and vascular endothelial growth factor (VEGF)) are released by platelets for the infiltration of inflammatory cells into the wound site.

Following the hemostasis phase, the inflammatory phase begins with recruiting neutrophils, monocytes, and lymphocytes to the wound site. With increased blood vessel permeability, neutrophils and monocytes migrate to the wound site, and local endothelial cells increase permeability by disrupting cell-cell interactions. The wound's initial white blood cell population comprises neutrophils (Petreaca, Yao, Liu, DeFea, & Martins-Green, 2007). Neutrophils are involved in the secretion of proteases and antimicrobial peptides to prevent microbial contamination at the wound site. They also protect against bacterial invasion with a "respiratory burst" by increasing ROS production (Wilgus, Roy, & McDaniel, 2013). On the second to third day of healing, monocytes become the dominant inflammatory cell population in the wound. Chemotaxis of monocytes to the wound site is mediated by chemokines secreted by neutrophils, monocytes, and keratinocytes at different stages of healing (Gillitzer and Goebeler, 2001; Wetzler, Kämpfer, Pfeilschifter and Frank, 2000). Monocytes at the wound site differentiate into macrophages. Macrophages play a role in removing apoptotic neutrophils and other dead cells. They also act as antigen-presenting cells and are responsible for the secretion of cytokines and growth factors (Novak and Koh, 2013). Growth factors activate endothelial cells, fibroblasts, and keratinocytes. Fibroblasts and keratinocytes stimulate cell proliferation, and VEGF makes wound healing possible by stimulating angiogenesis (Sabine A Eming, Krieg and Davidson, 2007). Lymphocytes, the last leukocyte type to reach the wound site, react specifically against microbes and other foreign substances in the wound (Martins-Green, Petreaca, & Wang, 2013).

During the proliferation phase, parallel to the inflammation phase, fibroblasts and endothelial cells migrate to the wound site (Lund et al. 1999). Proteases such as Serine, Cysteine, and Matrix metalloproteinase (MMP) are released to aid cellular migration through the fibrin clot and temporary matrix. The main events of this phase include the entry of fibroblasts, the deposition of extracellular matrix (ECM), the formation of new blood vessels, and re-epithelialization (Brix, Dunkhorst, Mayer, & Jordans, 2008). Fibroblasts are the primary cell type involved in the healing process and are responsible for producing fibronectin, hyaluronic acid, collagen, and proteoglycans. These substances help form ECM and keratinocytes (Martin & Leibovich, 2005). The initial fibrin matrix is replaced by granulation tissue, a dense combination of blood vessels, macrophages, and fibroblasts. This tissue is embedded in a loose matrix of fibronectin, hyaluronic acid, and collagen. During the formation of granulation tissue, new blood vessels develop from pre-existing vessels in a process known as angiogenesis. Angiogenic factors secreted by fibroblasts, macrophages, keratinocytes, and endothelial cells trigger angiogenesis (Li et al., 2014).

The remodeling stage is the stage in which the contraction rate of the wound increases, and the collagen is reshaped. In this stage, the last stage of

wound healing, collagen level is determined by MMP activity. MMP secreted by keratinocytes also regulates keratinocyte migration and re-epithelialization (Mott & Werb, 2004). MMPs, which are critical for wound healing, constitute a family of zinc-dependent endopeptidases that can degrade various macromolecular components of the ECM from proteases. MMPs degrade a range of ECM-related proteins, such as proteoglycans and collagen, and show a generalized proteolytic activity in the wound area (Saboo, Rathnayake, Vangaveti, & Malabu, 2016). Type IV collagen is synthesized by keratinocytes, and fibroblast regeneration forms the basement membrane immediately after the epithelial layer (Wojtowicz et al., 2014). Wound myofibroblasts and collagen balance determine the wound contraction process (Toriseva & Kähäri, 2009). Wound tensile strength increases rapidly between 1 and 8 weeks after injury. The result of wound healing is expressed by scar formation (Lau, Gobin, & West, 2006).

### 3. AGING AND WOUND HEALING

Aging is a degenerative process characterized by cellular dysfunction and disruption of homeostasis. With aging, changes are observed in all body functions. Due to aging, there is a gradual decline in tissue integrity, function, and regenerative capacity (López-Otín, Blasco, Partridge, Serrano, & Kroemer, 2013).

The combined effect of intrinsic and extrinsic aging manifests itself in aged skin. Intrinsic aging occurs genetically, independent of environmental influences. Extrinsic aging is the deterioration of the skin under the influence of environmental factors. It reflects effects such as exposure to UV light and environmental pollution on the skin structure. Combining intrinsic and extrinsic effects with aging leads to loss of skin functions, increased environmental sensitivity, and disruption of skin homeostasis. Under these factors, deterioration, dryness, pigment changes, wrinkles, sagging, and even benign or malignant tumors are observed in aged skin (Puizina-Ivic, 2008; Tobin, 2017).

The skin consists of three layers: epidermis at the outermost layer, dermis, and hypodermis at the bottom. The epidermis layer acts as a protective shield against external factors. It also contributes to maintaining the skin's moisture content by controlling water entry. With age, the skin's ability to retain moisture decreases as a result of changes in the epidermis layer. Skin with less moisture becomes drier and rougher. Another effect of aging is atrophy of the dermal-epidermal junction, even if the epidermal thickness does not change (Blair, Jones, Woessner, & Quinn, 2020; Gosain & Dipietro, 2004). In addition, the number of keratinocytes migrating to the skin surface is reduced in older individuals.

The dermis layer, which lies just below the epidermis, contains networks of collagen and elastin fibers that give the skin elasticity and firmness. The dermis

layer is analyzed in two structures: the superficial papillary dermis and the deep reticular dermis (Montagna & Carlisle, 1979). The surface contact between the epidermis and the dermis decreases with increasing age. The protrusions in the papillary dermis that maintain epidermal contact deteriorate, and surface contact decreases (Kurban & Bhawan, 1990). The number and function of antigen-presenting cells, such as fibroblasts, Langerhans cells, and mast cells in the dermis, are lost with age. At the same time, there is a decrease in protein content. While collagen synthesis decreases with age, collagen degradation increases. Elastin, responsible for skin elasticity, also shows a disorganized morphology in the aged dermis, leading to reduced skin elasticity (Shin et al., 2019). The dermis is the layer responsible for insulation and heat retention in the body. Skin aging can deplete the fat-storing cells in the adipose tissue, reducing the thickness of the skin and causing wrinkles to appear. It also leads to impaired temperature control in older people. In particular, the subcutaneous layer becomes thinner on some parts of the face and hands with age, while it becomes thicker on the thighs and abdomen (Karim & Aryani, 2021).

Structural and functional changes in the skin structure with aging increase the skin's susceptibility to injury. It also negatively affects the healing capacity of the skin. Delays in wound healing have been observed in various studies examining age-related changes (Gillian S Ashcroft, Mills, & Ashworth, 2002). The most surprising result of the studies is the finding that the effect of aging in healthy older adults causes a temporary delay in wound healing but does not cause an actual deterioration in the quality of healing (Gosain and Dipietro, 2004). In this context, healthy aging strategies also affect wound healing quality.

### 3.1. Changes in Wound Healing Phases During the Aging Process

Hemostasis Phase	Inflammation Phase	Proliferation Phase	Remodeling Phase
- Increased platelet aggregation	- Decreased capillary permeability	- Decreased size and number of fibroblasts	- Reduced collagen turnover
- Decreased collagen levels	- Decreased neutrophil diapedesis	- Delayed collagen synthesis	- Increased MMP's activity
- Increased growth factor	- Increased inflammation mediators	- Delayed granulation tissue	- Impaired scar formation
- Increased cytokines	- Impaired T-cell infiltration	- Delayed angiogenesis	
		- Delayed keratinocyte migrations	
		- Delayed re-epithelialization	

*Age-related changes in wound healing are shown in Table 1.*

**Table 1.** *Age-related changes in wound healing.*

### **Hemostasis Phase:**

Specific changes in the coagulation and immune systems with aging lead to alterations in the hemostasis phase of wound healing. With increasing age, changes in cell adhesion, migration, and functional responses are observed. Decreased collagen content in the skin and increased endothelial damage due to aging lead to increased adhesion of platelets to damaged endothelium and increased production of various growth factors and cytokines (Gillian S Ashcroft, Mills, & Ashworth, 2002; Khalid, Nawi, Zulkifli, Barkat, & Hadi, 2022).

### **Inflammation Phase:**

The inflammation phase, one of the most critical phases of wound healing, can be prolonged with aging. The prolonged inflammation phase leads to the formation of chronic wounds that heal later than the normal healing process of the wound. With increasing age, capillary permeability decreases, and diapedesis of neutrophils decreases. Decreased levels of nitric oxide, a vasoactive mediator due to aging, reduce capillary permeability and limit the diapedesis of neutrophils (Gosain & Dipietro, 2004). Aging leads to a significant decrease in the respiratory burst activity of neutrophils. Moreover, decreased burst activity leads to impaired phagocytosis of bacteria (Lipschitz & Udupa, 1986; Lord, Butcher, Killampali, Lascelles, & Salmon, 2001). Leukocytes, the second cell population in the wound area, show an age-related increase in the secretion of and response to many inflammatory mediators (Gosain & Dipietro, 2004). The other active cell community in the wound area is monocytes. Due to aging, the large size of monocytes makes it difficult to infiltrate the damaged area. Adhesion molecules are needed for their passage to the wound area. However, aging decreases these molecules significantly (Zhong, Simard, & Huot, 2018). Thus, monocytes that pass to the wound area in small numbers may mature and turn into macrophages in small numbers at the wound area. Macrophages play essential roles in the inflammatory phase. Macrophage depletion in wound tissue negatively affects granulation tissue formation and angiogenesis. It also leads to impaired synthesis of collagen and growth factors, resulting in delayed wound healing (Sgonc & Gruber, 2013; Vu et al., 2022). Infiltration of T cells, the last cell community of the inflammatory phase, is also disrupted, but the effects of this are not yet apparent (Sgonc & Gruber, 2013).

### **Proliferation and Remodeling Phase:**

The proliferative response of fibroblasts, keratinocytes, and vascular endothelial cells is delayed during the proliferation phase of wound healing with aging. In this case, delays occur in re-epithelialization, collagen synthesis, and angiogenesis in the proliferation phase, and a prolonged wound healing process is observed (Reed, Ferrara, & Vernon, 2001). With advancing age,

fibroblasts decrease in size and number. In addition, the response of fibroblasts to growth factors also decreases (M. D. West, 1994). Previous studies have also shown that re-epithelialization and wound contraction decrease with age (Bond et al., 2008; Consortium, 2020; Kim, Mustoe, & Clark, 2015). In healthy wound healing, the epidermis and newly developing blood vessels that need nutrition for proliferation play a crucial role. Therefore, angiogenesis plays a vital role in optimal wound closure. Although there are contradictions in the literature, it has been shown that angiogenesis decreases with age. Vascular endothelial cells are affected, and decreased angiogenic factors (FGF, VEGF, and TGF- $\beta$ ) lead to delayed angiogenesis with advancing age. Collagen synthesis, which plays an active role in wound closure, deteriorates with aging. MMPs provide collagen balance in wound healing; it has been determined that MMP levels increase with age. The balance of MMP activity is maintained by tissue inhibitors of metalloproteinases (TIMPs). Studies have shown that delayed healing in the elderly is due to overexpression of MMPs, under-expression of TIMPs, or both (Gillian S. Ashcroft, Horan, & Ferguson, 1997; Simonetti et al., 2013).

#### 4. MITOCHONDRIAL PERSPECTIVE ON WOUND HEALING IN AGING

Mitochondria are widely referred to as the cell's power source due to their innate functions to meet the cell's energy needs through oxidative phosphorylation (OXPHOS). Mitochondria also control cell life and death mechanisms and regulate cell metabolism, ion homeostasis, cell growth, and cell signaling (Jiang et al., 2023).

Mitochondria, responsible for OXPHOS, are the primary ROS producers and the first ROS target. Mitochondria affected by ROS are also thought to play a role in aging. Redox signaling plays a role in regulating cell function, but high concentrations of ROS can cause severe damage to mitochondria and other cellular components, leading to aging. Another theory based on the Free Radical Theory, which remains popular among aging theories, is the Mitochondrial Aging Theory. This theory suggests that oxidative damage that occurs during oxidative phosphorylation of mitochondrial macromolecules such as mtDNA, proteins, or lipids is responsible for aging (Miquel, Economos, Fleming, & Johnson Jr, 1980). As cells age, respiratory chains become less efficient, leading to increased electron leakage and reduced ATP production. It ultimately leads to higher ROS levels, exacerbating age-related damage (Cui, Kong, & Zhang, 2012). Loss of mitochondrial activity is likely a cause and consequence of aging.

Mitochondria are vital for maintaining healthy skin as they actively participate in various processes such as cell signaling, pigmentation, hair growth, and wound healing. Mitochondria involved in the skin's microbial defense increase glucose and ATP production in response to infection—

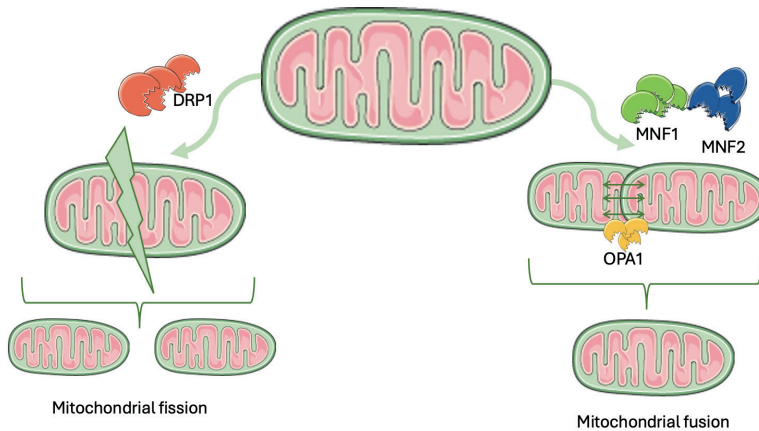
additionally, mitochondrial ROS production increases during defense against microbial infections (Stout & Birch-Machin, 2019). Mitochondrial function and ROS production help regulate stem cell differentiation. The continuous renewal of the skin epidermis depends on ATP produced by mitochondria. In addition, mitochondrial respiration and ROS signaling play a crucial role in the differentiation of keratinocytes, which are also important in wound healing. Although ROS act as essential signals in regulating cell functions, high ROS concentrations cause destructive effects in the cell. Therefore, it is crucial to maintain mitochondrial function and integrity in establishing ROS balance. Another essential condition for the skin is UV radiation and air pollution. UV radiation and environmental pollution can cause oxidative stress in the skin, which leads to increased levels of ROS and triggers skin aging. The first target of ROS within the cell is mtDNA, making it highly susceptible to oxidative stress (Birch-Machin & Bowman, 2016). According to the model proposed by Krutmann and Schroeder (2009), mtDNA loss occurs in dermal fibroblasts due to photoaging. mtDNA loss leads to insufficient energy supply. The loss of energy required for regeneration causes structural changes and loss of function in the skin (Krutmann & Schroeder, 2009). The combined effects of intrinsic and extrinsic factors cause skin aging. Studies also show that mitochondria play a role in many age-related skin healing disorders (Schiffmann et al., 2020; Xu, Li, Bjorklund, & Xu, 2022).

Mitochondria and ROS are essential in promoting wound healing. However, it is vital to consider the biphasic and dose-dependent roles of ROS and the different functions of mitochondria and mitochondrial ROS in various cell types. The most notable feature in aged skin is mitochondrial abnormalities. The increase in mtDNA deletions with age, accompanied by increased oxidative stress and loss of mitochondrial membrane potential, causes changes in mitochondrial function (Sreedhar, Aguilera-Aguirre, & Singh, 2020). The central regulators of mitochondrial functions are the balance of mitochondrial dynamics, mitochondrial biogenesis, and controlled mitophagy. The mitochondrial fusion and fission cycle constitute mitochondrial dynamics. In normal cells, mitochondrial fusion and fission must be in a dynamic balance that maintains the morphology and function of the cells (Lin, 2022).

The detachment of the damaged part of the mitochondria is defined as fission. The detached part of the mitochondria is then destroyed by autophagy, the cell's self-cleaning mechanism (Kraus & Ryan, 2017; Otera, Ishihara, & Mihara, 2013). The primary regulator of mitochondrial fission is Dynamin-related protein 1 (DRP1). The first step in mitochondrial fission is the binding of DRP1 from the cytoplasm to the outer mitochondrial membrane. It is mainly mediated by several outer mitochondrial membrane proteins, including mitochondrial fission factor (Mff), mitochondrial fission protein 1 (Fis1), and mitochondrial dynamics proteins (Di Nottia et al., 2021). Once recruited to the

outer membrane, DRP1 surrounds the mitochondria and induces constriction to separate the mitochondria through GTPase activity (Otera ve ark., 2013).

Mitochondrial fusion is a process that occurs with the cooperation of multiple proteins, which refers to the union of the outer and inner mitochondrial membranes (He & Maheshwari, 2023). There are three critical proteins regarding mitochondrial fusion: mitochondrial fusion protein 1 (Mfn1), mitochondrial fusion protein 2 (Mfn2), and optic atrophy 1 protein (OPA1) (Peng, Ramatchandirin, Pearah, Maheshwari, & He, 2022). Mfn 1 and Mfn2 are involved in mitochondrial outer membrane fission. OPA1 is involved in mitochondrial inner membrane fusion (C. Zhang et al., 2023). GTP is hydrolyzed by the cooperation of Mfn1 and Mfn2, resulting in membrane conformational changes leading to outer mitochondrial membrane fusion. OPA1, a transmembrane protein closely associated with the inner mitochondrial membrane, undertakes two primary functions. First, it triggers inner membrane fusion by mediating endosomal fusion. Secondly, it plays a role in preserving energy by maintaining the mitochondrial crista structure. Deletion of the OPA1 gene causes mitochondrial fragmentation, while overexpression of OPA1 contributes to mitochondrial elongation (Jiangnan Zhang, Qiao, & Luo, 2023).



**Figure 2.** *Mitochondrial Fission and Fusion. Figures used in the figure are illustrated using Servier Medical Art, licensed under a Creative Commons Attribution 4.0 Unported License (<https://creativecommons.org/licenses/by/4.0/>).*

The role of mitochondria is essential for wound healing to be completed healthily. Therefore, it is thought to play an active role in mitochondrial dynamics and maintaining mitochondrial health during wound healing. Recent studies



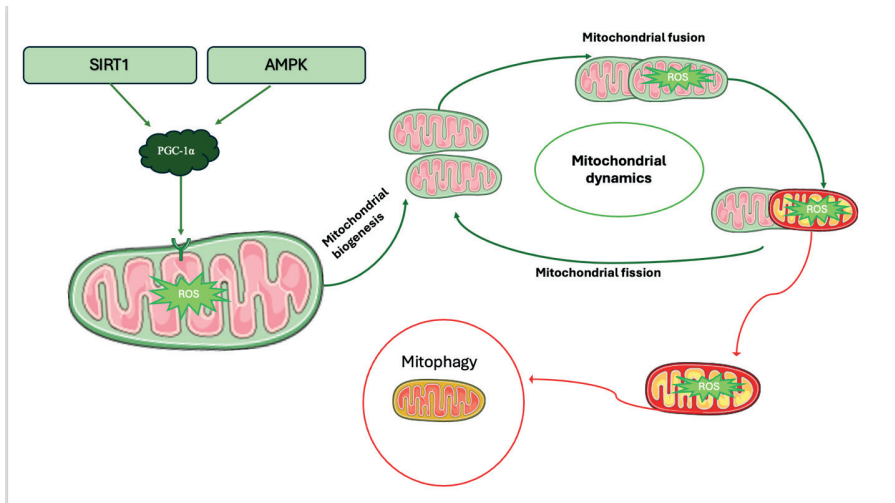
support this idea, and the importance of mitochondrial fission on wound healing has been advocated (Fu et al., 2020; Ponte et al., 2020). Immediately following injury, mitochondria undergo rapid and reversible degradation. The fission control protein DRP1 regulates wound-induced fragmentation of mitochondria (Ponte et al., 2020). It has yet to be elucidated how DRP1 senses damage and triggers mitochondrial fission. Fragmentation of mitochondria after injury has been examined in studies in different organisms following epidermal damage. Nevertheless, the studies are those other than those of human tissue, and differences in findings can be observed in human tissue. In vivo studies have shown that mitochondria have a fragmented phenotype in the differentiation of epidermal keratinocytes (Ipponjima, Umino, Nagayama, & Denda, 2020; Mellem et al., 2017). It is observed that mitochondrial fragmentation is thought to result from reduced energy requirements of keratinocytes. It also plays an active role in mitochondrial fragmentation, macrophage apoptosis, and cell proliferation. Therefore, properly regulating this physiological process is critical for healthy wound healing. Mitochondrial fragmentation is also known to promote macrophage apoptosis and cell proliferation. Proper regulation of these physiological processes is critical for well-coordinated wound healing (Parker et al., 2015; Seo, Yoon, & Do, 2018).

The effect of abnormalities caused by the imbalance in mitochondria dynamics on aging is undeniable. Mitochondria, critical in skin health with aging, also play an essential role in skin aging. It has been observed that Fis1 and DRP1 expression decreases in aging cells while Mfn protein levels increase (Gomes, Benedetto, & Scorrano, 2011; Lee et al., 2007). Additionally, decreased Fis1 levels increase the degradation of OPA1, triggering premature aging such as lysosomal accumulation (Lee et al., 2007). Ledrhem et al. (2022) focused on the importance of DRP1, which is involved in mitochondrial fission, in skin aging. They suggested that DRP1 expression in skin cells is inhibited through mitochondrial regulation of Sirtuin1 (SIRT1) activation, which, with increasing age, may alleviate skin aging (Ledrhem et al., 2022). Among environmental factors, the effect of UV exposure on keratinocyte cells has been shown to accelerate the aging of keratinocyte cells by inducing DRP1 translocation to promote fission in mitochondria (Jugé et al., 2016). Gupta et al. (2022) demonstrated in their study that UV exposure causes excessive mitochondrial fission by increasing the expression of proteins involved in mitochondrial dynamic processes, which leads to skin cell senescence as a result of activation of apoptotic signaling by promoting excessive ROS production (Gupta, Archoo, Naikoo, & Abdullah, 2022). The role of mitochondrial fusion in skin aging has yet to be fully elucidated. However, studies have shown that Mfn expression is increased in skin cancer and melanoma. It is thought that this may cause skin lesions by increasing mitochondrial fusion (Soares et al., 2019). The effects of mitochondrial dynamics in aging, especially skin aging, may play a critical role

during wound healing in aged skin. Imbalances in mitochondrial dynamics may negatively affect wound healing and cause delayed wound healing.

Mitochondrial biogenesis plays an active role in ensuring the energy supply required for cells. The process of synthesizing mitochondria in cells affected by toxin accumulation and mtDNA mutations, which can be activated under increasing stress conditions, is called mitochondrial biogenesis (C. Zhang et al., 2023). Peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1 $\alpha$ ) regulates mitochondrial biogenesis. PGC-1 $\alpha$  acts as a signaling molecule that directs mitochondrial DNA replication, transcription, and translation into proteins by translation factors (Chen et al., 2022).

Mitochondrial biogenesis decreases with age. There is a close connection with the sirtuin family, which plays an especially active role in aging. Activating protein kinase (AMPK) nicotinamide activates SIRT1 by increasing the level of adenine dinucleotide (NAD) and can modulate PGC-1 $\alpha$  (Abu Shelbayeh, Arroum, Morris, & Busch, 2023). Jing et al. (2021) showed that AMPK phosphorylation decreased, and SIRT-1 and PGC-1 $\alpha$  were significantly downregulated (Jing et al., 2021). Another sirtuin that affects mitochondrial biogenesis is SIRT3. Overexpression of SIRT3 leads to up-regulated expression of PGC-1 $\alpha$ . Sirt3 is vital in maintaining mitochondrial health through the SIRT3-AMPK-PGC-1 $\alpha$  pathways (C. Zhang et al., 2023). Increased PGC-1 $\alpha$  facilitates mitochondrial biogenesis, improves mitochondrial function, and delays skin aging caused by intrinsic and extrinsic factors.



**Figure 3.** SIRT1-AMPK-PGC-1 $\alpha$  axisn (Abu Shelbayeh vd. 2023). Figures used in the figure are illustrated using Servier Medical Art, licensed under a Creative Commons Attribution 4.0 Unported License (<https://creativecommons.org/licenses/by/4.0/>).

The role of mitochondrial biogenesis in wound healing has yet to be extensively studied. However, it has proven crucial for stress-induced metabolic signaling in injured skin fibroblasts and melanocytes (C. Zhang et al., 2023). Wong et al. (2022) showed that the impairment of re-epithelialization in the aged epidermis was caused by decreased PGC-1 $\alpha$  expression in their study. In addition, it has been suggested that PGC-1 $\alpha$  plays a regulatory role in response to agents causing damage during the healing. Also, PGC-1 $\alpha$  contributes to epidermal stem cell and skin repair (Wong et al., 2022).

Another vital regulator in maintaining the health of mitochondria is mitophagy. Mitophagy is an autophagy mechanism. Autophagy is a lysosome-dependent cellular degradation process in eukaryotic cells (Ahmed, Mendonca, Elhag, & Soliman, 2022). Cellular degradation can occur in mitochondria, peroxisomes, and endoplasmic reticulum, depending on the specificity of the substrates (Vargas, Hamasaki, Kawabata, Youle, & Yoshimori, 2023). Autophagy of damaged mitochondria is defined as mitophagy. Eliminating damaged mitochondria within the cell is essential for maintaining homeostasis (Bamshad et al., 2023). Mitophagy can occur through 3 different pathways: NIX/BNIP3-mediated mitophagy, PINK1/Parkin-mediated ubiquitin-induced mitophagy, and FUNDC1-mediated mitophagy (C. Zhang et al., 2023).

Mitochondrial dysfunction leads to disruptions in mitophagy, resulting in impaired cell energetics. Disruption of energy metabolism leads to cell aging. Given that skin tissue is constantly renewed, it is essential to maintain the balance of mitochondrial metabolism for energy supply. In damaged mitochondria, the balance of mitochondrial fission and fusion is disrupted, and ROS release increases. The damage to skin cells is alleviated to some extent by eliminating damaged mitochondria through mitophagy, providing the skin with the necessary energy, and preventing the release of ROS. However, there are conflicting studies on this subject. Liu et al. (2022) suggest it repairs damaged mitochondrial function by increasing mitophagy to restore cellular homeostasis (Liu et al., 2022). The current study also showed that mitophagy was increased by activation of the PINK/parkin pathway. In contrast to these studies, Chen et al. (2022) demonstrated that metformin attenuated UV-induced skin photoageing by suppressing mitophagy (Chen et al., 2022). In line with these studies, it should be kept in mind that mitophagy may have adverse effects in addition to its positive effects, and balanced mitophagy mechanisms should be kept in mind to maintain mitochondrial health.

Some studies suggest that mitophagy, eliminating damaged mitochondria, can impact skin aging. However, there is still controversy surrounding how to regulate mitophagy to delay skin aging. It is mainly because the relationship between mitophagy and apoptosis, a cell death process, is unclear. One theory suggests that regulatory mechanisms are activated by triggering mitophagy, which helps cells maintain homeostasis temporarily and delay apoptosis.

Another theory is that eliminating damaged mitochondria through enhanced mitophagy can improve mitochondrial function, restoring damaged cells and delaying skin aging. Despite these theories, it is still unclear how to manipulate mitophagy to improve skin aging. Therefore, the mechanism of mitophagy remains a crucial area for future research on mitochondria and skin aging (J. Zhang et al., 2023).

More studies in the literature are needed to investigate the effect of mitophagy on wound healing. Experimental studies have focused on the effect of mitophagy on cell migration during re-epithelialization. Studies conducted at different times have shown that the BNIP3-mediated mitophagy pathway plays a role in accelerating the migration of keratinocytes and that this occurs under the control of hypoxia (Junhui Zhang et al., 2019; Junhui Zhang et al., 2017). Feng et al. (2023) reported that hypoxia-induced mitophagy promoted the proliferation and migration of keratinocytes, which agrees with previous studies (Y. Feng et al., 2023). The effect of mitophagy on the inflammatory phase of wound healing has not been directly described, but mitophagy has been shown to alleviate inflammation by clearing damaged mitochondria. There is no study examining the effect of mitophagy in the inflammatory phase, which is a critical phase in wound healing. However, mitophagy is thought to alleviate inflammation by clearing damaged mitochondria (Guo et al., 2014; A. West et al., 2015). Feng et al. (2022) argued in their study that dysregulated mitophagy plays a critical role in the inflammatory phase of wound healing as it triggers chronic wounds. The study also showed that mitophagy promotes angiogenesis and the deposition of collagen fibers (Z.-h. Feng, Chen, Zheng, Zheng, & Zhao, 2022)

## 5. CONCLUSION

Aging is a degenerative process that affects all systems, leading to loss of function and disease. The most dramatic effects of aging are visible in the skin, which acts as the body's barrier. Wrinkles, sagging, and dryness increase with age. These aging-related changes cause significant visual changes and functional deficiencies, making the skin more susceptible to injury. Due to these effects, elderly individuals are prone to wound infection, trauma, and chronic wound development. Mitochondria, which play an active role in this process, directly affect the aging and wound healing processes. Maintenance of mitochondrial health depends on the balance of mitochondrial dynamics and mitophagy. Mitochondrial health affected by aging has critical consequences for wound healing. With aging, imbalances in mitochondrial dynamics cause skin aging and the formation of chronic wounds. It is also suggested that mitophagy, which plays an active role in controlling mitochondrial health, can reverse mitochondrial dysfunctions in the aging process and wound healing. In this context, controlling mitochondrial health will effectively regulate the effects of aging on the skin and close the wound-healing process healthily.

However, more studies are needed to eliminate the contradictions and elucidate the mechanisms fully.

Skin aging occurs due to the interaction of genetic and environmental factors. Physical protection is crucial in preventing environmental factors from causing skin aging. However, the impact of genetic aging should not be ignored. The most basic organelle in maintaining skin homeostasis is the mitochondria. Functional disruptions in mitochondria negatively affect skin health. Disruptions in the functioning of mitochondria, the powerhouses of cells, have been found to respond to injury and can affect signals involved in wound healing. Therefore, using skin care or nutritional products that contain specific agents capable of improving mitochondrial function and increasing the metabolic capacity of the skin can be a promising approach. Further research is required to fully comprehend the effect of aging on mitochondrial activities during the wound healing process.

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## *Chapter 4*

### **THE TOXICOLOGICAL RISKS OF MICROPLASTICS (MPS) IN VARIOUS AQUATIC ORGANISMS**

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Plastics are called synthetic organic polymers derived from petroleum and its derivatives and do not disappear naturally. Every year, 250 million tons of plastics are produced on our planet. In 2020, world plastic production reached 367 million tons. China is responsible for 32% of this production, Japan for 4%, and other Asian countries for the rest (Acarer, 2023). All this plastic waste is considered an emerging pollutant and poses a significant risk to marine biodiversity worldwide. According to NOAA (National Oceanic and Atmospheric Administration), microplastics are plastic parts smaller than 5 mm. They are a growing threat to marine biota, the aquatic environment, and the whole ecosystem (NOAA, 2023).

According to research, microplastic particles were found in the feces of *Callorhinus ursinus* (Northern fur seal). The analyses of fecal samples showed that northern fur seal species were exposed to MPs throughout the Eastern Pacific Ocean (Donohue et al., 2019). Plastic materials accumulate in natural habitats such as soils, rivers, lakes, and seas. MPs are very heterogeneous particles that vary in size, shape, color, chemical composition, density, and other properties. MP particles, generally in amorphous form, can also be spherical, cylindrical, oval, and fibrous fibers of various sizes. Of course, with industrial development, plastic materials benefit our lives. However, it is impossible not to be affected by the permanent damage of plastics that lasts many years. Essentially, it is a significant threat to wildlife. Some studies on microplastics in the sea show significant risks along the food chain, from algae to humans. Microplastics are easily transported to the upper trophic levels in the food chain. MPs were found even in benthic fish, filtration-fed mussels and clams, crabs, and aquaculture (Mathalon & Hill, 2014; Van Cauwenberghe & Janssen, 2013). All kinds of invertebrates, fish, fish larvae, reptiles, birds, and mammal species that swallow plastic parts of different sizes as nutrients lose their lives. The ingestion of MPs by aquatic organisms also causes physical damage to the organism's tissues and organs. Microplastic particles have been found in mussels, clams, crabs, and even aquaculture produced by farms.

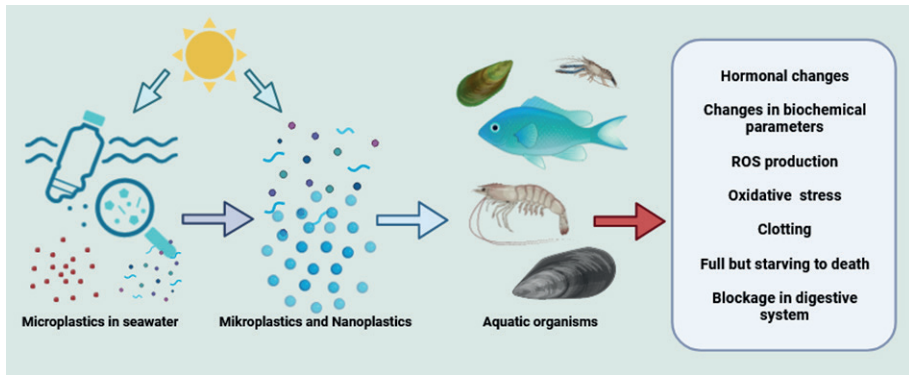
Today, microplastics are an increasingly severe environmental and human health problem. In aquatic environments, microplastic particles can be listed as fiber, polyester, and nylon. Nylon can be considered the first plastic product that enters our daily lives. Plastics are made of polyethylene and consist only of carbon and hydrogen atoms. Nylon, which we frequently use in almost every item in our daily life, consists of carbon, hydrogen, and nitrogen atoms (Ziani et al., 2023; Li et al., 2019). These materials are high molecular weight and highly persistent in the environment because they are not biodegradable. Plastic wastes are separated into microscopic pieces due to photochemical and mechanical processes during their slow natural degradation for many years (Acarer Arat, 2024). MPs are divided into two groups: primary and secondary. The primary group is usually in cosmetic products such as toothpaste,

exfoliating creams, and detergents. Secondary microplastics are caused by the abrasion of large plastic parts by environmental effects such as sunlight, water, and wind, and they crumble to small dimensions. Macro-sized plastics, respectively, are first turned into microplastics and then into nanoplastics with continued disintegration. MPs are not filtered during wastewater treatment and are generally released directly into fresh and saltwater environments such as rivers, lakes, and seas. A study conducted in 2019 reported that the amount of microplastic poured into the sewage due to soap and cosmetics consumption was 2.4 mg per person per day (Zhao *et al.*, 2019).

### **Toxic Effects of Microplastics:**

Plastic polymers may exhibit different degrees of toxicity. Therefore, the adverse effects of microplastics depend on the type of plastic, its chemical composition, and the absorption of chemical pollutants with plastic affinity. For example, Rochman *et al.* (2015) found more histological abnormalities in freshwater oysters (*Corbicula fulminea*) due to exposure to polyvinyl chloride (PVC) or polystyrene (PS) rather than PET or polyethylene. Aquatic organisms accumulate microplastics primarily in the digestive systems. For some aquatic invertebrate species, ingestion of microplastics may not be through nutrition alone. (Li *et al.*, 2015). Microplastic particles were found in the gills of some crabs, too. Microplastics form a cocktail of polymers and additives capable of absorbing all environmental chemicals, including environmental pollutants in the sea. Given their tiny size (<5 µm), microplastics are ingested by various marine organisms as food. (Rummel *et al.*, 2016b). Microplastics have been observed in many fish species caught in oceans, seas, and freshwater (Alomar *et al.*, 2017; Bessa *et al.*, 2018; Jabeen *et al.*, 2017; Morgana *et al.*, 2018; Pazos *et al.*, 2020). A study conducted in Chile identified and classified various microplastics in the stomach and intestinal contents of six economic fish species. Researchers reported that they found more microplastic particles in coastal fish species (71%) that they caught from different trophic levels, such as ocean and coastal habitats, than in oceanic species (29%) (Pozo *et al.*, 2019). Microplastics are found in all aquatic environments. Among the most commonly found microplastics are tire wear particles (TWP). The effects of TWP release on marine ecosystems are a significant concern. In a study, the effects of TWP particles on the filtration rate of *Mytilus edulis* were examined. The study showed that high levels of plastic particles in the environment harmed the filtration rate of *M. edulis* (Skrubbeltrang Thomsen *et al.*, 2024). Since mussels are filtration-fed organisms, they are suitable model organisms to investigate the accumulation of microplastics and environmental hazards. Some genotoxic effects may also occur due to aquatic organisms' exposure to microplastics (Janet *et al.*, 2022). Microplastics can cause DNA damage, resulting in DNA strand breaks (Gedik *et al.*, 2022b). The micronucleus frequency has been observed in mussels exposed to microplastics and various

environmental pollutants (Hoellein *et al.*, 2021). DNA damage (strand breaks) caused by microplastics has also been reported in *Scrobicularia plana* hemocytes (O'Donovan *et al.*, 2018). In another study, microplastic particles of six commercial mollusk species (*Mytilus galloprovincialis*, *Ruditapes decussatus*, *Crassostrea gigas*, *Hexaplex trunculus*, *Bolinus brandaris*, *Sepia officinalis*) were investigated. In the study, the bioavailability of organisms and consumer risks in the food chain have been tried to determine, and the resulting microplastic types are fibers, fragments, and films, respectively. The most common fibers are polymers such as polyethylene and polypropylene. A study conducted in the Bizerte Lagoon region of Tunisia on the high risks of microplastic particles was reported in mollusk species living in the area (Abidli *et al.*, 2019).

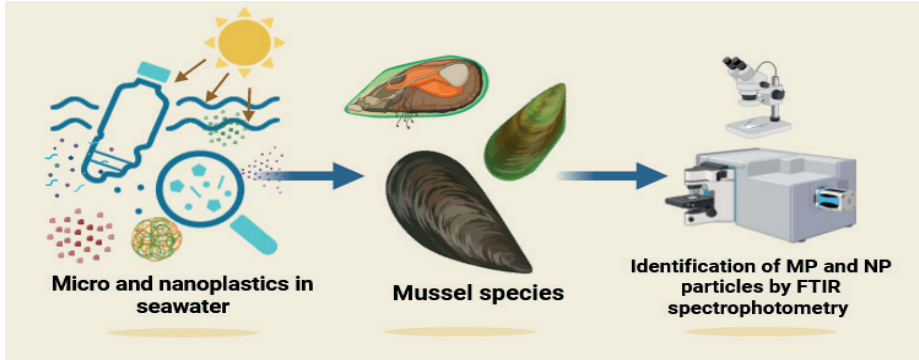


**Figure 1:** Biochemical and physiological effects of microplastics on aquatic organisms.

The toxicological risks associated with microplastic ingestion for organisms arise from the material itself and the ability to absorb and concentrate environmental pollutants from seawater and then transfer them through food chains. In addition, microplastics can affect ecological processes (Guzzetti *et al.*, 2018). For example, in 2016, 9 million tons of plastic material was produced in our country, and approximately half a million tons of plastic waste is disposed of into the environment annually (Başaran Kankılıç *et al.*, 2023). As in the world, plastics constitute more than 80% of the solid wastes reaching the sea in our seas (Aytan *et al.*, 2016). Some of the mussels in Europe can contain about 90 types of microplastics. Although the consumption of mussels varies widely between countries and generations, an average mussel consumer can swallow about 11,000 microplastic particles per year. If inhaled or ingested by humans, microplastics may accumulate in adipose tissue and show localized particle toxicity. Chronic exposure is predicted to be of more significant concern due to the accumulation effect that may occur over time. Although microplastics are known to have the potential to affect human health adversely, it is essential to be able to determine current exposure levels and loads (Mofokeng, 2023). Moreover, a significant number of environmentally hazardous heavy metals,



pesticides, and chemicals such as Bisphenol-A are added to the microplastics, which can accumulate along the trophic food chain (Avio et al., 2015; Prokic et al., 2019; Cheang et al., 2018). The discharged urban wastewater contains microplastic and microbead particles, especially from synthetic fibers and cleaning materials from washing machines. Therefore, wastewater plants can be effective in removing microplastics from wastewater. Monitoring the pollution level in marine ecosystems of all plastic products, including microplastics, and investigating the harmful environmental effects caused by them is a long-standing legal obligation in European Union countries (Farrell & Nelson, 2013; Provencher et al., 2018). In 2014, it was reported that the amount of plastics that drift in water in the world's seas is seven trillion tons, of which 4.85 trillion tons are microplastics. (Prokic et al., 2019). Microplastics were also detected in many seafood samples taken from markets. This finding suggests that aquaculture is also affected by microplastic pollution. A study conducted in Xiangshan Bay showed that microplastics from aquaculture accounted for 55.7% of microplastics in seawater (Cheung et al., 2018 ). Microplastics can adsorb different chemical pollutants such as heavy metals, pesticides, and toxic dyes. The possibility of plastic parts to adsorb different chemical contaminants has been proven under laboratory conditions. Therefore, aquatic organisms face an ever-increasing risk. Different particle polymers, such as polyvinyl chloride, polyethylene, polypropylene, and polystyrene, have also been reported to have high absorption capacity (Ugwu et al., 2021). Van Cauwenberghe and Janssen found that farmed mussels had higher microplastic concentrations (178 microfibers) than natural mussels (126 microfibers). In addition, Rochman et al. (2015) described the presence of microplastics (> 500 microns) in fish sold commercially in Indonesian markets. In addition, microplastic particles were reported in 28% of processed fish in the USA (California). Australian scientists reported that they found microplastic fragments in the digestive systems of blue mussels in their measurements on the beaches of South Australia in 2022. In this research conducted at Flinders University, they reported that the MP fragments found in mussels mostly came from textile-based fibers, fishing lines, and large floating plastic pieces. Researchers state that the microplastic pieces found, although microscopic, can harm the environment, marine life, and potentially humans (Janet et al., 2022). It has been determined that some permanent organic pollutants (POPs), polychlorinated biphenyls (PCBs), organo-halogenated pesticides, nonylphenol, PAHs, and dioxins are found in plastic wastes on different beaches of the world (Aramendia et al., 2024). It is still unclear whether microplastics adsorb pollutants and whether they are a potential chemical source in the food chain. Avio et al. (2015) showed that microplastics can effectively adsorb organic contaminants such as pyrene from the marine environment.



**Figure 2:** Identification of microplastics in aquatic organisms.

They have also reported this chemical's potential transfer and bioaccumulation in mussel tissues. Prolonged exposure to microplastics has demonstrated various genotoxic effects, such as micronucleus frequency in hemocytes. High levels of microplastics were found in nine mussel species from a fish market in China (Li *et al.*, 2015). This situation has been associated with the abundance of microplastics resulting from intensive anthropogenic activities. In addition, increased microplastic pollution has been reported in river mouth waters and freshwater systems. (Zhao *et al.*, 2015; Su *et al.*, 2016). Species that are not economically important are at least as crucial as economically valuable species (Joyce & Falkenberg, 2023). In a study conducted with non-commercially important mussel species in Iskenderun Bay (Turkiye), soft tissues of *Brachidontes pharaonis* species mussels were used. At the same time, this study is the first to report the abundance of microplastics in the soft tissues of the bivalve *Brachidontes pharaonis* in Iskenderun Bay. (Yücel and Kılıç, 2023). According to the data obtained from the study, severe microplastic pollution exists in the region. The predominant polymers found were fibers, polypropylene, and polyethylene. The findings also show the impact of anthropogenic activities, especially on marine biota.



**Figure 3:** *Microplastics and Nanoplastics Cycle.*

**Conclusion and Recommendations:** Microplastics are an increasingly severe environmental and human health problem. There are few studies on risk assessment of MPs in our country yet. This prevents a realistic understanding of the microplastic risk. Microplastics are easily transported to the upper trophic levels in the food chain. Microplastics may adversely affect the health and development of both natural species and aquaculture in marine life. MPs were found even in fishes living in the benthic and pelagic zone, mussels and clams fed by filtration, crabs, and aquaculture produced on farms. This is a growing problem for the future, not only for the aquatic environment but also for human health. When the literature is examined, it is seen that there are significant risks, including people at the end of research about MPs.

Further research is needed to fully identify potential adverse risks to aquatic organisms related to microplastic pollution, which we will observe even further negative impacts in the future. In 2016, the United Nations report stated that more than 800 species of aquatic organisms are exposed to microplastics by swallowing or circulating. It was determined that 220 of these 800 species ate microplastic wastes in nature. Plastic nutrition occurs at different trophic levels, including marine mammals, fish, invertebrates, and fish-eating birds. As a result of microscopic examination, plastic particles were generally deposited in the digestive tract of the aquatic organism.

Micro and nano-plastics accumulate in the animal's body or travel from the intestinal system to the circulatory system or surrounding tissues. Specific regulations are being made to limit the industry's use and distribution of primary microplastics. For example, since July 2018, the United States has banned manufacturing microplastic products. In particular, the restriction on selling cosmetic products that can produce microplastic is planned to be implemented as soon as possible (UNEP, 2014). Specific regulations in the UK and France prohibit the sale and use of cosmetics and cleaning products containing microbeads. Plastic and nylon bags are banned in China, Australia, and many European countries. Bags are taxed in Canada, Germany, the United Kingdom, the Netherlands, and Ireland. In summary, since it is impossible to eliminate plastics, which have become an indispensable part of our lives, it is necessary to produce nature-friendly alternatives to reduce and stop the amount of plastic entering the marine environment, to reduce its consumption both individually and on a country basis, and to raise environmental awareness from an early age, especially through education and awareness activities. There is a global and urgent need to effectively handle plastic waste, to continue multidisciplinary scientific studies in this field, and to develop new technologies to remove existing plastics from the sea.

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## *Chapter 5*

### **THE ROLE OF TWO TRITERPENES IN IMMUNE SYSTEM AGING**

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## 1- INTRODUCTION

In recent years, there has been a change in the demographic structure of our country and the world due to various reasons. The elderly population, defined as aging, has been observed to exceed 10.0% of the total population. In Turkey, the elderly population is increasing faster than other age groups, resulting in a decrease in the proportion of children and young people in the total population and an increase in the proportion of the elderly. Although Turkey still has a relatively young population compared to countries with a higher proportion of elderly citizens, the number of elderly individuals is quite significant. In 2017, the elderly population in Turkey was 6,895,385, but it has since increased by 22.6% in the last five years, reaching 8,451,669 in 2022. The percentage of elderly individuals in the total population was 8.5% in 2017 and is projected to increase to 9.9% by 2022. (Kol, E. (2022). According to population projections, the elderly population rate is predicted to be 12.9% in 2030, 16.3% in 2040, 22.6% in 2060 and 25.6% in 2080 (data.tuik.gov.tr, 2023). Moreover, it is worth noting that Turkey's population is aging faster than many developed countries, particularly those in Europe. According to projections, it is estimated that it will take 25 years for Turkey's elderly population rate to increase from 7% to 14%. In comparison, this period is 115 years for France, 85 years for Sweden, and 69 years for the USA. (Çuhadar, S. G. (2020). This situation underscores the importance of implementing innovative country strategies supported by scientific data. These strategies can benefit both the economy and human well-being. Green chemistry or its components to delay aging has garnered attention recently.

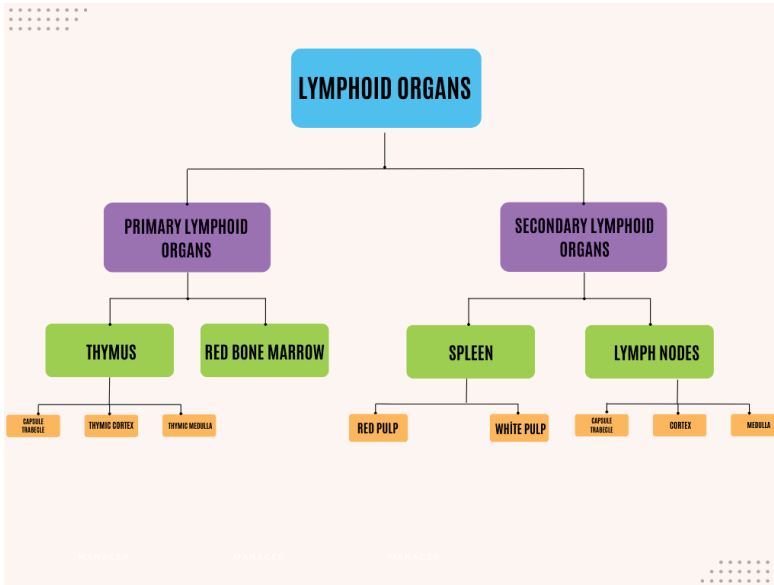
Aging is an unavoidable process that occurs in every individual and triggers cellular responses that cause many phenotypic changes. The process involves slowing and stopping of cell growth, chromatin reformation, changes in metabolic programming, impaired autophagy and disruption of the balance of proinflammatory factors, accompanied by a gradual decrease in metabolic functions. Additionally, ageing is a significant risk factor for various health conditions, including cancer, diabetes, cardiovascular diseases, and neurodegenerative disorders (McHugh, D., & Gil, J. (2018). With the studies carried out so far, signs of aging have been identified, and promising clues have been collected that target the aging process and delay many diseases that occur with aging. Although studies on aging are viral, it is necessary to focus more on strategies related to healthy aging and introduce some innovations.

For this reason, research should focus on ensuring healthy aging and reducing diseases that develop due to aging. The importance of healthy aging models is evident, especially in studies conducted on laboratory animals. Recent studies draw attention to the use of green chemistry or its components to delay aging. Double and triple antioxidant combinations may be appropriate

to reduce low-grade inflammation in aging. In this review, dual uses of saponin and Squalene will be evaluated.

## 2- IMMUNOSENESCENCE

With aging, there is an increase in the number and uncontrolled release of cytokines, an increase in inflammatory reactions, and an increase in autoimmunity, which is defined as the body's abnormal response to its antigens. This condition is defined as "immunosenescence". In immunosenescence, changes occur in the number of both acquired and innate immune cells and, accordingly, in their functions. The quantity of circulating naïve B and T cells declines, while the quantity of memory cells increases or stays constant. The most significant markers of these changes are mitochondrial dysfunction, glucose accumulation, reactive oxygen species (ROS) accumulation, and an increase in proinflammatory cytokines. Aging causes changes in the immune system's metabolism, which can lead to the development of cancers, autoimmune diseases, and neurodegenerative diseases in elderly patients. It is important to note that these evaluations are objective and supported by scientific evidence (Doran, M. F., Pond, G. R., Crowson, C. S., O'Fallon, W. M., & Gabriel, S. E. (2002). (Jemal, A., Siegel, R., Ward, E., Murray, T., Xu, J., & Thun, M. J. (2007). Immunosenescence: It is characterized by defects in calcium-mediated signaling, mitochondrial dysfunction, naïve/memory cell imbalance, inflammation, and thymic atrophy (Liu, Z., Liang, Q., Ren, Y., Guo, C., Ge, X., Wang, L., & Han, X. (2023). Aging cells secrete factors known as senescence-associated secretory phenotype (SASP) and stimulate chronic inflammation. The accumulation of factors resulting from chronic inflammation also accelerates aging (Liu, Z., Liang, Q., Ren, Y., Guo, C., Ge, X., Wang, L., & Han, X. (2023). Thus, a vicious cycle is created in which aging and inflammation trigger each other (Arai, Y., Martin-Ruiz, C. M., Takayama, M., Abe, Y., Takebayashi, T., Koyasu, S., Von Zglinicki, T., 2015). The vaccine response decreases in the aged immune system, and susceptibility to infections increases. To explain aging in the immune system, the effects on organs such as the thymus, spleen, and lymph nodes, to which aging is directly related, need to be addressed.



*FIGURE 1. Representation of Lymphoid Organs*

The thymus is surrounded by a thin capsule originating from connective tissue. The capsule is inserted into it, dividing it into compartments. Each section is called a lobule. Lobules: It has dark and light-colored areas called cortex and medulla. The thymus is a central lymphatic organ responsible for producing the first functional T lymphocytes. It is worth noting that thymocyte counts and hormone secretion levels tend to increase during early human development but then gradually decline over time as development continues. Thymus degeneration occurs with aging. In thymus atrophy, epithelial spaces disappear over time, and the perivascular space fills the aged thymus. This reduces the frequency of naïve T cells. It increases peripheral differentiated memory T cells and reduces naïve T cells to migrate to the brain (Wertheimer, A. M., Bennett, M. S., Park, B., Uhrlaub, J. L., Martinez, C., Pulko, V., Nikolich-Zugich, J., 2014). Studies have found that young adults who underwent thymectomy in early childhood experienced premature immunosenescence compared with adults of the same age, and older individuals showed altered T cell profiles. This provides strong evidence for the role of the thymus in the aging of the human immune system. In addition, in a study on old mice, Increased levels of phosphorylated p53 binding protein were seen in senescent cells in the thymus. This increase indicates oxidative stress and DNA damage, which leads to cellular aging (Liu, Z., Liang, Q., Ren, Y., Guo, C., Ge, X., Wang, L., Han, X. (2023), (Aw, D., Silva, A. B., Maddick, M., Von Zglinicki, T., & Palmer, D. B. (2008).

The spleen is surrounded externally by a capsule made of fibrous connective tissue. Unlike other lymphoid organs, the spleen does not have a cortex and medulla. Due to its anatomical appearance, the spleen consists of two parts: The White Pulp, which consists of lymphoid follicles with the central arteriole at its periphery, and the Red Pulp, which appears red due to the erythrocytes in its structure. The spleen is a secondary lymphoid organ that supports immunity. It contains approximately 25% of the body's lymphocytes, macrophages, dendritic, and plasma cells. In addition, it undertakes tasks such as antibody production, activity of B and T cells suitable for antigens, and elimination of apoptotic cells (Berger, A., German, J. B., Chiang, B. L., Ansari, A. A., Keen, C. L., Fletcher, M. P., & Gershwin, M. E. (1993). In the aging process, major changes develop in the cellular composition and the spleen's microarchitecture. In the normal white pulp region, the demarcated T and B cell areas lose clarity. During the aging process, the cellular content and microarchitecture of the spleen undergo significant changes. The clarity of the demarcated T and B cell areas in the normal white pulp regions is lost. As a result, marked changes occur in marginal zone macrophages' organizational and regulatory functions (Aw, D., Hilliard, L., Nishikawa, Y., Cadman, E. T., Lawrence, R. A., & Palmer, D. B. (2016). It has been observed through various studies that as individuals age, there is a decrease in the proportion of T cells in the spleen while the number of plasma cells increases. Furthermore, research has reported specific impairments, such as reduced phagocyte ability of macrophages in the marginal zone, impaired functions of microenvironment-mediated antigen-presenting cells, and impaired migration of B cells. (Turner, V. M., & Mabbott, N. A. (2017).

Lymph nodes are oval-shaped structures surrounded by a fibrous capsule ranging from 0.1 to 2.5 cm in length. They consist of a capsule, outer cortex, and inner medulla. Lymph nodes are crucial in localizing T and B cells and initiating immune responses. The quantity, functionality, and availability of lymph nodes decrease significantly with age. In elderly individuals, there may be a decrease in fibroblastic reticular cells, leading to a compressed and less reticular stromal network. There may also be fat accumulation and fibrosis in their lymph nodes (Li, X., Li, C., Zhang, W., Wang, Y., Qian, P., & Huang, H. (2023). Stimulation of stromal cells can decrease replication potential, affecting the balance of naive T cells. Senescent cell buildup and inflammation in lymph nodes affect the movement and enlistment of immune cells (Budamagunta, V., Foster, T. C., & Zhou, D. (2021). (Becklund, B. R., Purton, J. F., Ramsey, C., Favre, S., Vogt, T. K., Martin, C. E., Surh, C. D. (2016). Studies have shown that aging negatively affects lymph nodes, causing a decrease in their number and morphological degeneration, such as fat accumulation, loss of lymphoid tissue, and fibrosis. This can lead to decreased immune function and an increased risk of infection (Ahmadi, O., McCall, J. L., & Stringer, M. D. (2013).

The brain consists of several anatomic areas, including the frontal, occipital, parietal lobes, the hippocampus, the cerebellum, and the brainstem. It contains pyramidal nerve cells that are 20-50µm long and Betz cells that are 120 µm long and 60 µm wide. The frontal lobe is responsible for cognitive thinking and motor activity based on past experiences. Additionally, dopamine-sensitive neurons are present in the frontal lobe. Dopamine is a system related to attention, long-term memory, and planning. As individuals age, there is a decrease in attention due to a reduction in dopamine receptors (Lee, C. K., Weindruch, R., & Prolla, T. A. (2000), (Madden, D. J. (2007)). The parietal lobe is responsible for the brain's orientation function and is located in front of the occipital lobe and behind the frontal lobe. It combines and interprets stimuli from the sense organs and enables object manipulation. It has been observed that neuron loss in the parietal lobe may occur with aging. The hippocampus is a vital component of the brain that is responsible for long-term memory and orientation.

Furthermore, the hippocampus is involved in determining appropriate behavior in various environments. According to research, individuals with damaged hippocampi may experience difficulty learning and may forget information immediately. Dysfunction in the hippocampus has been associated with elevated levels of glucocorticoids. Research has shown that as people age, there may be a decrease in the number of neurons, synaptic connections, new neuron formation, and dendritic atrophy (Driscoll, I., Hamilton, D. A., Petropoulos, H., Yeo, R. A., Brooks, W. M., Baumgartner, R. N., & Sutherland, R. J. (2003).

The kidneys are a pair of organs located retroperitoneally on the posterior wall of the abdomen. They have a characteristic shape, with an upper and lower pole, a lateral convex, and a concave medial border. The renal pelvis and renal vessels are located in the medial border, with a prominent depression known as the 'Hilus'. Kidney function may decline with age, leading to kidney failure. This decline can be influenced by race, genetic makeup, environment, and gender. Physiological and morphological changes in glomeruli lead to a decrease in GFR (Glomerular Filtration Rate) in both rats and humans (Baylis, C. (2008), (Zhou, X. J., Rakheja, D., Yu, X., Saxena, R., Vaziri, N. D., & Silva, F. G. (2008).

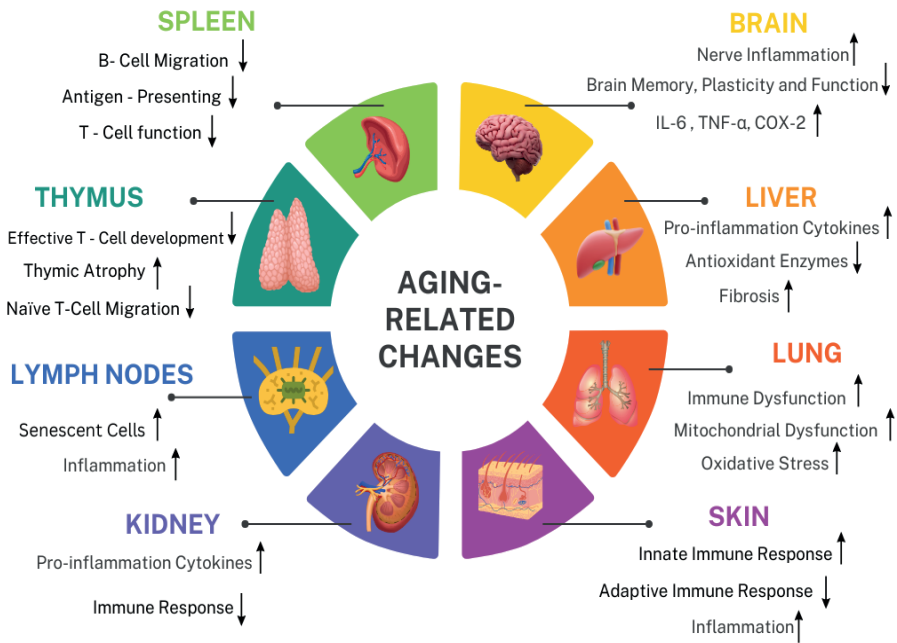
The liver is a vital organ that carries out diverse functions. It weighs around 1500 grams, equivalent to approximately 2% of the body weight. These cells are arranged in rows of ribbons that measure 25-40 µm in diameter. The liver primarily comprises hepatocytes, commonly called liver cells, which make up roughly 67% of the cells in the liver. It has been observed that the synthesis of antioxidant enzymes primarily occurs in the liver. However, as individuals age, the liver tends to decrease in size and blood flow, reducing the metabolic activity of parenchymal cells. This decrease in metabolic activity includes gene

expression, mitochondrial respiration, and xenobiotic metabolism (Seo, A. Y., Hofer, T., Sung, B., Judge, S., Chung, H. Y., & Leeuwenburgh, C. (2006). Furthermore, it is widely acknowledged that the liver's regeneration capacity also decreases with age (Timchenko, N. A. (2009). It has been observed that the increase in ROS/RNS levels causes oxidative damage to DNA, RNA, and proteins due to changes in redox homeostasis.

Additionally, with aging, mutations in the mitochondrial genome lead to oxidative DNA damage over time. The literature has recently presented studies investigating the relationship between aging and oxidative protein and DNA damage in the liver. According to a study, it was found that the group of aged rats had higher levels of protein carbonyl (PCO), which are protein oxidation parameters, and malondialdehyde (MDA), the end product of lipid peroxidation, in comparison to the young control group (Aydin, S., Atukeren, P., Cakatay, U., Uzun, H., & Altuğ, T. (2010).

Lung aging is a complex process that involves various molecular and physiological changes. These modifications may impact lung function, regeneration capacity, and susceptibility to acute and chronic lung ailments. During lung aging, there may be significant changes in respiratory muscle strength and a reduced capacity to expel mucus or foreign particles from the lungs. (Cho, S. J., & Stout-Delgado, H. W. (2020).

The skin is a multi-layered organ that plays a crucial role in protecting the body. Its effectiveness is attributed to the close intercellular communication between its layers. During the aging process, it has been observed that metabolically senescent cells tend to accumulate in the skin, despite their inability to divide. As a result, these cells secrete sequestrants, known as SASPs, which have been found to disrupt the skin's architecture significantly. Upon comparing senescent dermal fibroblasts to non-aging dermal fibroblasts, it has been noted that the latter tend to secrete more sequestrants. The excessive secretion mentioned may disrupt the differentiation of keratin-producing cells, leading to impaired skin barrier functions. This, in turn, may trigger an increase in the production of IL-6, a proinflammatory cytokine (Choi, E. J., Kil, I. S., & Cho, E. G. (2020).



**FIGURE 2.** Aging-related changes in some tissues

#### 4. SAPONIN

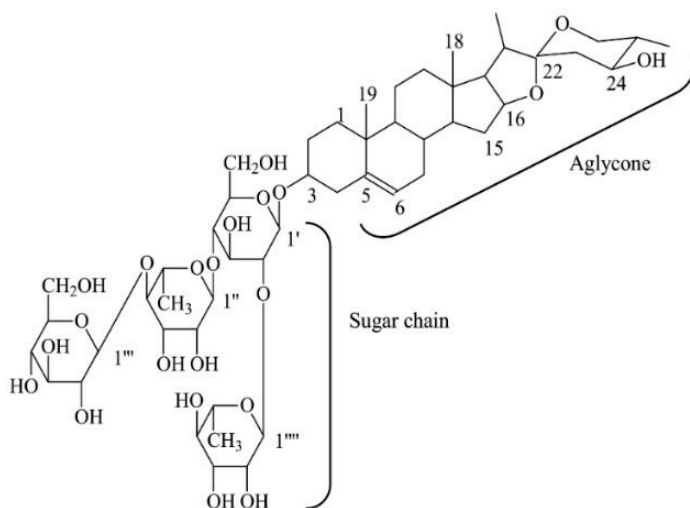
Saponin is one of the natural compounds that has many beneficial effects. Saponin: It is derived from the Latin word 'sapo', meaning soap (Rao, A. V., & Sung, M. K. (1995). They show antibiotic and antifungal activity. Another remarkable feature is that they form complexes with cholesterol (Yesilada, E. (1995). According to the report, it affects the emulsification of fat-soluble substances in the digestive tract. This includes the formation of micelles that contain saponins, bile acids, fatty acids, diglycerides, and fat-soluble vitamins (Cheeke PR (1999). It has been shown that lipid metabolism is affected in humans and various animals fed with saponin-containing plants or saponin extracts. It has also been reported to reduce human serum cholesterol levels (Bingham, R. (1978). When used in low doses, saponins have an effect that increases the immune power of the vaccine (adjuvant effect). It is known that they show this effect by stimulating the immune system and increasing the synthesis of antibodies against antigens (Ilsley, S. E., Miller, H. M., & Kamel, C. (2005). (Oda, K., Matsuda, H., Murakami, T., Katayama, S., Ohgitani, T., & Yoshikawa, M. (2000). It is suggested that saponins are localized in the spleen after oral administration and then gradually enter the circulation and exert an adjuvant effect (Nagai, T., Suzuki, Y., Kiyohara, H., Susa, E., Kato, T., Nagamine,



T., Yamada, H. (2001). It has been reported that saponins, in addition to their stimulating effects on specific immunity, also affect some nonspecific immune reactions such as inflammation and monocyte proliferation (uncontrolled proliferation and increase in the number of cells) Delmas, F., Di Giorgio, C., Elias, R., Gasquet, M., Azas, N., Mshvildadze, V., Timon-David, P. (2000). Saponins are commonly used in clinical drug development due to their various pharmacological activities, including immunomodulatory, neuroprotective (Dong, J., Liang, W., Wang, T., Sui, J., Wang, J., Deng, Z., & Chen, D. (2019), anti-oxidative, anti-inflammatory (Augustin, J. M., Kuzina, V., Andersen, S. B., & Bak, S. (2011), (Wang, T., Di, G., Yang, L., Dun, Y., Sun, Z., Wan, J., Yuan, D. (2015), antimicrobial (Augustin, J. M., Kuzina, V., Andersen, S. B., & Bak, S. (2011), antitumor (Lacaille-Dubois, M. A. (2005), anti-apoptotic (Yang, B. R., Cheung, K. K., Zhou, X., Xie, R. F., Cheng, P. P., Wu, S., Lee, S. M. Y. (2016). anti-diabetic (Uzayisenga, R., Ayeka, P. A., & Wang, Y. (2014), and anti-cancer (Vuong, Q. V., Hirun, S., Chuen, T. L., Goldsmith, C. D., Murchie, S., Bowyer, M. C., Scarlett, C. J. (2015) effects. It is important to note that all evaluations presented are objective and supported by evidence. Saponins have a triterpene nucleus with one aldehyde and two oligosaccharide chains. It has been shown that the mixture of triterpene saponins also exhibits significant anti-inflammatory, anti-edema, and capillary-protective effects. Saponins are commonly used in medical applications to relieve hemorrhoids, varicose veins, and rheumatic pains (Hu, J. N., Zhu, X. M., Han, L. K., Saito, M., Sun, Y. S., Yoshikawa, M. & Zheng, Y. N. (2008). Research has shown that the aldehyde group interacts with T cell surface receptors, facilitating costimulation (Marciani, D. J. (2003). This knowledge has aided in the development of synthetic analogs of naturally derived saponins with preserved immune activity and reduced toxicity (Burakova, Y., Madera, R., McVey, S., Schlup, J. R., & Shi, J. (2018); (Fernandez-Tejada, A., Tan, D. S., & Gin, D. Y. (2016). Saponins are known as natural anti-diabetic agents with low toxicity (Wang, T., Di, G., Yang, L., Dun, Y., Sun, Z., Wan, J., Yuan, D. (2015). They also have pharmacological potential such as reducing serum cholesterol levels, immunomodulatory potential by regulating cytokine expression, and cytotoxic effect on tumor cells (Jeepipalli, S. P., Du, B., Sabitaliyevich, U. Y., & Xu, B. (2020). According to recent *in vivo* and *in vitro* studies, it has been suggested that saponins possess immunotherapeutic properties and can stimulate the cell-mediated immune system and enhance antibody production (Rajput, Z. I., Hu, S. H., Xiao, C. W., & Arijo, A. G. (2007), (Sarikahya, N. B., Nalbantsoy, A., Top, H., Gokturk, R. S., Sumbul, H., & Kirmizigul, S. (2018).

Additionally, saponins have been found to induce the production of cytokines such as interleukins and interferons, which may mediate their immunostimulatory effects. It is believed that saponins interact with antigen-presenting cells to induce many of these responses (Haridas, V., Arntzen, C.

J., & Gutterman, J. U. (2001), (Yui, S., Ubukata, K., Hodono, K., Kitahara, M., Mimaki, Y., Kuroda, M., Yamazaki, M. (2001). Saponins play an important role in cellular immunity. It provides this function with its carbonyl and aldehyde functional groups. The immune response involving carbonyl and aldehyde groups is based on a covalent reaction between amino and carbonyl groups on Antigen-presenting cells (APCs) and T cells (Cui, X., Ma, X., Li, C., Meng, H., & Han, C. (2023). This results in the formation of reversible intercellular Schiff (Rhodes, J. O. H. N. (1989). A study found that three steroidal saponins isolated from *P. odoratum* can promote the in vitro proliferation of mouse spleen cells (Tatsuno, I., Gottschall, P. E., & Arimura, A., 1991).



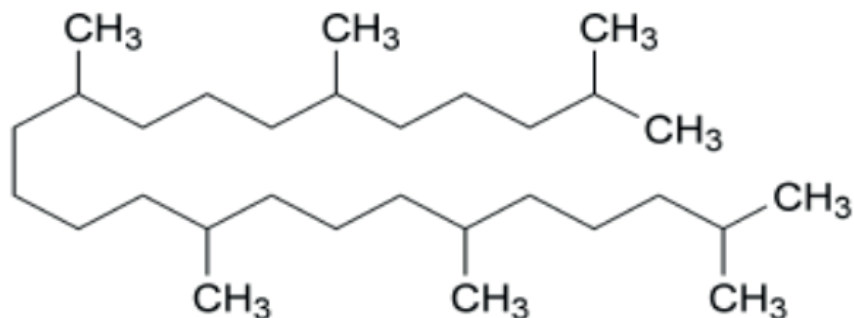
**FIGURE 3.** Chemical Structure of Saponin (Chaieb, I. (2010).

## 5. SQUALENE

Another natural compound is Squalene. Dietary sources of Squalene include shark liver, olive oil, and walnuts. Squalene is involved in the medical treatment of cancer. It also lowers low-density lipoprotein (LDL) cholesterol and triglyceride levels; it has a high-density lipoprotein (HDL) cholesterol-raising effect (Chua, N. K., Howe, V., Jatana, N., Thukral, L., & Brown, A. J. (2017). Squalene, an intermediate product of cholesterol metabolism, has been shown to have antioxidant and anti-aging effects on the skin and a protective effect against colon and lung cancer (Smith, T. J., Yang, G. Y., Seril, D. N., Liao, J., & Kim, S. (1998). Squalene; There are studies on its facilitating effect of oxygen reaching the cellular level and its contribution to improving organ functions through aerobic metabolism. It has also been shown that Squalene can be used in nutraceuticals and therapeutic agents to control oxidative stress

and numerous age-related diseases. Squalene has potent biological effects on the immune system. Studies support its antioxidant, anti-inflammatory (Wei, J., & Feng, J. (2010), and anti-atherosclerotic properties (Lou-Bonafonte JM, Martínez-Beamonte R, Sanclemente T, Surra JC, Herrera-Marcos LV, Sanchez-Marco J, Arnal C, Osada J. (2018). The detoxification activity of Squalene against various chemical agents such as hexachlorobiphenyl, hexchlorobenzene, arsenic, theophylline, phenobarbital, and strychnine has been demonstrated in many experimental models (Kamimura, H., Koga, N., Oguri, K., & Yoshimura, H. (1992), (Richter, E., & Schäfer, S. G. (1982). In a study investigating the protective effect of Squalene against H<sub>2</sub>O<sub>2</sub>-induced DNA damage, a significant dose-dependent decrease in H<sub>2</sub>O<sub>2</sub>-induced DNA damage was detected as a result of 24-hour incubation with increasing amounts of Squalene in the MCF10A cell line (Warleta, F., Campos, M., Allouche, Y., Sánchez-Quesada, C., Ruiz-Mora, J., Beltrán, G., & Gaforio, J. J. (2010). Due to its dietary benefits, biocompatibility, inertness and many advantageous properties, Squalene is widely used in nutritional, nutraceutical, pharmaceutical and therapeutic fields for disease management and treatment (Bozbulut, R., & Akbulut, G. (2016).

Squalene is an isoprenoid compound with a structure similar to that of vitamin A,  $\beta$ -carotene, coenzyme Q10, vitamin E, and vitamin K. It contains six double bonds, exhibits radical solid scavenging capacity, and protects the skin from the harmful effects of lipid peroxidation (Kohnoa Y., Egawab Y., Itohb S., Nagaokab S., Takahashia M., Mukai K. (1995). In an experimental model investigating the effect of Squalene on isoproterenol-induced myocardial infarction, it has been observed that using Squalene before isoproterenol application reverses the effect of lipid peroxidation and preserves GSH levels. Additionally, the levels of glutathione-dependent antioxidant enzymes (GPX or GST) and antiperoxidative enzymes (CAT and SOD) were found to be protected by squalene application (Farvin, K. S., Anandan, R., Kumar, S. H. S., Shiny, K. S., Sankar, T. V., & Thankappan, T. K. (2004). In a separate study, the anti-inflammatory activity of Squalene on lipopolysaccharide (LPS)-stimulated rodent peritoneal macrophages and human blood monocytes and neutrophils was investigated. The environment of LPS-treated murine peritoneal macrophages exhibited a significant increase in nitrites, an indicator of nitric oxide (NO) production, and this effect was almost abolished by squalene (Cárdeno, A., Aparicio-Soto, M., Montserrat-de la Paz, S., Bermudez, B., Muriana, F. J., & Alarcón-de-la-Lastra, C (2015). In an experimental study investigating the effects of Squalene on radiation, it was reported that squalene application was the determining factor for longer lifespan in mice fed 2% squalene for 14 days (Storm, H. M., Oh, S. Y., Kimler, B. F., & Norton, S. (1993).



**FIGURE 4.** Chemical Structure of Squalene (Matyas, (2004).

## 6. CONCLUSION

Aging is an inevitable process encompassing several interrelated aspects, including chronological, psychological, social, and biological factors. As individuals age, there is a gradual decline in physiological capacity and the ability to respond to environmental factors. This, in turn, leads to an increased sensitivity and susceptibility to various diseases. The immune system is one of the most affected systems in the aging process. Immune aging is a complex process involved in many age-related diseases, including changes in immune cells, cytokine secretion, and cell dysfunctions (Salminen, A., Kaarniranta, K., & Kauppinen, A. (2019). Recently, healthy aging has emerged as a topic of interest. Various studies are being conducted to reveal the causes of aging, to treat aging, or to reduce its symptoms. The most popular methods for healthy aging today are calorie restriction, exercise, vitamins and antioxidant supplements.

The use of antioxidants to modulate the immune system is a common practice. Similarly, potent antioxidants are essential for eliminating the damage caused by aging. Recently, there has been a notable increase in interest in triterpenes, particularly in saponin and Squalene, triterpenes. This review will provide scientists with fundamental information about the capacity of these two triterpenes to enhance sub-acute oxidative and aging damage and to reinforce the anticipated pharmacological properties. In order to elucidate the relationship between aging and the immune system, it is essential to reveal the changes that occur in aged organs, primarily the thymus and spleen.

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## *Chapter 6*

### **ALLOSYME VARIATIONS OBSERVED IN DIFFERENT GROUPS OF LIFE**

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## INTRODUCTION

Each living species on earth constitute the global ecosystem together with living space and other living organisms which share the living space. All living species on earth constitute biodiversity (Kışlalıoğlu & Berkes, 1994). According to the 2011 research of the United Nations Environmental Protection Program, although 8 million 700 thousand living species have been identified today, it is estimated that the number of living species in natural life may reach 100 million.

With the classification of living organisms, it has become possible to recognize many and different types of living organisms (“Bilimsel sınıflandırma,”).

Systematic information was obtained based on morphological, anatomical and behavioral characters until the end of the 1950s. Criticism of the characteristics of traditional characters led to the search for other alternative techniques. After the 1960s, macromolecules that precisely determine the similarity rate between individuals began to be used frequently in systematic studies (“Taksonomi (biyoloji),”).

With the combined use of classical and molecular systematics easier, accurate and precise information can be obtained about the living organisms (Elven, Bachmann, & Gusarov, 2010).

The first of molecular systematic studies focused mostly on proteins. While protein electrophoresis and histochemical staining were initially used, allozyme electrophoresis and isozyme electrophoresis have become increasingly popular among systematists because they are effective techniques for generating systematic data from macromolecules. Although studies on DNA markers are on the agenda in population genetics today, allozymes continue to be used extensively (Crawford & Ornduff, 1989; “Taksonomi (biyoloji),”).

Allozymes can be studied in living organisms by the gel electrophoresis method. According to the results obtained from electrophoretic studies, it is revealed that invertebrate animals generally show more variation than vertebrate animals due to polymorphism and heterozygosity.

Although genetic polymorphism appears to be very common in natural populations, it is not a universal phenomenon. Today, loss of genetic variation is revealed in populations that are on the verge of extinction. Cheetahs (*Acinonyx jubatus*) and Polar bears (*Ursus maritimus*) are species in danger of extinction in the wild (Hartl, 1988).

## BIOLOGICAL DIVERSITY

The number of species of living organisms on earth is quite high, and when extinct species that can be recognized from their fossils are added to this number, it reaches quite large numbers. Approximately 1 million 500 thousand



animal and 800 thousand plant species exist in the ecosystems. According to the 2011 research of the United Nations Environmental Protection Program, although 8 million 700 thousand living species have been identified today, it is estimated that the number of living species in natural life may reach 100 million.

The greatest diversity of living organisms in the animal kingdom is seen in Invertebrate animals. While the number of species and population size is gradually increasing in Insects that are hexapod Invertebrates as well as Bacteria that belong to the kingdom Monera, it is decreasing in Vertebrate animals.

With the classification of living organisms, it has become possible to recognize many and different types of living organisms (“Bilimsel sınıflandırma”).

### **SYSTEMATIC**

Classification; It is the arrangement of organisms that are alive or have become extinct on Earth in similar groups.

Systematic is derived from the Greek word *systema*. The word systematic appeared for the first time in the 1st edition of Linne’s work *Systema Naturae*, published in 1735. The modern definition of systematic was made by Simpson in 1961.

Taxonomy, on the other hand, has methods and principles developed to examine living organisms with a very high number of species in an orderly manner. Taxonomy is derived from the combination of the Greek words *taxis* (ranking) and *nomos* (name) and means the ordering of names. According to Simpson, taxonomy is a classification that includes principles, methods and rules. Taxonomy was first used to classify plants by the French botanist Augustin Pyramus de Candolle in 1813 (“Bilimsel sınıflandırma”).

### **MOLECULAR SYSTEMATICS**

Systematic information was obtained based on morphological, anatomical and behavioral characters until the end of the 1950s. Criticism of the characteristics of traditional characters led to the search for other alternative techniques. After the 1960s, macromolecules that precisely determine the similarity rate between individuals began to be used frequently in systematic studies (Elven et al., 2010; “Taksonomi (biyoloji”).

Molecular systematic genetics is a sub-branch of genetics that examines the structure and functions of genes, which carry the genetic information necessary for the development of living organisms and transmit them from generation to generation, at the molecular level. Molecular genetics uses methods of molecular biology and genetics. Molecular systematics is the field

of genetics that enables the processing of molecular information, the study of samples with methods and principles, and the correct scientific classification with rules (“Moleküler genetik”).

Since molecular systematics can clearly determine the similarity rate between individuals, living organisms can be grouped step by step, and the similarities between groups also contribute to explaining evolution. Molecular systematics provides information about how similarities and differences between groups occur and change.

Molecular systematics can achieve much more accurate results by using protein and nucleic acid compounds, building block sequences and gene isolation information in detecting differences between living organisms. With molecular systematic studies, the phylogenetic and taxonomic importance of secondary metabolism products can be clearly revealed (Elven et al., 2010).

In molecular systematic studies, the difference between the amino acid or nucleotide sequences of a macromolecule such as a protein or DNA with the same function belonging to two different species indicates the evolutionary distance between species. After two different species, separated from a common ancestor, begin to evolve, many mutations spontaneously occur in their DNA. The numbers of sequence differences caused by mutations in the primary structure of the macromolecule are used as criteria by evolution researchers. The presence of a large number of sequence differences reveals that living organisms evolved by diverging from their ancestors and each other a long time ago, while a small number of them reveals that the individuals are close relatives and, moreover, they may belong to the same species (Sato et al., 2009).

With the combined use of systematic and molecular systematics, information about the living world can be obtained more easily, accurately and precisely.

### **Uses of Molecular Systematics**

- Molecular marker systems
- Investigation of phylogenetic relationships
- DNA purification
- DNA fingerprint
- DNA sequence analysis
- DNA mapping
- Recombinant DNA technology
- Determination of paternity by DNA test

- Biochemistry

### Molecular Marker Systems

It has been developed in recent years to detect species that cannot be systematically identified or are difficult to distinguish from each other (Erguden, 2007).

Molecular marker: It is a piece of DNA that relates to any gene or gene region in the genome, has no biological role and can be inherited to future generations (Ridout, Donini, Ridout, & Donini, 1999).

Molecular markers have some properties (Botstein, White, Skolnick, & Davis, 1980; Hatzopoulos et al., 2002; Helentjaris, King, Slocum, Siedenstrang, & Wegman, 1985; Williams, Kubelik, Livak, Rafalski, & Tingey, 1990). These;

- Polymorphic,
- Codominant,
- Distributes throughout the genome,
- Reliable,
- Reproducible,
- Easy to apply,
- Can be analyzed easily and quickly,
- It is not affected by the environment and other loci,
- Data can be exchanged between laboratories.

Molecular markers are divided into two groups: DNA markers and protein markers (9)(Erguden, 2007).

### Protein Markers

Allozymes and isozymes are used as protein markers (Erguden, 2007).

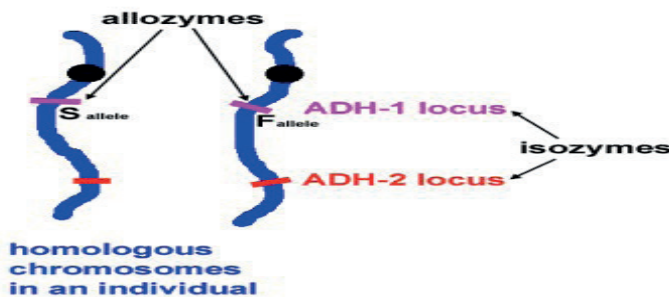


Figure 1 Allozyme and isozymes ("Allozyme,")

Allozymes are enzymes in different forms encoded by different alleles on the same gene locus. Isozymes contain different molecular structures encoded by different genes located on different gene loci in different tissues. Although isozymes catalyze the same chemical reaction, they are enzymes that differ from each other in terms of their amino acid sequences, the affinity of the substrate to the enzyme, and the regulation of enzyme activity. Allozymes are a subset of isozymes (figure 1), (Buth, 1984; Lehninger, Nelson, & Cox, 1993; Nelson & Elisens, 1999).

### **Allozyme Studies**

The first molecular systematic studies were largely related to proteins. In first applications, protein electrophoresis and histochemical staining were used. Allozyme and isozyme electrophoresis has become increasingly popular among systematists as it is an effective technique for generating systematic data of macromolecules. Although studies on DNA markers are on the agenda in the field of population genetics today, allozymes continue to be used extensively (Crawford & Ornduff, 1989; “Taksonomi (biyoloji),”).

The use of allozymes in systematic and population genetics began in 1966 with the study of scientists named Hubby and Lewontin, in which they examined the degree of iatrogenic heterozygosity and the amount of change in natural populations of *Drosophila pseudoobscura* and continues to be widespread today (Hubby & Lewontin, 1966; Lewontin & Hubby, 1966).

### **Areas of Use of Allozymes**

- Population genetics
- Phylogenetics

Enzyme electrophoresis methods are frequently used in studies to detect genetic variation within and between species. Genetic distances between different populations of a species or species are calculated by analyzing allele frequencies (Crawford & Ornduff, 1989; Gottlieb, Warwick, & Ford, 1985). By determining and calculating gene frequencies, homozygotes and heterozygotes, the lineage development and evolutionary history of a species or higher taxonomic groups can be determined. In addition, calculations are also used to investigate and compare the evolutionary relationships between populations (Kandemir, Kence, & Kence, 2000).

### **Advantages of Allozymes**

- Found in many organisms (about 100, mostly in animals)
- Expressing of data as codominant (Gómez, 1998)
- Disadvantage of Allozymes
- Low number of alleles in loci (Gómez, 1998)

Different electrophoretic methods have been developed to work with different types of proteins. Allozymes can be studied in living organisms by the gel electrophoresis method (Parlak, 2007).

### **Allozyme Electrophoresis**

- How many different loci enzymes consist of,
- Number of alleles in loci,
- Allele frequencies at the population level,
- Genetic heterozygosity,
- Reveals individual enzyme phenotypes.

Genetic information obtained from electrophoresis answers the questions of whether the genes in the compared samples belong to the same or different gene pools and how different the compared gene pools are from each other (Bulut, 2007).

Allozymes migrate differently on the gel due to size, shape, or both. Among the mutations in the DNA region coding for a particular enzyme, point mutations can mostly create an allozyme allele. If an amino acid is substituted in a protein molecule, the electrophoresis migration rate will change as the net charge will be affected or may lead to a conformational change. The new allele is initially encountered at a very low frequency in the population. The allele may survive depending on its physiological performance and frequency of transmission to subsequent generations. Alleles that occur or disappear randomly in populations indicate the level of diversity of populations (Buth, 1984).

### **Allozyme Electrophoresis Technique**

**Storage of samples:** Due to enzyme sensitivity and long-term research, samples are stored at -70 °C.

**Homogenization of samples:** Samples are homogenized in the extraction liquid.

**Preparation of the gel:** The gel, which must be prepared in certain proportions, is dissolved in the gel buffer specific to the enzymes that are the examination sample.

**Electrophoresis:** Electrode buffer specific to enzymes is filled into the compartments of the electrophoresis tank. The electrophoresis method specific to the enzyme being examined is applied. When an electric current passes through them, proteins with a negative electrical charge move to the positive electrical side of the electric field, in other words, from the cathode to the anode.

**Histochemical staining:** Various reaction mixtures are prepared for each of the enzymes and staining is performed.

**Gel fixation:** The gel is washed with fixing liquid after the enzyme bands become visible.

**Evaluation of the results:** After viewing the formed bands, band patterns are drawn. The results are evaluated by assigning different letters to the enzymes according to their fast and slow mobility, starting with the one closest to the anode. After bands, band patterns are drawn.

Electrophoresis allows discrimination based on differences between two proteins rather than analysis of two proteins. Proteins that act together under the same electrophoretic conditions are most likely similar.

Allozymes look different from each other when they move on the gel because of their different physical properties, such as electrical charge and size. Differentiation of different alleles of a particular gene is achieved through the appearance of the allozyme as a result of its mobility on the gel. By comparing the bands provided, genetic variation between different populations can be detected (Erguden, 2007).

In a monomeric enzyme containing a single polypeptide unit, the different location of each band in the activity region is a direct result of the presence of a different polypeptide encoded by the F allele, which has a fast electrophoretic mobility, or the S allele, which has a slow electrophoretic mobility (figure 2).

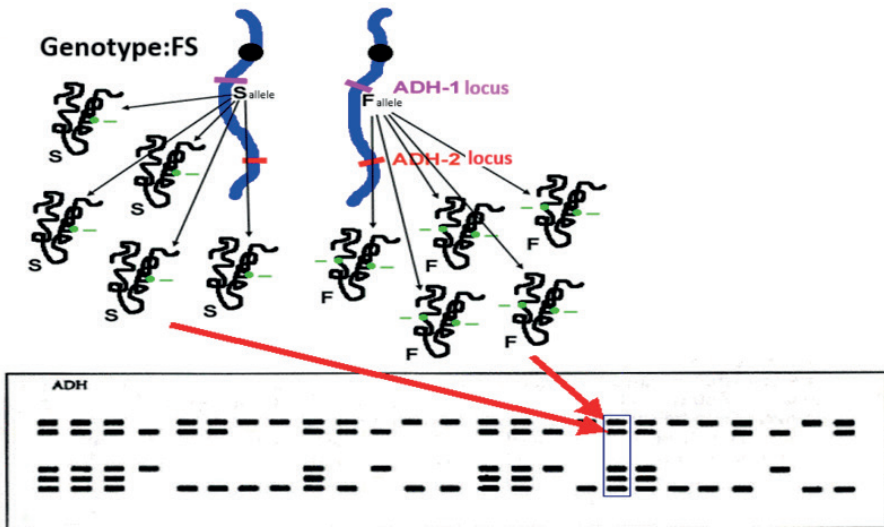


Figure 2 Electrophoretic image of Monomeric Alcohol dehydrogenase enzyme (“Allozyme,”)

Figure 3, different band locations in the activity region of a dimeric enzyme containing 2 polypeptide units in its quaternary structure indicate different

combinations of these polypeptide units. For polypeptide subunits coded by 2 different alleles such as F and S in an individual, there are three situations: FF dimer with fast electrophoretic mobility, SS dimer with slow electrophoretic mobility, and FS dimer showing enzyme bands of both alleles (Atanassova, Brookes, Loxdale, & Powell, 1998).

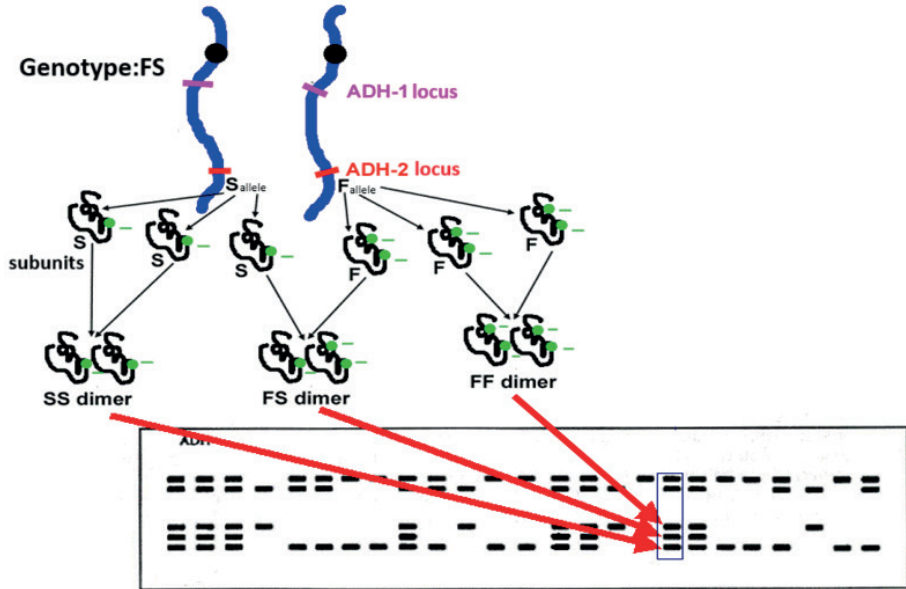


Figure 3 Electrophoretic image of dimeric Alcohol dehydrogenase enzyme (“Allozyme.”)

### Advantages of Allozyme Electrophoresis

- The information available is unbiased and easy to calculate.
- The information possessed is the result of hereditary variation and is not affected by phenotypic features such as physiological changes.
- It allows the analysis of many individuals at the same time.
- It allows the separation of heterozygous and homozygous individuals.
- Provides variants that can be used as molecular markers in systematic and population ecology researches. Variants create genetic polymorphisms at different loci in the genome.
- Compared to other molecular methods, results are obtained in a shorter time and are less costly (Erguden, 2007).

### Disadvantages of Allozyme Electrophoresis

- It allows examination of only structural genes.
- Some allozymes that vary may be under selection pressure, resulting

in a weak genetic marker.

- It cannot always detect the many distinctions that are likely to exist between genes. For example; Some amino acid changes may not change their electrical charges. Two researchers may make different interpretations of this situation as follows: Proteins with electrophoretic mobility are different or proteins with the same mobility do not have similar amino acid sequences.
- Since some amino acid changes may not change their electrical charges, researchers can make contradictory interpretations, such as proteins with different electrophoretic mobility are different or proteins with the same mobility do not have similar amino acid sequences.
- It may sometimes be difficult to explain the loci or alleles of some enzymes.
- Some enzymes can only be located in certain tissues.
- Since an intact sample is required for enzymatic activity determination, the need for live or frozen samples constantly arises.
- May require studying a large number of samples. For example; Since the size of Nematodes is very small, it is very difficult to determine their individual genotypes (Erguden, 2007).

### **Polymorphism**

Polymorphism occurs as a result of fragment placement, deletion, inversion or displacement in chromosomes. Regulation of gene expression, morphology, biochemistry, development and behavior are affected by polymorphism. Allozymes have been successfully used to detect polymorphisms, which are the source of phenotypic variations in the evolutionary process (Britten, 1986).

By allozyme electrophoresis, the presence of alleles of a gene and therefore its genetic polymorphism can be determined. Based on the data obtained, the variation in a population can be calculated mathematically. In population genetics, if a polymorphic population has two or more genotypes for a certain gene locus and the frequencies of each of them in the population are above 1%, the population is said to be polymorphic for the gene. In this case, for example, if there are two different alleles for a certain character in the population, the frequency of one of them will be 99% and the other will be 1%. To calculate the degree of polymorphism, the number of polymorphic loci in a population is proportioned to the total number of loci examined.

Since the gel shown in figure 4 shows monomorphism, it is concluded that all individuals have the enzyme with the same electrophoretic mobility. In the gel showing allozyme polymorphism, it is observed that 8 individuals are homozygous for the F allele, which has fast electrophoretic mobility, while



2 individuals are homozygous for the S allele, which has fast electrophoretic mobility, and 6 individuals are heterozygous for the F/S alleles, which show the enzyme bands of both alleles (Hartl, 1988).

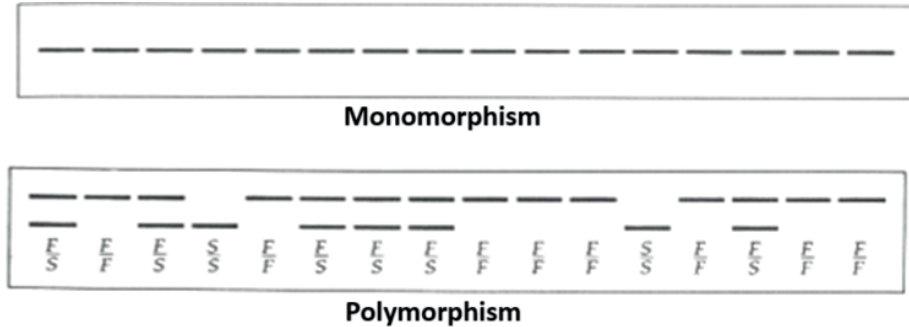


Figure 4 Monomorphism and polymorphism (Hartl, 1988).

### Heterozygosity

The degree of polymorphism calculated to show variation in populations is only useful for certain purposes in the field of population genetics. Since the number of loci showing variation may vary depending on the number of individuals examined in the population, it is not considered a reliable method to calculate variation in the population except for certain purposes. The safer method is to calculate the degree of heterozygosity.

To calculate the degree of heterozygosity (H), the average frequency of individuals who are heterozygous for each locus in the population is divided by the average of these frequencies for all loci. If more than one population of a species is examined, the average of the heterozygosity levels of each population is taken (Hartl, 1988).

### Environmental Expansion Theory

Many researchers suggest that there is a relationship between heterozygosity and variation in the environment in which the population is located. Environmental grain theory tries to explain variation in species by establishing a relationship between the size of the organism itself and the size of the environment in which it lives. There are two types of environment theory: coarse-grained and fine-grained (Hartl, 1988).

#### Coarse-grained environment theory

The parts of the environment in which the organism lives are much larger than the organism itself. Because the organism is small, it does not have the opportunity to choose from these many parts. For example; A garden consisting of many different types of flowers is a coarse-grained environment for an insect. In this environment, an individual of a particular insect species will spend its entire larval stage in just one flower. Therefore, it will gain

experience only with the same plant. Another insect of the same species will be able to gain vital experience in another plant. Thus, although there is multiple selection in the environment, different individuals of the same species may have gained experience in different environmental environments. Thus, high genetic variation can be expected in this type of species, as the chance of success of different alleles increases in different environments (Hartl, 1988).

**Fine-grained environmental theory**

The proportion of the parts of the environment in which the organism lives is small relative to its size and activities. Therefore, the organism can recognize the entire mosaic environment it is in and gain experience there. For example; A particular meadow is a fine-grained environment for a horse. Because the horse can wander and graze everywhere in this meadow. It has no other choice for itself other than the grass here. But a very large meadow with grasses on one side and thorns and other plants on the other is a coarse-grained environment for this horse.

According to the environmental extent theory; Genetic variation will be less in species with large bodies, greater mobility and populations consisting of a small number of individuals (e.g. Mammals).

On the other hand, genetic variation may be greater in species with small bodies, less mobility, limited to certain environments and large populations (e.g. Aphids).

Many plant species have ecotypes that differ morphologically and physiologically in different microhabitats. Studies have found that long-living populations show genetic variation (e.g. Hieracium umbellatum).

As a result of electrophoretic studies, it is seen that genetic polymorphisms are very common in natural populations. According to the results obtained, it is revealed that Invertebrate animals generally show more variation than Vertebrate animals due to polymorphism and heterozygosity.

*Table 1 shows a comparison of genetic diversity among different living groups.*

Organism	Number of Species	Number of Loci	Polymorphism	Heterozygosity
Drosophila	28	24	0.529	0.150
Wasp	6	15	0.243	0.062
Other insects	4	18	0.531	0.151
Marine invertebrates	14	23	0.439	0.124
Snail	5	18	0.437	0.150
Fish	14	21	0.306	0.078
Frog	11	22	0.336	0.082

Reptile	9	21	0.231	0.047
Bird	4	19	0.145	0.042
Mammal	30	28	0.206	0.051
Autonomous plant	33	14	0.179	0.058
Interspecific matching plant	36	11	0.511	0.185

Table 1 Comparison of genetic diversity among different living groups (Ayala & Kiger Jr, 1980)

In Chart 1, while allozyme variations among other living groups are at similar high values, a 70% decrease in the polymorphism and heterozygosity values of *Drosophila* populations is observed (Hartl, 1988).

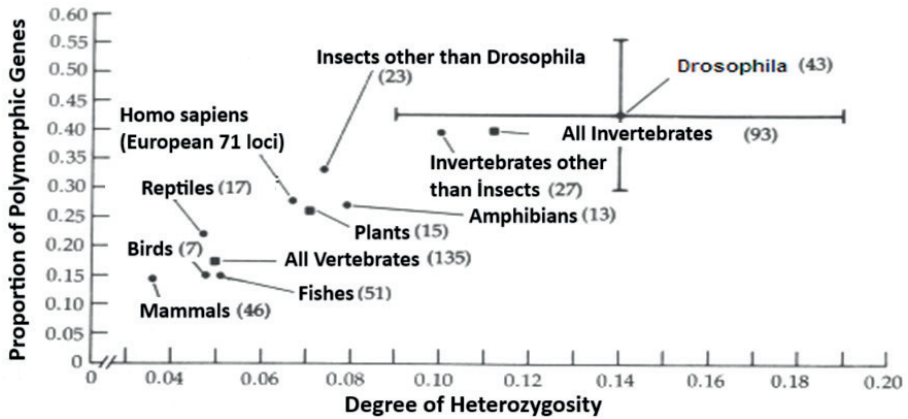


Chart 1 Allozyme variations in different living groups (Hartl, 1988).

## EXAMPLES FROM ALLOSYME STUDIES

In their study in 2009, Burcu KOÇAK MEMMİ and her colleagues investigated the resistance levels against Malathion and the enzyme activities and esterase polymorphisms of carboxyl esterases, cholinesterases, acetyl esterases and other esterases in *Drosophila melanogaster* natural populations obtained from 10 different localities.

In the study where esterase allozymes were investigated by starch gel electrophoresis in *Drosophila* Hatay-Dörtyol natural populations, carboxyl esterase (EST 6) polymorphism was found. The frequencies of fast (FF), slow (SS) and heterozygous (FS) alleles of the esterase enzyme were determined. The fast allele of *Drosophila* is most frequently found in the Antalya-Central, Balıkesir-Ayvalık, İzmir-Çandarlı, Hatay-Erzin, Eskişehir-Central, Kocaeli-Kerpe, Antalya-Serik and Antakya-Central populations, and the slow allele is most frequently found in the İzmir-Central population. . When the fast and

slow allele frequencies were evaluated in the Hatay-Dörtyol population, it was concluded that they were equal (MEMMİ, 2009).

In their study in 2007, Şafak BULUT and colleagues evaluated the morphometric, karyological analysis and allozyme variations of *Meriones tristrami blackleri*, *Meriones tristrami lycaon* and *Meriones tristrami intraponticus* subspecies in Manisa (Turgutlu), Karaman (Karadağ) and Kastamonu (Tosya).

Enzyme studies in populations of *Meriones tristrami* subspecies were first performed on Glyceraldehyde 3 phosphate dehydrogenase (G3PDH), Alpha glycerophosphate dehydrogenase ( $\alpha$ -GPDH), Phosphoglucomutase, Aconitase, Glucose 6 phosphate dehydrogenase, Superoxide dismutase (SOD), Phosphogluconate dehydrogenase, Mannose 6 phosphate isomerase, Aldolase, Malic enzyme, Lactate dehydrogenase, Isocitrate dehydrogenase, Glucose phosphate isomerase, Fumarase, Hexokinase enzymes. 24 gene loci were studied in 35 samples of *Meriones tristrami* subspecies. 4 of these loci (G3PDH,  $\alpha$ -GPDH-1,  $\alpha$ -GPDH-2 and SOD) were found to be polymorphic and heterozygous.

In the samples studied, the fixation index value was 0.67, indicating approximately 7% genetic difference. The average heterozygosity value of the loci showing polymorphism in the populations is 0.016. The genetic distance is between 0.000 and 0.002, which is a very low value. It has been determined that gene flow between populations is high (Bulut, 2007).

In their study in 2008, Ufuk GÜNDÜRÜ and his colleagues revealed *Mus macedonicus* and *Mus domesticus* species living in 7 different localities and the genetic differentiation between different populations of these species by allozyme electrophoresis analysis. Isocitrate dehydrogenase (IDH-1, IDH-2), Malic enzyme, Phosphoglucomutase (PGM), Fumarate hydratase, Superoxide dismutase (SOD-1, SOD-2), Glucose 6 phosphate isomerase (GPI), Xanthine dehydrogenase, Glucose 6 phosphate dehydrogenase, Malate dehydrogenase (MDH-1, MDH-2), Carbonic anhydrase (CA-1, CA-2), Glutamate oxaloacetate transaminase (GOT-1, GOT-2), Adenylate kinase, Esterase, Lactate dehydrogenase (LDH-1, LDH-2, LDH-3, LDH-4, LDH-5) enzymes were studied in *Mus macedonicus* and *Mus domesticus* populations.

A total of 35 *Mus* samples from Edirne, Kırklareli, Tekirdağ, Bolu, Düzce, Zonguldak and Bartın localities were evaluated. 23 loci in 14 enzyme systems were detected by starch and polyacrylamide gel electrophoresis. 5 of these loci (PGM, IDH-2, GPI-1, CA-1, CA-2) were found to be polymorphic. Since the isocitrate dehydrogenase-2 locus has three alleles (A, B, C), it is the distinctive locus of *Mus domesticus* and *Mus macedonicus* species.

When the data on loci in *Mus* populations were examined, it was determined that the average value of the fixation index was 0.3693 and the gene flow value, which expresses genetic differentiation between populations, was 0.426.

In the dendrogram created based on genetic similarity values, it is seen that there are two main clusters consisting of *Mus domesticus* and *Mus macedonicus* populations, and each of the main clusters is divided into two subclusters. While the first subcluster of *M. macedonicus* includes the Bolu population, and the second subcluster includes Edirne, Tekirdağ, Kırklareli and Düzce populations, the first subcluster of *M. domesticus* includes the Bolu population, and the second subcluster includes Tekirdağ, Zonguldak and Bartın populations (Gündürü, 2008).

In their study in 2009, Şengül HASKILIÇ and her colleagues evaluated the morphological and genetic variations of the *Crocidura* genus in 21 different localities by morphological and allozyme electrophoresis analyses.

Glycerol 3 phosphate dehydrogenase, Lactate dehydrogenase (LDH), Malate dehydrogenase, Malic enzyme, Isocitrate dehydrogenase, Phosphoglucose dehydrogenase (PDG), Glucose 6 phosphate dehydrogenase (G6PD), Aspartate aminotransaminase, Hexokinase (HK), Creatine kinase (CK), Phosphoglucosmutase (PGM), Esterase, Acid phosphatase (ACP), Leucine aminopeptidase, Fumarase, Mannose 6 phosphate isomerase (MPI), Glucophospho isomerase (GPI) enzymes were studied.

Allozyme analyzes were performed on 77 samples using cellulose acetate gel electrophoresis. Of the 25 loci detected, 14 (LDH-1, LDH-2, LDH-3, LDH-4, LDH-5, PGD, G6PD, HK, CK-c, CK-m, PGM, ACP, MPI, GPI) were found to show polymorphism.

It was determined that the genetic differentiation value was 0.1854 and the genetic distance between the populations was found to be very low, ranging between 0.000 and 0.068. The mean expected heterozygosity and observed heterozygosity values were between 0.000-0.107 and 0.000-0.140, respectively. Morphological and allozyme analysis results show that 21 individuals of the *Crocidura* population belong to the *Crocidura suaveolens* species.

The first of the two main clusters in the dendrogram created using the genetic distance matrix includes Yenifakılı/Yozgat, and the second cluster includes twenty other localities other than Yenifakılı/Yozgat locality. It was determined that the genetic distance values between the Yenifakılı / Yozgat population and other populations were larger, while other localities had smaller values among themselves. There are no major differences between populations (Haskiliç, 2009).

## LOSS OF GENETIC VARIATION

Although genetic polymorphism appears to be very common in natural populations, it is not a universal phenomenon. Today, loss of genetic variation is revealed in populations that are on the verge of extinction (Hartl, 1988).

### Cheetah

Cheetahs (*Acinonyx jubatus*) are one of the species in danger of extinction. Until 20 thousand years ago, four cheetah species lived in Asia, Europe, North America and Africa. A single cheetah species has spread from Southwest Asia to India and a large part of Africa for the last few centuries. Today, fewer than 20 thousand cheetahs are alive, most of which are distributed in the southern and eastern parts of Africa. One of the biggest challenges facing cheetahs is decreasing genetic diversity.

In their study in 1985, Stephen O'Brien and his colleagues investigated genetic diversity in 55 Cheetah populations in Southeast Africa. By determining the polymorphism and heterozygosity values to be zero in the 52 gene loci identified, they concluded that there was no genetic diversity in Cheetah populations. In studies conducted on 7 wild cat species other than Cheetahs, the results showing that polymorphism with allele frequencies in 48-50 gene loci ranged from 8% to 20.8% and heterozygosity ranged from 0.029 to 0.072, represent the typical normal level of genetic diversity for Mammals.

In the study conducted by Stephen O'Brien and his colleagues in 1986, they examined 49 enzyme systems of 30 individuals of *Acinonyx jubatus raineyi*, an East African subspecies. As a result of the research, it was determined that since only 2 of the loci examined were polymorphic, they could be considered almost monomorphic. Cheetahs were found to have 4% polymorphism and 0.0014 heterozygosity, showing some genetic diversity. It was determined that 98 *Acinonyx jubatus jubatus* subspecies living in southern Africa showed 2% polymorphism and 0.0004 heterozygosity in a single gene locus (Gur; Hartl, 1988).

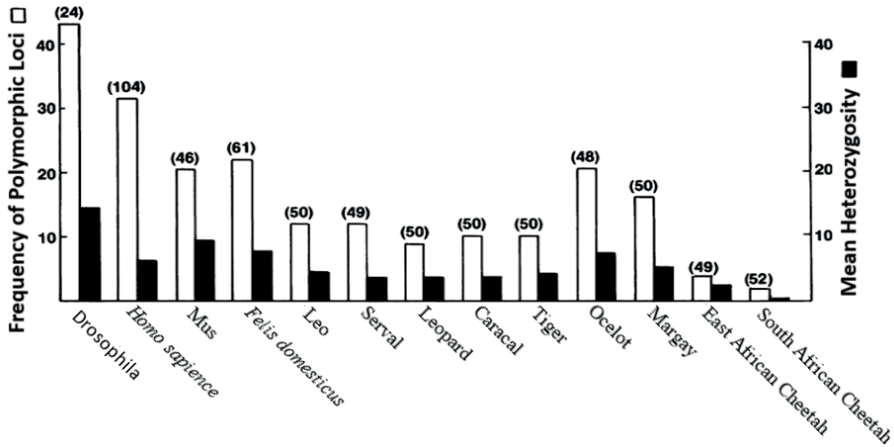


Chart 2 Comparison of cheetah genetic diversity with different living groups (O'Brien et al., 1987).

The loss of genetic variation in cheetah populations living in the southern and eastern parts of Africa is at dangerous levels (chart 2). While many mammal species became extinct around 10 thousand years ago, there is evidence that the genetic diversity of the cheetah population decreased as a result of genetic drift resulting from the bottleneck effect. It is thought that the repeated shrinkage in cheetah populations caused the allele diversity in the gene pool to decrease.

While more than 10% of cats survive even as a result of the most deadly diseases, in 1982, 27 of 42 cheetahs who contracted Feline Infectious Peritonitis, originating from a virus that is not a very lethal strain, in the Wildlife Safari Park, died, and most of the other Cheetahs became ill and produced high levels of antibodies against the disease agent. None of the Lions got sick and all of them developed antibodies against the disease.

Cheetahs, being solitary organisms, are extremely unlikely to encounter this disease before. The cheetah population has become susceptible to the disease because special alleles located at various loci of its genes have repeatedly spread randomly in the population as a result of the bottleneck effect and eventually replaced alleles that would resist the disease.

If the Cheetah population can survive in its habitat at a suitable size and for a long enough period of time, it can create the genetic diversity it needs to adapt to environmental changes (Gur).

### Polar bear

Another species in danger of extinction in the wild is Polar bear (*Ursus maritimus*). It is known that the Polar bear population is estimated to be around 20,000, but their numbers decrease above  $-50^{\circ}\text{C}$ . Polar bears, which can live in the entire polar region, are especially prevalent in the Canadian

Arctic archipelago, Northern Alaska, Wrangel Island and Western Alaska, North-Central Siberia, Svalbard-Franz Josef Land and Greenland (“Kutup ayısı”).

Allendorf et al. in 1979, followed by Larsen et al. in 1983, reported in their studies that Polar bear populations showed little genetic diversity as a result of allozyme electrophoresis.

In similar studies conducted by Cronin et al. in 1991 and Scribner et al. in 1997, they determined that there was a small amount of genetic variation among Polar bear populations in the Beaufort and Chukchi seas by protein electrophoresis analysis.

Scientists named Paetkau and Strobeck determined the heterozygosity and polymorphism values of eight bear populations in North America in their study in 1994. Research results reveal that while Black and Brown bear populations have similar genetic variation, the Polar bear population shows less genetic variation (table 2).

	Population	Heterozygosity	Polymorphism
<i>Ursus arctos</i>			
Kluane NP	102	%76	1/260.000.000
Richardson Mts.	238	%76	1/290.000.000
Coppermine	76	%60	1/780.000
Seward Peninsula	30	%72	1/15.000.000
Alasca Peninsula	28	%53	1/28.000
Kodiak Island	68	%27	1/93
Yellowstone	108	%56	1/152.000
<i>Ursus americanus</i>			
Banff NP	64	%82	1/7.200.000.000
New foundland Island	46	%43	1/1.300
<i>Ursus maritimus</i>			
Hudson Gulf	60	%63	1/1.300.000

Table 2 Genetic diversity values of eight bear populations in North America (Waits, 1999)

In their study in 1999, Paetkau and his colleagues found the genetic distance value between Polar bear populations to be extremely low. They also reported that there was a significant correlation between Polar bear population movement and genetic data around the world.

Researchers named Paetkau and Strobeck reported in 1998, in their study on casein and major histocompatibility complex genes, which show significant allelic variation, that Polar bears showed less genetic variation within their populations than Black and Brown bear populations. However; The degree of heterozygosity on a hypothetical population was calculated as 0.68 for Polar bears, 0.66 for Brown bears, and 0.72 for Black bears.



Paetkau and his colleagues reported in their study in 1999 that Polar bears belong to an evolutionary lineage. They concluded that the large decrease in the Polar bear population was mostly due to small genetic distances or lack of genetic distance between Polar bear populations. While changes in the Polar bear population have remained low throughout geological time, the reason for today's difference is the decrease in genetic diversity as the gene pool has been depleted as a result of the inbreeding ability of Polar bears being affected ("Polar Bears International," ; Waits, 1999).

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