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miRNA-3Age: A novel miRNA-based aging marker and its association with lifestyle and dietary characteristics

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List of Abbreviations

miRNAs……microRNAs
WHO……World Health Organization
CA……...Chronological age
BA……Biological age
AFAR……..American Federation for Aging Research
FI……..Frailty Index
DNAmage……..DNA methylation age
HDL……High Density Lipoprotein
ncRNAs……...non-coding RNAs
UTR……...untranslated region
mRNAs……messenger RNAs
NF-κB……...nuclear factor-κB
TLR……...Toll-like receptor
ROS……reactive oxygen species
C/EBPβ……..CCAAT/enhancer-binding-protein-ß
NRF2……...nuclear factor erythroid 2-related factor 2
SOD……superoxide dismutase
HMOX1……..heme oxygenase-1
TP53INP1……tumor protein p53-inducible nuclear protein 1
FOXO3……..Forkhead box O3
HIF-1α……Hypoxia-inducible factor 1-alpha
DNMT1……DNA methyltransferase 1
SIRT1……..Sirtuin 1
NOX4……..NADPH oxidase 4
NLRP3……..(NOD)-like receptor protein 3
IRAK……Interleukin-1 receptor (IL-1R) associated kinase
MyD88……...Myeloid differentiation primary response 88
PTEN……phosphatase and tensin homolog
PDCD4……..programmed cell death protein 4
IL……Interleukin
TGF-β……transforming growth factor ß

TNF-α……...tumor necrosis factor-alpha
CDKI……...cyclin-dependent kinase inhibitor
pRb……...retinoblastoma protein
MAPK…….mitogen-activated protein kinase
cDNA…….complementary DNA
qPCR…….qualitative polymerase chain reaction
MAE……..mean absolute error
MedAE……...median absolute error
CI……...confidence interval
OR……odds ratio
NIAAA……..National Institute on Alcohol Abuse and Alcoholism
CHD……Coronary Heart Disease
CRP……..C-reactive protein
MD…….Mediterranean diet
EPA……eicosapentaenoic acid
DHA……...docosahexaenoic acid
EGCG……..epigallocatechin-3-gallate

Abstract

As life expectancy rises dramatically, the disparity between Lifespan and Healthspan becomes a pressing issue, underscoring the urgent need for practical aging markers. Horvath's discovery of the DNA methylation age highlights the significance of epigenetic clocks in assessing biological age, presenting a crucial benchmark for contemporary aging studies. This thesis hypothesizes that age-associated microRNAs can serve as reliable epigenetic biomarkers of aging and provide a novel approach for interventions to extend Healthspan.

A comprehensive literature review identified miRNAs, notably miR-21, miR-24, and miR-155, closely linked to crucial aging processes such as inflamm-aging. The study employed a dataset comprising a quantitative analysis of the ΔCt levels of these miRNAs in capillary blood samples from a cohort of 539 adults. The miRNA-3Age model for estimating biological age was developed using a linear model and modern machine-learning techniques. The model's effectiveness was assessed using metrics such as mean absolute error (10.4 years), median absolute error (9 years), and the correlation between biological and chronological age ($\rho = 0.13$ ($p= 0.002$)).

Furthermore, this is the first study to highlight the relationship between lifestyle, behavioral, and dietary characteristics, and miRNA age. The difference between miRNA-3Age and chronological age (BA-CA), was defined as a new binary variable (biologically younger and biologically older). A logistic regression model identified a significantly higher chance of being biologically younger for daily alcohol consumption (OR= 2.85, p= 0.0004), moderate exercises (OR= 1.69, p= 0.036), moderate fish consumption (OR= 2.30, p= 0.00005), daily whole grain consumption (OR= 1.80, p= 0.032), and daily green tea intake
(OR= 1.54, p= 0.024). Additionally, a significantly lower chance of being biologically younger was observed for smoking (OR= 0.4, p= 0.0004), regular fast-food intake (OR= 0.4, p= 0.00005), and elevated stress levels (OR= 0.58, p= 0.005).

The study underscores the complexity of identifying aging biomarkers and building a functioning aging estimator. Furthermore, this research establishes a link between miRNA

expression and lifestyle, providing new insights into the aging process and highlighting the potential of miRNAs as targets for anti-aging strategies to extend Healthspan.

Zusammenfassung

In den letzten zwei Jahrhunderten hat die Lebenserwartung signifikante Zuwächse verzeichnet, während die Zeitspanne, die man in vollkommender Gesundheit lebt, nicht im gleichen Maße zugenommen hat. Diese Diskrepanz führt dazu, dass die letzten Jahre vermehrt in Krankheit verbracht werden und unterstreicht die dringende Notwendigkeit, zuverlässige Marker für den Altersprozess zu identifizieren. Horvaths Entdeckung der DNAm Aging Clock hebt die Bedeutung epigenetischer Marker zur Bewertung des biologischen Alters hervor und stellt einen wichtigen Maßstab für die zeitgenössische Alternsforschung dar. Die folgende Arbeit stellt die Hypothese auf, dass altersassoziierte mikroRNAs als zuverlässige epigenetische Biomarker des Alterns dienen und einen neuen Ansatz für Anti-Aging Interventionen liefern können.

Anhand einer umfassenden Literaturrecherche wurden miRNAs identifiziert, die mit wichtigen Alterungsprozessen wie inflamm-aging in Verbindung stehen, wobei insbesondere in der folgenden Untersuchung miR-21, miR-24 und miR-155 hervorgehoben wurden. Für diese Studie wurde ein Datensatz verwendet, welcher unteranderem die ΔCt-Werte dieser drei miRNAs in Kapillarblutproben einer Kohorte von 539 Erwachsenen enthielt. Das miRNA-3Age-Modell zur Schätzung des biologischen Alters wurde unter Verwendung eines linearen Modells und moderner machine-learning Techniken entwickelt. Die Effektivität und Genauigkeit des Modells wurde anhand von Metriken wie dem MAE (10.4 Jahre), MedAE (9 Jahre) und der Korrelation zwischen biologischem und chronologischem Alter ($\rho = 0.13$ ($p= 0.002$)) bewertet.

Zudem ist dies die erste Studie, welche die Beziehung zwischen Lebensstil, Verhaltensweisen sowie diätetischen Eigenschaften und dem miRNA-Alter hervorhebt. Der Unterschied zwischen miRNA-3Age und chronologischem Alter (BA-CA) wurde als neue binäre Variable definiert (biologisch jünger und biologisch älter). Ein logistisches Regressionsmodell identifizierte eine signifikant höhere Wahrscheinlichkeit, biologisch jünger zu sein bei täglichem Alkoholkonsum (OR = 2,85, p = 0,0004), moderater Bewegung (OR = 1,69, p = 0,036), mäßigem Fischkonsum (OR = 2,30, p = 0,00005), täglichem Vollkornkonsum (OR = 1,80, p = 0,032) und täglichem Grünteekonsum

(OR = 1,54, p = 0,024). Darüber hinaus wurde eine signifikant geringere Wahrscheinlichkeit, biologisch jünger zu sein, bei Rauchern (OR = 0,4, p = 0,0004), regelmäßigem Fast-Food-Konsum (OR = 0,4, p = 0,00005) und erhöhtem Stressniveau (OR = 0,58, p = 0,005) beobachtet.

Die Forschung unterstreicht die Komplexität der Identifizierung von Biomarkern für das Altern und des Aufbaus eines funktionierenden Altersschätzers. Durch die Verknüpfung von altersbedingten Veränderungen in der miRNA-Expression und Lebensstilfaktoren bieten die Ergebnisse neue Forschungsansätze für die Entwicklung von Anti-Aging-Strategien zur Verlängerung der Gesundheitsspanne.

1. Introduction

1.1 Aging and Healthy Aging

Aging is a natural process marked by the accumulation of degenerative processes, leading to a decline in mental and physical capacity. It also increases the risk of a variety of non-communicable diseases, including cancer, cardiovascular diseases, neurological diseases, and diabetes, as well as an increased risk of mortality and eventually death (Chalise et al., 2023; López-Otín et al., 2013; Wagner et al., 2016). Aging is primarily characterized by what is known as the *Hallmarks of Aging* (Figure 1). These hallmarks comprise fundamental biological processes identified as potential targets for aging intervention. They include, but are not limited to, mitochondrial dysfunction, accumulation of somatic and mitochondrial DNA mutations, epigenetic alterations, (immune-)senescence, oxidative stress, loss of protein homeostasis, aberrant intracellular communication, shortened telomere length, dysregulated nutrient sensing, and stem cell depletion (López-Otín et al., 2013; Rivero-Segura et al., 2020; Wagner et al., 2016).

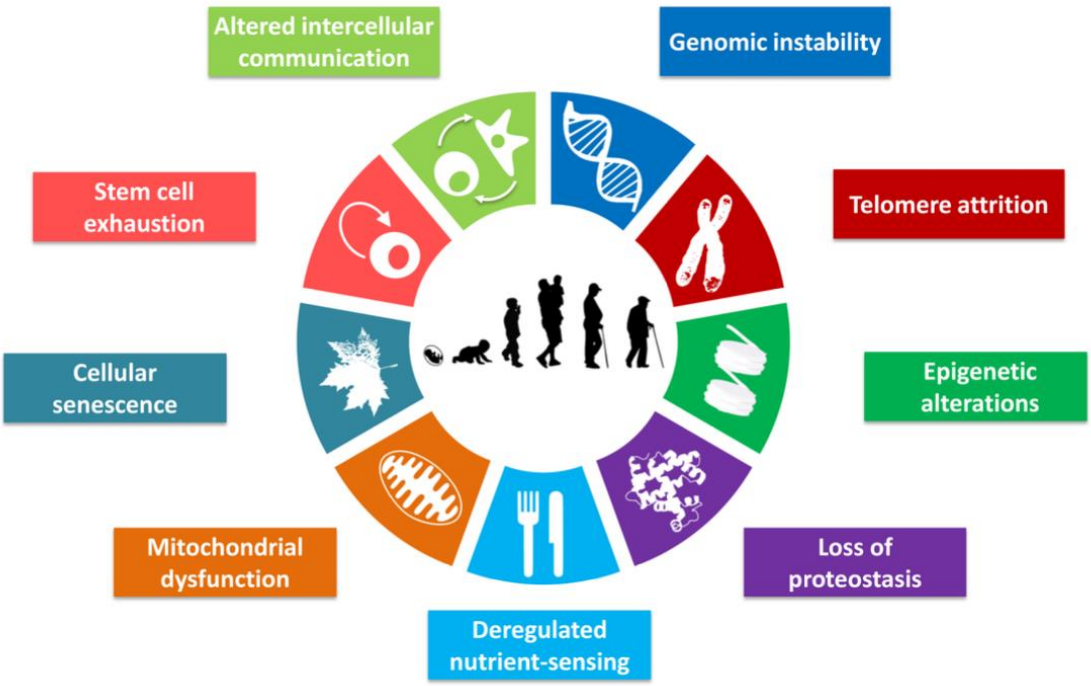

Figure 1: The hallmarks of aging, depicting key biological processes contributing to aging (Reprinted by López-Otín et al., 2013, p.37).

Over the past two centuries, advancements in water and food quality, hygiene, housing, lifestyle, immunization, antibiotics, and medical care have significantly increased life expectancy (Partridge et al., 2018). Most developed nations are experiencing a doubling of the average lifespan due to a drastic reduction in mortality rates (Partridge et al., 2018). In 1950, no country had more than 11% of its citizens aged 65 and above. By 2000, this percentage had risen to 18%, projected to increase to 38% by 2050 (Rudnicka et al., 2020). This surge in average lifespan since the mid-twentieth century has led to a demographic shift, expanding the elderly population from around 130 million (5% of the world population) in 1950 to an estimated 1.6 billion (17% of the world population) by 2050 (Bell et al., 2019). This trend is reflected in the European Union, where the number of people aged 80 and above is expected to grow significantly, reaching around 75 million by 2100, compared to just under 30 million in 2023. Additionally, the population aged 20-64 is projected to decrease steadily over the same period (Figure 2) (Eurostat, 2023).

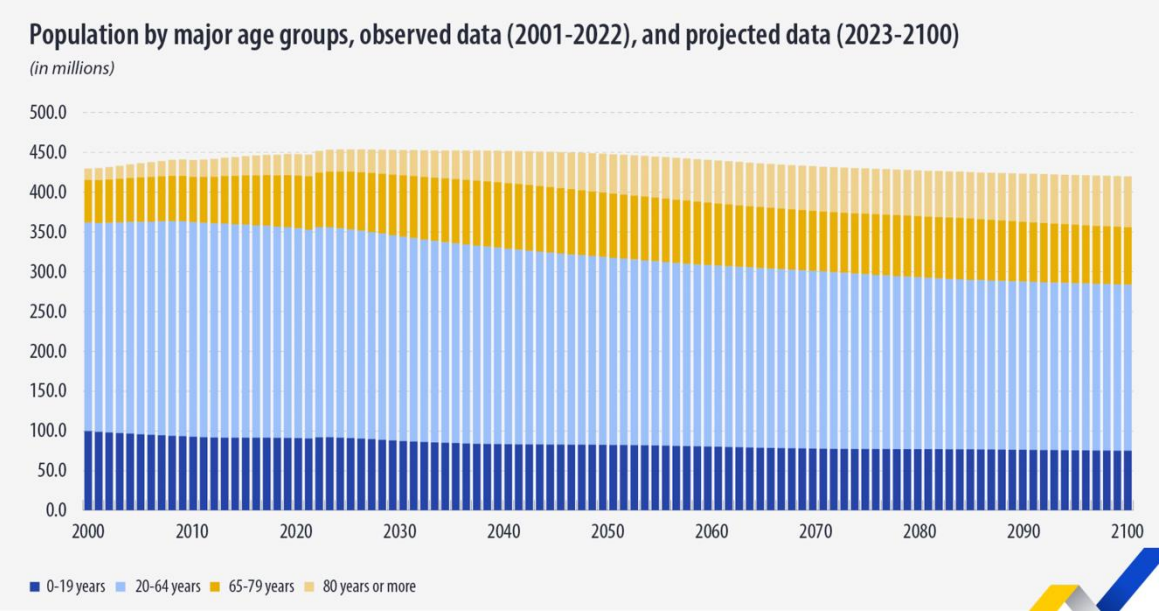

Figure 2: Population distribution (Year 2000-2100) in Europe by age groups, highlighting the significant increase in the elderly population (Reprinted by Eurostat, 2023).

According to the World Health Organization (WHO), healthy aging involves the development and maintenance of the capability to function effectively in old age. Functional ability here refers to a person's capability to do what they value. It includes meeting basic needs, learning, growing and making decisions, being mobile, building and maintaining relationships, and contributing to society (Rudnicka et al., 2020).

Despite the previously described gains in lifespan, the healthy, disease-free lifespan, also called *Healthspan*, has not increased in the same way, with only a 4.6-year increase in healthy life expectancy accompanying a five-year increase in total life expectancy from 2000 to 2015. This discrepancy is responsible for the fact that 16-20% of life today is spent in late-life morbidity, with the range for women being significantly higher than for men (Partridge et al., 2018).
The global aging population has become a critical medical and social demographic challenge worldwide, as age is the significant risk factor for chronic diseases, necessitating prioritized care and the axiomatic need to find methods to help people stay healthy and free of disability as they age (Chalise et al., 2023; Ng et al., 2020; Partridge et al., 2018).
To extend quality of life and increase Healthspan, it has been proposed that the slowing of aging could be achieved by promoting health interventions (Chalise et al., 2023).

Emerging evidence suggests that our body's various alterations as we age are linked to a person's epigenetics (Campisi & Vijg, 2009; Oberdoerffer & Sinclair, 2007). Epigenetic changes occur naturally in all cells and tissues at different stages of life, and they refer to phenotypic modifications that do not alter the DNA sequence. These changes include DNA methylation, acetylation, 3D genome architecture, histone modifications, and noncoding RNAs like microRNAs (López-Otín et al., 2013). The environment and lifestyle choices are the main culprits for these changes, which are highly heritable (Buck Louis et al., 2017). Since epigenetic alterations are theoretically reversible, they offer an effective target for anti-aging treatments to extend the Healthspan (López-Otín et al., 2013).

1.2 Biomarkers of Aging

Maximizing a person's Healthspan makes the measurement of aging-related pathology essential (Bell et al., 2019). Chronological age (CA) denotes the years alive since birth. It is recorded by the simple time flow and is considered an imperfect surrogate measure of the

aging process that cannot accurately evaluate the aging status (Baker & Sprott, 1988; Rivero-Segura et al., 2020). Estimating age requires the development of a new health marker that can accurately predict a person's or organ's functional capacity and how it changes during a lifetime (Baker & Sprott, 1988).

Chronological age remains the most substantial risk factor for age-related diseases and mortality, but it is essential to differentiate between time course and biological aging (Levine et al., 2018). Biological age (BA), also called physiological age, describes a human's overall health condition at a particular date in relation to their chronological age (Rivero-Segura et al., 2020). BA can be determined by incorporating biomarkers of aging, i.e., age-related variables associated with health status, into existing health indicators (Kim et al., 2022).

Since the 1980s, researchers have recognized the need for valid and reliable biomarkers to understand, slow, halt, or reverse aging. Baker and Sprott determined an accurate biomarker of aging as a: "[…] biological parameter of an organism that either alone or in some multivariate composite will, in the absence of disease, better predict functional capability at some late age than will chronological age" (Baker & Sprott, 1988, p.1).

Based on Baker and Sprott's work, the American Federation for Aging Research (AFAR) has proposed the following criteria for a biomarker of aging:

"1. It must predict the rate of aging. In other words, it would tell exactly where a person is in their total life span. It must be a better predictor of life span than chronological age.

2. It must monitor a basic process that underlies the aging process, not the effects of disease.

3. It must be able to be tested repeatedly without harming the person. For example, a blood test or an imaging technique.

4. It must be something that works in humans and in laboratory animals, such as mice. This is so that it can be tested in lab animals before being validated in humans" (T. E. Johnson, 2006, p.1).

Biomarkers of aging can be categorized into the following four groups: blood- or blood-component-based markers, DNA-based markers, physical function and anthropometry, and novel biomarkers (Wagner et al., 2016). Current methods include physical age (based on clinical measures like diastolic and systolic blood pressure, total cholesterol, albumin, and C-reactive protein), functional age (cognitive age and the Frailty Index (FI)), telomeric age, and DNAm age (weighted average of methylation levels) (Benetos et al., 2001; Horvath, 2013; Levine, 2013; Lin, 2022). However, as of today, a single method has yet to be recognized as the golden standard for measuring aging (Lin, 2022). Despite the comprehensive understanding of the biological underpinning of aging, accurately quantifying this complex process remains challenging for current biomarkers (Wagner et al., 2016).

1.3 Omics-based Biomarkers of Aging

Emerging research suggests that a group of omics biomarkers associated with aging could encompass the various physiological factors that impact an individual's health throughout life (Rivero-Segura et al., 2020). Studies at the multi-omics level combine various factors such as transcriptome, epigenome, extracellular RNAs, telomere length, proteome, metabolome, and microbiome. This multi-faceted approach could offer a more holistic view for developing healthy aging interventions for the next generation (Rivero-Segura et al., 2020). By leveraging these comprehensive biomarkers, the novel health-function-centered paradigm may be utilized to promote healthy aging (Rivero-Segura et al., 2020).

Among the omics-based biomarkers, two stand out the most: DNA methylation age (DNAm age) and telomere length (Levine et al., 2018). DNA methylation is a post-replicative modification in cytosines within CpG islands and is commonly assigned to the *Epigenomic* field (Horvath, 2013). Over time, these regions across the genome experience hypo- and hyper-methylation changes, which can impact biological age (Levine et al., 2018). The corresponding physiological age estimator, the *Epigenetic clock*, also called DNAm age, was first described and named by researcher Steve Horvath in 2013. Horvath's DNAm age is linked to a range of age-related diseases, like Parkinson's disease, Werner syndrome, neuropathology, and physical and cognitive fitness (Horvath & Raj, 2018). Additionally, Levine's DNAm Phenoage, a life expectancy predictor and a further development of the

original DNAm age, has a highly predictive accuracy for coronary heart diseases, cause-specific death, and the time of death itself (Levine, 2013).

Secondly, telomere length is a commonly used biomarker of aging (Rivero-Segura et al., 2020). Telomeres are protective caps at the end of eukaryotic chromosomes. They consist of a repetitive DNA sequence (5'–TTAGGG–3'). With each cell division, telomeres become shorter, and as age increases, the shortening range also increases. This can lead to chromosome instability, senescence, and DNA damage (Rivero-Segura et al., 2020).

On the other hand, experts worldwide are focusing on further identifying potential biomarkers to complete the omics set. One promising biomarker source is the metabolome due to its high sensitivity and specificity (Rivero-Segura et al., 2020). Metabolomics refers to the complete set of low molecular weight metabolites, providing a direct functional physiological readout. It comprises proteins involved in deficits in nutrient recognition, protein, and lipid metabolism, such as polyunsaturated fatty acids and total fatty acids, NAD+ levels, small HDL, and glucose (Rivero-Segura et al., 2020).

Moreover, the first *Proteomic* studies have revealed that with time, proteins involved in homeostasis, apoptosis, immune function, and iron transport increase significantly as a person ages (Rivero-Segura et al., 2020). Proteomic studies include the complete set of proteins expressed under different pathological or physiological conditions by the cell, tissue, genome, or organism at a specific time. A. A. Johnson et al. (2020) identified 23 proteins with increased expression in older subjects and created the first Proteomic age estimator (A. A. Johnson et al., 2020).

Finally, *Transcriptomic* studies identified eight genes (GFBP3, LRRN3, CRIP2, SDC, IDS, TCF4, GATA3, HN1) linked to healthy aging (Rivero-Segura et al., 2020). These genes capture around 71% of the transcriptional variation and are broadly associated with proliferation, adhesion, differentiation, and inflammatory markers (Kochunov et al., 2013).

As scientific research progresses into the Transcriptomic spectrum, the focus has shifted towards miRNAs. Their role in regulating key biological pathways implicated in aging positions miRNAs as potential cornerstone biomarkers for capturing the multifaceted nature of aging. Additionally, evidence shows that the correlation between the Epigenetic clocks

and Transcriptomic-based biomarkers of aging is relatively low, with correlation coefficients between 0.1 and 0.33, indicating that each method describes a different facet of aging (Rivero-Segura et al., 2020).

Given this, it is essential to develop a functional miRNA-based age-estimator that can complement existing biological age calculators and thus further advance the development of a multidimensional approach to an omics biomarker set.

1.4 Extracellular RNAs: microRNAs

The aging process is related to higher levels of transcriptional noise, significantly affecting ncRNAs, including microRNAs, which influence lifespan by targeting longevity or regulating stem cell behavior (López-Otín et al., 2013). miRNAs are a category of small single-stranded noncoding RNAs that are about 21-23 nucleotides long (Noren Hooten et al., 2010). They can influence gene expression by binding to the 3' untranslated region (UTR) of target messenger RNAs (mRNAs) (Machida et al., 2015). Gain and loss of function studies in Caenorhabditis elegans and Drosophila melanogaster were the first to show miRNAs' contribution to the aging process (Boehm & Slack, 2005; Ibáñez-Ventoso & Driscoll, 2009). Since then, various studies have identified several miRNAs at cell, tissue, and organism levels that accelerate or decelerate aging in humans and are associated with age-related diseases such as cancer, diabetes, obesity, and cardiovascular diseases (Khee et al., 2014; Rivero-Segura et al., 2020).

1.4.1 Inflamm-aging miRNA

The molecular inflammation hypothesis of aging suggests that an increase in oxidative stress triggers the activation of redox-sensitive transcription factors during aging (Halper et al., 2015). These factors then enhance the expression of pro-inflammatory genes in various cell types. Consequently, aging is characterized by a persistent inflammatory state, commonly called *inflamm-aging*, where mediators like proteases, cytokines, chemokines, and growth factors are continuously present for years at levels higher than baseline (Olivieri et al., 2013). Age-related chronic inflammation is primarily caused by the gradual immune cell activation and the accumulation of pro-inflammatory senescent cells with a pro-inflammatory phenotype (Olivieri et al., 2013). Scientific research has confirmed that a relatively large number of miRNAs regulate inflamm-aging, mainly by influencing the nuclear factor-κB (NF-κB) signaling pathway or Toll-like receptors (TLRs).

For example, activation of the TLR signaling leads to a rapid and significant co-induction of miR-155, miR-21, and miR-146a (Halper et al., 2015; Olivieri et al., 2013).

1.4.1.1 miR155

miR-155 is intricately linked to the body's immune response and is predominantly found in organs such as the liver, spleen, and thymus (Tacke et al., 2019). It plays a pivotal role in immune cell development and the regulation of systemic inflammatory processes (Tacke et al., 2019). By regulating the expression of multiple proinflammatory mediators, miR-155 is implicated in the body's response to infection, highlighting its broader role beyond aging (Tacke et al., 2019). Analysis of global miRNA expression profiles in adult women revealed that miR-155 is among the most up-regulated miRNAs in older age groups, suggesting its potential role as a biomarker of aging (Sredni et al., 2011).

Aging, in general, is associated with increased reactive oxygen species (ROS) generation and a concomitant decline in the effectiveness of antioxidant defenses, leading to cellular dysfunction, abnormality, and cell death, promoting organ and tissue aging (Onodera et al., 2017). MiR-155 emerges as a critical player in this process, with studies showing its induction of ROS generation and the upregulation in the bone marrow of aged mice (Lettieri-Barbato et al., 2022; Onodera et al., 2017). This effect is mediated by suppressing CCAAT/enhancer-binding-protein-ß (C/EBPβ), a transcription factor that regulates the expression of nuclear factor erythroid 2-related factor 2 (NRF2), superoxide dismutase 1 (SOD1), and heme oxygenase-1 (HMOX1) (Lettieri-Barbato et al., 2022). The overexpression of miR-155 in aged subjects has been shown to suppress antioxidative genes, triggering increased ROS production and highlighting a crucial link between miR-155, inflammation, and oxidative stress (Onodera et al., 2017).

On the other hand, reduced levels of miR-155 could lead to increased tumor protein p53-inducible nuclear protein 1 (TP53INP1), a marker of p53-mediated growth arrest (Wang et al., 2020). The miRNA targets transcripts in different cellular contexts, including Forkhead box O3 (FOXO3) and Hypoxia-inducible factor 1-alpha (HIF-1α), thereby influencing ROS levels and further contributing to cellular aging (Lettieri-Barbato et al., 2022).

1.4.1.2 miR21

MicroRNA 21 has emerged as a novel biomarker of healthy aging, exhibiting a distinctive expression pattern across the lifespan. Its circulating levels were found to be increased during the aging process. However, intriguingly, they are found to be decreased in healthy individuals above 80 years and centenarians (Bu et al., 2017). This pattern suggests that reduced levels of miR-21 may confer benefits for longevity, presenting this miRNA not only as a marker for biological aging but potentially as a target for interventions aimed at promoting healthy aging (Lettieri-Barbato et al., 2022; Olivieri et al., 2017).

Furthermore, miR-21's expression correlates with various age-related diseases, including neurodegenerative disorders like Alzheimer's and Parkinson's disease, type 2 diabetes mellitus, and hypertension, underscoring its utility in identifying and understanding the molecular underpinnings of these conditions (Olivieri et al., 2021). For instance, Hijmans et al. (2018) observed a significant correlation between reduced levels of miR-21 in plasma and systolic blood pressure.

Evidence suggests that miR-21 links two fundamental age effects: cellular senescence and inflamm-aging (Halper et al., 2015). For example, studies could show that miR-21 is significantly enriched in extracellular vesicles released from senescent endothelial cells (Mensà et al., 2020). These vesicles can transfer miR-21 to neighboring young cells, suggesting a mechanism by which cells can propagate senescence signals to their environment (Mensà et al., 2020). Moreover, by targeting DNA methyltransferase 1 (DNMT1) and Sirtuin 1 (SIRT1) levels, miR-21 influences epigenetic modifications crucial for maintaining genomic integrity (Lettieri-Barbato et al., 2022; Mensà et al., 2020).

Furthermore, miR-21 targets genes involved in antioxidant defense, such as SOD2, and may activate enzymes that produce ROS, like NADPH oxidase 4 (NOX4) (Lettieri-Barbato et al., 2022). This action hints that miR-21 might also contribute to oxidative stress, further promoting cellular senescence through higher stress levels (Hinkel et al., 2020).

On the other hand, miR-21 plays a crucial role in regulating the body's inflammatory response by affecting the NF-κB and (NOD)-like receptor protein 3 (NLRP3) pathways (Olivieri et al., 2021). Both in vitro and in vivo studies on animals have shown how miR-21

can either promote or inhibit these pathways, which are essential for controlling inflammation. This leads to the conclusion that miR-21 can be considered a molecule that modulates pro- and anti-inflammatory processes (Olivieri et al., 2021).

For instance, miR-21 works as an anti-inflammatory agent that downregulates the expression of interleukin-1 receptor-associated kinase (IRAK) and myeloid differentiation primary response 88 (MyD88), components of the TLR signaling pathway, as well as transcripts of the tumor suppressor genes, such as phosphatase and tensin homolog (PTEN) and programmed cell death protein 4 (PDCD4) (Olivieri et al., 2013). PDCD4, for example, takes part in the activation of NF-κB and enhances the production of proinflammatory interleukin-6 (IL-6), thereby limiting the production of the anti-inflammatory IL-10 (Olivieri et al., 2021; Sheedy, 2015).

Conversely, it has been shown that the lack of transforming growth factor ß (TGF-β) signaling in aging organisms leads to the up-regulation of miR-21, accompanied by increased production of tumor necrosis factor-alpha (TNF-α) and interferon-γ. Under these circumstances, miR-21 can act as an agonist for single-stranded RNA-binding TLRs, activating the NF-κB pathway and the secretion of inflammatory molecules (Halper et al., 2015; Olivieri et al., 2013). Additionally, miR-21 is an endogenous ligand of TLR8, a receptor on intracellular vesicular membranes (Olivieri et al., 2021). TLR8 is known for its role in the immune response by recognizing single-stranded RNA, particularly GU-rich sequences (Olivieri et al., 2021). Circulating miR-21 interacts with TLR8 on immune cells, activating the NF-κB pathway and triggering the secretion of key inflammatory cytokines, TNF and IL-6 (Munk et al., 2017).

1.4.2 Senescence miRNA: miR24

Senescence, a concept first described by Leonard Hayflick in the 1960s, delineates a fundamental biological process where normal cells cease to proliferate after a finite number of divisions yet remain metabolically active (Hayflick, 1965). This phenomenon underscores the body's natural limit to cell replication, serving as a critical mechanism to prevent the propagation of damaged cells, thereby safeguarding genomic integrity (Hayflick, 1965). However, as time passes, the buildup of senescent cells plays a role in the aging process and age-related diseases (Hayflick, 1965).

MicroRNAs are pivotal in regulating cellular senescence, especially stress-induced senescence, exerting their influence through the alteration of gene expression (Bu et al., 2017). These small, non-coding RNAs recognize and bind to complementary sequences typically found in target mRNAs' 3’ UTR, leading to a sequence-specific silencing mechanism (L.-H. Chen et al., 2010; Munk et al., 2017).

MicroRNA 24 has become a critical regulator in the complex cellular aging process. Specifically, miR-24 targets a range of senescence-associated genes such as MKK4, $p16^{INK4a}$, E2F2, and H2AX, each playing a critical role in modulating cell cycle progression and DNA repair mechanisms (Noren Hooten et al., 2010). In addition, the miRNA microarray analysis from salivary exosomes of both young and elderly participants revealed miR-24 as a new indicator of aging (Machida et al., 2015).

Several senescence-associated miRNAs, including miR-24, influence the p16 pathway, inhibiting cell proliferation (L.-H. Chen et al., 2010; Khee et al., 2014). P16 is a cyclin-dependent kinase inhibitor (CDKI) that controls the cell cycle. The primary target of CDK is the retinoblastoma protein (pRb). In its active state, CDKs phosphorylate pRbs, leading to its inactivation. This inactivation releases E2F, a family of transcription factors regulating the gene expression required for cell cycle progression, specifically the transition from the G1 to the S phase (L.-H. Chen et al., 2010). Research shows that as cells undergo repeated division during the aging process, miR-24 levels decrease. This decline is noteworthy since miR-24 targets p16 mRNA for repression by binding to two specific sites on the mRNA: One in the coding region and another in the 3’ UTR. By targeting these sites, miR-24 represses the translation of p16 mRNA, reducing corresponding protein levels (L.-H. Chen et al., 2010; Khee et al., 2014). In conclusion, the inverse relationship between miR-24 and p16 suggests that the decrease in miR-24 with age contributes to the accumulation of p16, therefore promoting cellular senescence (Khee et al., 2014). Furthermore, Munk et al. (2017) could confirm the inverse correlation of miR-24 and p16 levels in osteoarthritis-associated senescence, thereby linking miR-24's regulatory function directly to pathological conditions associated with aging.

In addition to modulating the p16 pathway, miR-24's impact extends to the cellular response to oxidative stress and DNA damage, pivotal elements in the aging process (Noren Hooten

et al., 2010). Noren Hooten et al. (2010) suggest that miR-24 coordinately modulates the histone variant's H2AX expression. This double-strand response protein recruits and retains DNA repair factors to master the increased DNA damage and oxidative stress associated with aging. This modulation is critical in maintaining genomic integrity under stress conditions (Noren Hooten et al., 2010). Further, Bu et al. (2016) have elaborated on this by demonstrating that overexpression of miR-24 enhances oxidative stress-induced senescence in human diploid fibroblasts, which leads to upregulated expression of p53, a critical regulator of the cell cycle and apoptosis. It was concluded that enforced overexpression of miR-24, to compensate for the low level due to aging, promotes oxidative stress and induces premature senescence in cells. This suggests that miR-24 can accelerate the aging process by enhancing the effects of oxidative stress, triggering early senescence (Bu et al., 2016; Noren Hooten et al., 2010).

Finally, it is worth noting that miR-24 has been found to significantly impact the immune system's aging (Machida et al., 2015). In 2015, Machida and his team discovered that miR-24 targets specific genes that can profoundly affect the mitogen-activated protein kinase (MAPK) signaling pathway. This pathway regulates genes involved in the production of inflammatory cytokines and chemokines at both the transcriptional and post-transcriptional levels (Machida et al., 2015). The rise in miR-24 levels might play a role in increasing vulnerability to age-related alterations in immune and inflammatory conditions, underscoring the broader implications of miR-24 in aging beyond its direct effects on cell cycle and DNA damage response mechanisms (Machida et al., 2015).

2. Objectives

Aging is a multifaceted process characterized by complex interactions among biological and molecular mechanisms (Wagner et al., 2016). Despite many proposed theories, none of them have been able to explain the phenomenon of aging entirely (Bell et al., 2019; Horvath & Raj, 2018). Recognizing the limitations of singular biomarkers in encapsulating the multifactorial nature of aging, current research has shifted towards a holistic approach, integrating omics-based biomarkers to provide a more nuanced understanding of aging processes. Notably, noncoding RNAs have been shown to complement epigenetic age models such as DNAm age and have been instrumental in unveiling previously unknown aging aspects (Huan et al., 2018). Furthermore, a recent genome-wide assessment of 800 different miRNAs revealed that they are differentially expressed with ongoing years, mostly decreasing in number (Noren Hooten et al., 2010).

Building upon existing research, the present study investigates the relationship between capillary blood miRNA expression and age. It was hypothesized that miRNA ΔCt values could serve as a basis for developing an age estimator, employing chronological age as a marker for biological aging. To the current knowledge, this constitutes the initial effort to establish a miRNA-based calculator grounded in miRNAs significantly associated with age or age-related phenomena across various studies. This study aims to construct and subsequently validate the calculator to determine a person's biological age and primarily addresses the feasibility and accuracy of this model. The findings are intended to support the statistical model development of a miRNA aging clock analogous to the Horvath aging clock, thereby contributing to the broader field of aging research.

Furthermore, the thesis explores the interaction between lifestyle factors and biological age, introducing a novel perspective on epigenetic alterations that are theoretically reversible (Peters et al., 2015). The possibility of reversibility presents exciting opportunities for developing new and effective anti-aging treatments. By examining the correlation this study aims to provide insights into strategies that could serve as initial steps toward developing comprehensive therapies or lifestyle modifications to enhance Healthspan and prevent age-associated diseases.

3. Methods

3.1 Cohort Recruitment and Data Collection

The cohort was recruited between 2015 and 2023 by an Austrian company specializing in personalized nutrition. The baseline data of 1151 subjects included variables related to lifestyle, behavior, medical history, diet, and nutrition collected through self-complete questionnaires and capillary blood sampling.

The lifestyle and dietary characteristics derived from the original questionnaire were recoded to simplify the number of subcategories and enhance the interpretability of the data.

Alcohol Consumption was categorized as "Seldom to Never" ("*never*", "*only on occasions*", and "*1-2 times per month*"), "Weekly Intake" ("*3-5 times per month*" and "*1-2 times per week*"), "Daily Drinker" ("*daily*"), and "Heavy Drinkers" ("*several times per day*").

Smoking Status was recoded as "No" ("*non-smokers*" and "*former smokers*", regardless of quit time) and "*Yes*" ("*current smokers*", further distinguished by quantity smoked).

Stress Levels subcategories were consolidated into a single one, combining the original categories "*Low*" and "*Moderate*".

Physical Activity and **Exercise** were maintained as initially defined.

Fruit and Vegetable Intake was classified as "Seldom to Never" ("*occasionally*" and "*every other day*"), "Moderate" ("*daily*"), and "High" ("*several times per day*" up to "*5 times per day*").

Milk and Milk Products were grouped into "Never" ("*never*"), "Seldom to Every Other Day" ("*occasionally*" and up to "*every other day*"), and "Daily" ("*daily*" and "*several times per day*").

Fish Consumption categories were defined as "Seldom to Never" ("*rarely*" and "*almost never*"), "Moderate" ("*once per week*" and "*2-3 times per week*"), and "High" ("*4 or more times per week*").

Meat Intake was categorized as "Seldom to Never" ("*rarely*" and "*almost never*"), "Moderate" ("*1-6 times per week*"), and "High" ("*daily*" and "*several times per day*").

Whole Grain Consumption followed a similar pattern, with "Seldom to Never" ("*rarely*" and "*almost never*"), "Moderate" ("*1-6 times per week*"), and "Daily" ("*daily*" and "*several times per week*").

Fast Food Intake was classified into "Seldom to Never" ("*never*" and "*rarely*") and "Moderate to High" ("*several times per week*" up to "*daily intake*").

Variables such as **Sweets** ("*seldom to never*", "*several times per week*"), **Liquids** *("< 1 liter*", "*1-2 liters*", "*> 2 liters*"), **Coffee** ("*No*", "*Yes*"), and **Green Tea** ("*No*", "*Yes*") were deemed straightforward and were not utilized.

As the aim was to discover a general aging marker applicable to a broad population, it is essential to set specific exclusion criteria to create a "healthy" -baseline and avoid certain factors that might influence the relationship between age and miRNA expression.
The chosen criteria encompassed underage individuals (Austrian law: <18 years) and conditions that might affect miRNA ΔCt values, such as Arthritis (Xiao et al., 2018; Zhu et al., 2019), inflammatory gastrointestinal diseases (W.-X. Chen et al., 2014), depression (R. Ding et al., 2023), high blood pressure (Huang et al., 2017), diabetes mellitus types 1 and 2 (L. Ding et al., 2016; Santos et al., 2019), thyroid dysfunction (Caglar & Cayir, 2019), and gout (Bohatá et al., 2021).

To summarize, the subsequent dataset for the present thesis included 539 out of the 1151 participants.

3.2 ΔCt Values Determination and miRNA Selection

miRNA's ΔCt values were determined using a safety lancet Extra 18G for capillary blood sampling on a *Whatman*® protein saver card, with saver cards dried and stored at -20°C. The RNA extraction method uses the principle of binding RNA molecules to magnetic beads, separating them from other components through a series of washing steps.

The extraction occurs over two days. On day one, the saver cards are excised and incubated overnight in a protease solution using a *ThermoMixer*. The subsequent extraction on day two utilizes the *KingFisher™ Duo Prime Magnetic Particle Processor* (96-well deep setting) and the *MagMAX FFPE DNA/RNA Ultra kit* (Applied Biosystems™ by Thermo Scientific™). For the detection and quantification of miRNAs, the previously extracted RNA is transcribed into complementary DNA (cDNA), offering more stability and abundance for detection. The cDNA synthesis for miRNA analysis uses the *TaqMan™ Advanced miRNA cDNA Synthesis kit* and the *SimpliAmp™ Thermal Cycler* (both by *Applied Biosystems™* by *Thermo Scientific™*). A negative control sample with nuclease-free water was included in each run. The qualitative polymerase chain reaction (qPCR) mix, comprising miRNA assay, nuclease-free water, and *TaqMan™ Fast Advanced Master Mix* for qPCR, is prepared for each miRNA individually. Following the transfer of reaction mixes to a 96-well plate, the *QuantStudio™ 3 Real-time PCR System* executes the reaction per the manufacturer's protocol.

The resulting data is analyzed using *Applied Biosystems® qPCR analysis modules* (*Thermo Scientific™*), employing the ΔCt method with miR-93 as endogenous controls for statistical calculations. For the statistical analysis, the ΔCt resulted in the normalization of the mean Ct of each sample minus the mean Ct of the housekeeping miR-93, as in the following equation:

$$\Delta Ct = mean\ Ct(sample) - mean\ Ct(miR-93)$$

Equation 1

The original dataset included 31 different miRNAs associated with a broad range of biochemical processes, which required a selection to determine the ones most relevant to this study. The selection process was initiated with a comprehensive search on PubMed using keywords such as "miRNA and age", "miRNA aging", "age-related miRNA", "inflamm-aging miRNA", and "senescence miRNA".

This preliminary investigation provided an array of studies, identifying six potential miRNAs associated with aging that were present in the current dataset: miR-19b, miR-20a,

miR-21, miR-24, miR-146a, and miR-155 (ElSharawy et al., 2012; Huan et al., 2018; Noren Hooten et al., 2010).

A subsequent review was conducted for the three miRNAs that were included in the dataset in a sufficient manner: miR-155, miR-21, and miR-24. Specific research with keywords like "miR-155 and biological age", "miR-155 and aging", "miR-21 and biological age", "miR-21 and aging", "miR-24 and biological age", and "miR-24 and aging", helped narrow down the research focus. This literature review gained insights into these selected miRNAs' roles in aging processes, providing a solid basis for further analysis.

3.3 Model Development and Validation

The dataset was systematically partitioned into independent testing and training subsets, following an 80/20 ratio, resulting in 433 subjects allocated to the training set and 106 to the testing set. By splitting the whole dataset into a training and testing set, the model aims to demonstrate high replicability. The 80/20 split was chosen to ensure an appropriate representation of the participants in both partial datasets. Furthermore, a random shuffling procedure was employed to circumvent any potential sequence bias. All the following statistical analyses, up until the evaluation, were conducted using the training set to ensure an unbiased validation later using the testing set.

The Mann-Whitney U test was used to determine whether the critical variables' chronological age and miRNA's ΔCt values differ between the testing and training sets. Furthermore, depending on the type of the variable, a t-test (for metric variables), a Mann-Whitney U test (for metric variables, which are not normally distributed), or a Chi^2 test (for continuous variables) was used to identify whether any clinical or lifestyle determinants differed between the two datasets. However, undetected technical factors contributing to a difference between the training and testing datasets cannot be entirely excluded.

Before generating the age estimator, a scatter plot was created to obtain a rough overview of the correlation between the ΔCt values of the individual miRNAs (miR-21, miR-24, miR-155) and age within the dataset (Figure 3-5). Following a detailed examination for normal

distribution, the Spearman rank correlation test was considered the appropriate method for the corresponding statistical analysis.

Moving on to the age estimator, Horvath states that the model development requires three crucial decisions:

1. the statistical prediction method
2. the outcome measure, and
3. the covariates (in this case, miR-21, miR-24, miR-155) (Horvath & Raj, 2018).

The following age estimator, later referred to as miRNA-3Age, uses chronological age as a surrogate for biological age, as studies show that chronological age is highly correlated with biological age (Horvath & Raj, 2018). Further, Horvath states that chronological age is: "[…] arguably a near-optimal surrogate of biological age during development […]" of aging clocks (Horvath & Raj, 2018, p. 6).

In the context of miRNA-3Age, the study uses a multiple linear regression model as the method of choice. The variables are fitted in a linear equation (Equation 2) with miRNA ΔCt values as the independent variables and chronological age as the dependent one as

$$Y = ß_0 + ß_1 * x_1 + ß_2 * x_2 + ß_3 * x_3$$

Equation 2

where Y is the predicted biological age, $ß_0$ is the intercept, $ß_1$ is the coefficient for miR-21, $ß_2$ for miR-24, and $ß_3$ for miR-155, while x_1 stands for miR-21's ΔCt value, x_2 for miR-24's ΔCt value, and x_3 for miR-155's ΔCt value.

The miRNA prediction model underwent rigorous training exclusively on the training sample set, a critical step to ensure an unbiased validation of the model's predictive capabilities. To achieve this, a five-times repeated ten-fold cross-validation approach was implemented on the training set, a staple in applied machine learning for its efficacy in assessing model performance (Nakatsu, 2021). This method systematically divides the

training set into ten subsets; the model is then trained on nine subsets and validated on the tenth. This cycle is performed ten times, rotating the validation subset each time, allowing every data portion to be used for training and validation (Nakatsu, 2021). To further solidify the reliability of the model evaluation, this ten-fold process is repeated five times with different random shuffling of the data, which helps average the error prediction rate and provides a robust performance measure (Nakatsu, 2021). This comprehensive cross-validation optimized the hyperparameters for the biological age prediction model.

The prediction mean absolute error (MAE) and R^2 were calculated to examine the final model's linear regression performance and generalization ability. In regression models, R^2 is a standard metric to quantify to what extent variation in the dependent variable can be explained by the independent ones (Eastman, 1984). R^2 ranges from zero to one (or 0-100%), where zero indicates that the model does not describe any of the variability of the response data around its mean, and one means that the model explains all of it. A higher R^2 value implies that the model accurately predicts the dependent variable as it accounts for a larger amount of variance (Eastman, 1984).

The final model was then applied to the testing cohort, thereby examining generalization ability using the mean absolute error. The MAE represents the average absolute differences between the model's predictions (biological age) and the actual observations (chronological age). A lower MAE indicates a model's greater precision in forecasting outcomes and can be calculated using the following equation:

$$MAE = \frac{x}{y}\sum_{i=0}^{n} | x_i - x |$$

Equation 3

Where *n* is the number of errors, and | *xi* – *x* | is the absolute error.

After evaluating the generalizations and regression performance, the miRNA-3Age was calculated for the whole cohort (n= 539) using the optimal parameters conducted via cross-validation.

The next step was to determine the predictive accuracy. Horvath states that two essential measures can be used here. The first is the "age correlation", which describes the correlation coefficient between biological and chronological age (Horvath, 2013). After checking for normal distribution, the Spearman rank was selected to determine this parameter. The second one is the median absolute error (MedAE), the median absolute difference between biological and chronological age (Horvath, 2013).

The median absolute error is one of the most used measures of model accuracy (Salih et al., 2023) and can be calculated as in the following equation

$$MedAE\ (y, \hat{y}) = median\ (\ |y_1 - \hat{y}_1|\ , \ldots , |y_n - \hat{y}_n|\ )$$

Equation 4

where y represents the predicted biological age, whereas $\hat{y}$ chronological age.

3.4 miRNA-3Age and Lifestyle Choices

Finally, the study examined the relationship between miRNA-3Age and various lifestyle choices.

The difference between miRNA-3Age and chronological age was defined as "biological age acceleration". The residual index, BA-CA, was used to identify whether a person is biologically younger (negative value) or biologically older (positive value), following a method utilized by Professor Tze Pin Ng and his research group (Ng et al., 2020). The difference was summarized in a new binary variable called "Aging".

To determine the correlation between miRNA-3Age and the different lifestyle and dietary characteristics, the odds ratio (OR) and 95% confidence interval (CI) were estimated with a logistic regression model. Biologically older was set as the reference category, so the model presents the chances of being biologically younger for all individual regression tests.

RStudio 2022.02.0 was used for all statistical analyses, and the p-value was set to 0.05.

4. Results

4.1 Characteristics of the Study Population

Table 1 delineates the demographics of the 539 participants included in the following analysis.

Of all study participants, 64% identified as women, amounting to 346 individuals. Since the questionnaire asks explicitly about gender and not sex in an open question format, it is impossible to determine in retrospect how many of the 64%, or 346 participants, are biologically female or identify as women. In general, it can be stated that only "woman" and "man" (in German: "Frau" und "Mann") were handwritten answers to the corresponding question, and no other gender identities were mentioned among the participants.

The cohort spanned a broad age spectrum, from 18 to 91 years, averaging 48.81 ± 13.02 years. Correspondingly, it is noteworthy that the age distribution remained consistent between participants identifying as women and men, indicating uniformity in the two groups (t-test: p= 0.2033). Moreover, the cohort had an average BMI of 24.73 ± 5.21 $\frac{kg}{m^2}$, ranging from 16.61 to 64.56 $\frac{kg}{m^2}$. Lastly, the mean ΔCt values of the chosen miRNAs averaged 3.50 ± 0.74 for miR-21, 2.89 ± 0.56 for miR-24, and 7.89 ± 0.77 for miR-155.

For validation purposes, the cohort was divided into separate training and testing sets, with 433 and 106 participants, respectively.

The mean age within these subsets was 48.85 years for the training group and slightly less, at 48.61 years, for the testing group (Mann-Whitney U test: p= 0.867). Furthermore, the mean ΔCt values for the three miRNAs show no significant difference between the two datasets. The average miR-21 ΔCt value in the training set was 3.48 and 3.59 in the testing set (Mann-Whitney U test: p-value= 0.096). For miR-24, the mean ΔCt value was 2.88 (training set) and 2.93 (testing set), with a non-significant Mann-Whitney U test confirming uniformity (p-value= 0.24). Lastly, the mean ΔCt for miR-155 stands at 7.86 in the training set vs. 7.98 in the testing set (Mann-Whitney U test: p-value= 0.33).

Other clinical and lifestyle determinants exhibited no significant variations between the two datasets, reinforcing the internal consistency of the study's parameters.

Table 1: Descriptive characteristics of the 539 study's participants.

	Study Population
Total	539
Gender: woman	346 (64%)
Age range [years]	18 – 81
Chronological Age [years]	48.81 ± 13.02
Biological Age [years]	48.78 ± 2.38
BA-CA [years]	0 ± 12.87
Biologically older	243 (45.1%)
miR-21 [ΔCt]	3.50 ± 0.74
miR-24 [ΔCt]	2.89 ± 0.56
miR-155 [ΔCt]	7.89 ± 0.77
BMI [$\frac{kg}{m^2}$]	24.73 ± 5.21

Note: Data are presented in n (%), mean ± SD, or range (min–max score). BMI: body mass index.

4.2 miRNAs in Relation to Chronological Age

After an extensive literature review, various studies identified miRNAs correlated with chronological age. Consequently, this prompted an investigation into whether such correlations could be observed within the present dataset.

The results of the Spearman rank test are presented in Table 2. The ΔCt values for miR-21 showed a statistically significant negative association with chronological age (ρ= -0.12, p= 0.011, R^2= 0.020). Although the test demonstrates a statistically significant result, a coefficient of -0.12 indicates a relatively weak correlation. Furthermore, the low R^2 of 0.020 suggests that miR-21's ΔCt value explains only about 2% of the variance in chronological age, implying that while there is a notable association, a large portion of the age variance remains unexplained by miR-21 alone.

Conversely, the ΔCt value for miR-24 indicated a positive but weak correlation with chronological age (ρ= 0.04, p= 0.340, R^2= 0.001). Although minor, the positive correlation

coefficient ρ suggests a trend of decreasing expression with increasing age. However, the notably low R^2 and high p-value imply that miR-24 does not contribute highly to age variance and that its association with age is not statistically significant.

Lastly, miR-155 exhibited a negative correlation with age (ρ= -0.07, p= 0.130, R^2= 0.006), suggesting increased expression over time. Like miR-155, miR-24 is not statistically significant, and the meager R^2 value indicates that it explains less than 0.6% of the variance.

In summary, it can be said that among the three, only miR-21 yielded a statistically significant correlation, though the R^2 values for all miRNAs were notably low, ranging from around 0.1% to 2% (Table 2).

Table 2: Results of Spearman rank correlation test between miRNA ΔCt values and chronological age.

miRNA	**Correlation Coefficient ρ**	**R^2**	**p-value**
miR-21	-0.12	0.020	**0.011**
miR-24	0.04	0.001	0.340
miR-155	-0.07	0.006	0.130

Note: Higher miRNA ΔCt values indicate lower expression levels. Therefore, negative correlation coefficients ρ indicate a positive association between miRNA expression and chronological age. Significant p-values are shown in boldface.

Figures 3 to 5 visually represent the relationships, with a distinct yellow trend line highlighting the corresponding linear correlation between the miRNA's ΔCt values and age. A closer analysis of the figures reveals a broader dispersion in data points for miR-21 compared to the more consolidated points of miR-24 and miR-155. Values for miR-21 and miR-24 ΔCt are clustered between one and six, whereas miR-155 values lie roughly between four and eleven. The observed dispersion in the data points, particularly for miR-21, suggests a more significant variability in its expression with age.

Furthermore, it's noteworthy that miR-24 and miR-155 present a higher scattering than miR-21, yet without significant outliers. This consistency in data points lends credibility to the observed trends and suggests that extreme values do not influence the relationships.

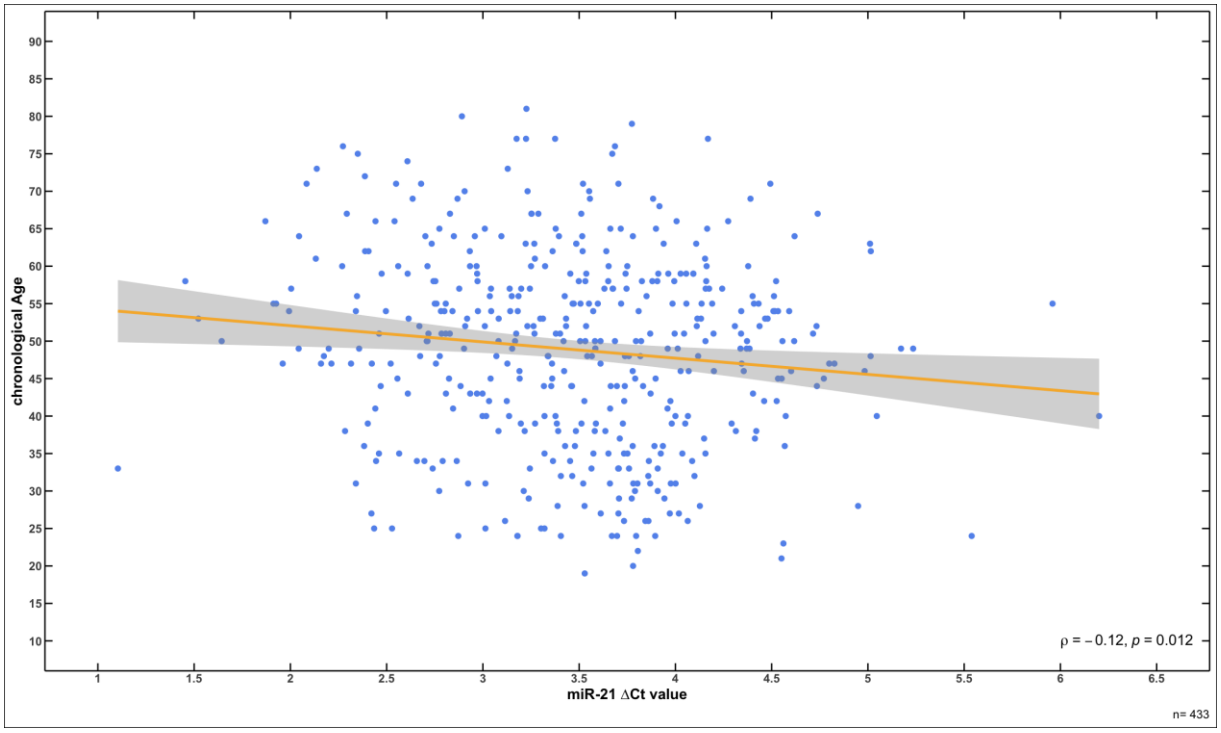

Figure 3: Scatter plot between miR-21 ΔCt and chronological age. The yellow trend line highlights the type of relationship, with the gray zone indicating the standard deviation.

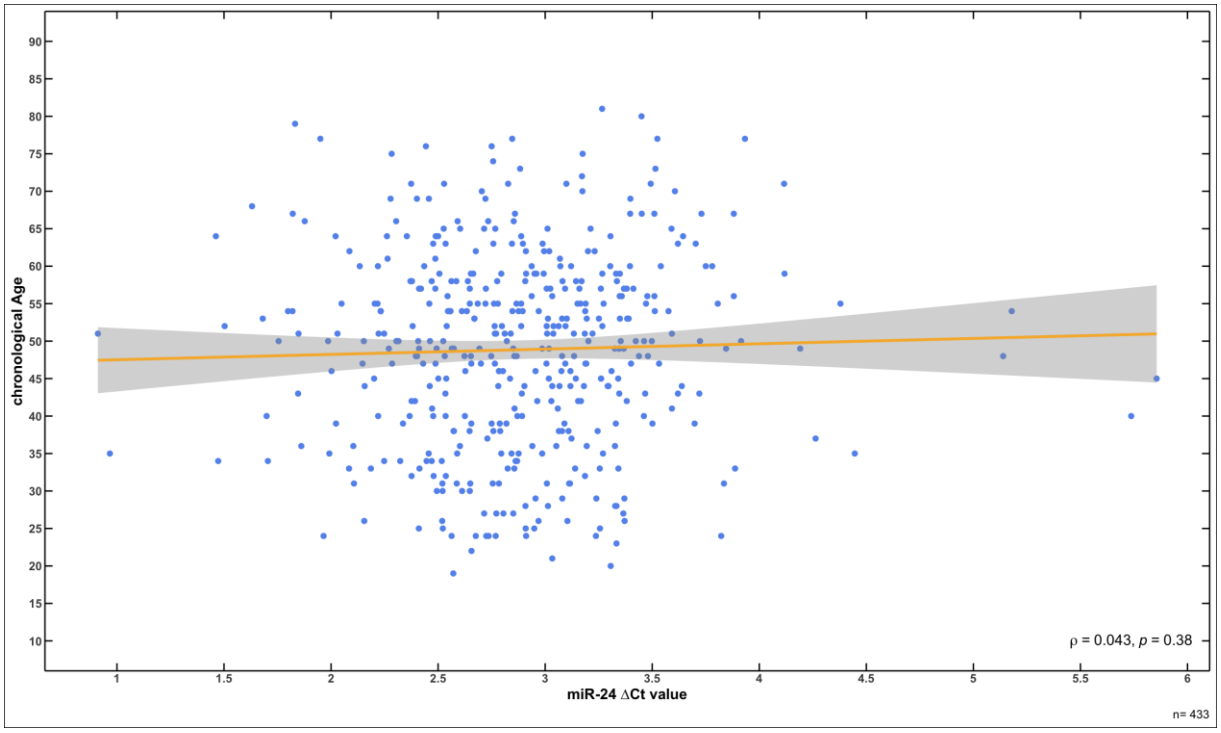

Figure 4: Scatter plot between miR-24 ΔCt and chronological age. The yellow trend line highlights the type of relationship, with the gray zone indicating the standard deviation.

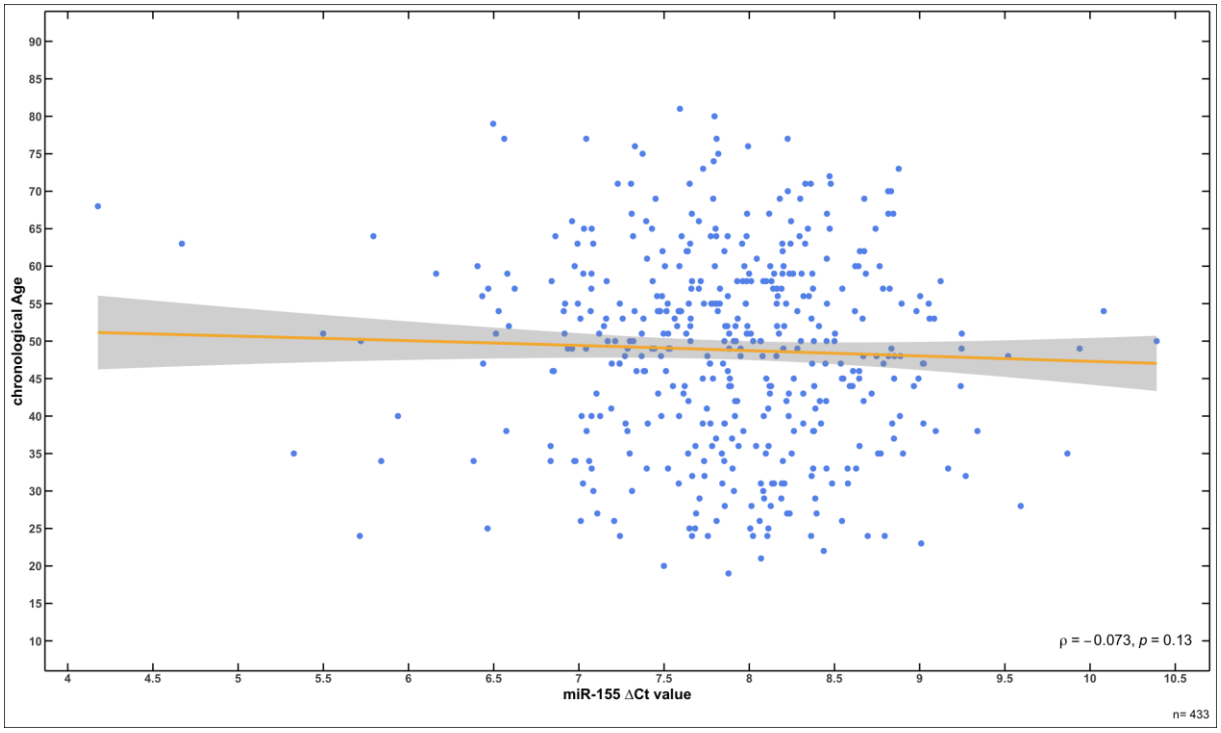

Figure 5: Scatter plot between miR-155 ΔCt and chronological age. The yellow trend line highlights the type of relationship, with the gray zone indicating the standard deviation.

4.3 miRNA Age Prediction

A multivariate analysis was performed in the quest for age prediction despite the non-significant results for the correlation with miR-24, miR-155, and chronological age. This was driven by the hypothesis that a combined model of the three miRNAs could still produce significant results. Additionally, a Spearman rank test revealed that all three miRNAs are significantly associated with one another, strengthening the hypothesis to combine the miRNAs in a single age prediction model. For example, a notable correlation between miR-24 and miR-155 is indicated by a correlation coefficient ρ of 0.5 ($p < 2.2E^{-16}$). However, the other two pairings present a lower but still highly significant ρ of 0.12 for the correlation between miR-21 and miR-155 ($p= 0.005$) and 0.2 for miR-21 and miR-24 ($p= 2.5E^{-5}$).

The training for this combined model was exclusively conducted using the training sample set, with a 5-times repeated 10-fold cross-validation approach. The exact procedure can be found in Methods 3.3.

The Intercept ß$_0$ is set to 66.73 years [53.83, 79.63] with a highly significant p-value of less than $2E^{-16}$. This suggests that if all ΔCt values (x_1, x_2, x_3) were zero, the anticipated biological age would hover around 66.73 years (Table 3).

Delving deeper, the regression coefficient ß$_1$ for miR-21 stands at -2.33 [-4.02, -0.64] (p= 0.007), suggesting that with every unit increase in the ΔCt value of miR-21, the predicted biological age decreases by 2.33 years, given that other factors remain unchanged (Table 3).

Secondly, miR-24's positive coefficient ß$_2$ positioned at 2.96 [0.47, 5.46] (p= 0.020) indicates that each unit rise in its ΔCt value leads to an increase of 2.96 years in the predicted age, again holding other factors steady (Table 3).

Lastly, for miR-155, the coefficient ß$_3$ is -2.34 [-4.11, -0.54] (p= 0.011), denoting that for each unit increase in its ΔCt value, the predicted biological age decreases by 2.34 years, under the assumption that other variables are kept constant (Table 3).

Table 3: Results of the linear model (chronological age ~ miR-21 + miR-24 + miR-155), performance metrics, and predictive accuracy.

	Regression Coefficient	**95% CI**	**p-value**
Intercept	66.73	[53.83, 79.63]	**0**
miR-21	-2.33	[-4.02, -0.64]	**0.007**
miR-24	2.96	[0.47, 5.46]	**0.020**
miR-155	-2.34	[-4.11, -0.54]	**0.011**
Performance Metrics			
R^2		0.051	
MAE [years]		10.4	
Predictive Accuracy			
MedAE [years]		9	
Age Correlation ρ		0.13 (p= **0.002**)	

Note: Higher miRNA ΔCt values indicate lower expression levels. Therefore, negative regression coefficients indicate a positive association between miRNA expression and chronological age. Significant p-values are shown in boldface.

The results can be summarized as the following linear equation:

$$Y = 66.73 - 2.33 * x_1 + 2.96 * x_2 - 2.34 * x_3$$

Equation 5

where, x_1, x_2, and x_3 are miRNA ΔCt values, and Y is the anticipated miRNA-3Age.

Key metrics such as the mean absolute error and the R^2 were derived to evaluate the biological age model's performance. The recorded MAE is set to 10.4 years, suggesting that, on average, the predicted biological age deviates from the actual chronological age by approximately 10.4 years without considering the direction of this discrepancy. Switching the focus to the $R^{2,}$ the value of 0.051 illustrates that roughly 5.1% of age variance is attributable to the predictors miR-21, miR-24, and miR-155 (Table 3).

The biological age for all subjects was determined using optimal parameters identified through cross-validation along with the miRNA's ΔCt values. The miRNA-3Age spans from 40 to 60 years, indicating a significantly smaller range than the chronological age, with 18 to 91 years. In addition, the standard deviation in biological age was more consistent than chronological age, averaging 48.78 ± 2.38 years (Table 1). Notably, the biological age, like age in general, did not show gender-specific differences (t-test: p= 0.6204).

The concept that the difference between biological and chronological age indicates "biological aging" is supported by various studies (Huan et al., 2018; Ng et al., 2020). By calculating the residual index, BA-CA, it becomes possible to determine whether someone is biologically younger or older than their chronological age (Method 3.4). The data showed that out of 539 subjects, 243 (45.1%) were found to be biologically older, whereas 296 subjects (54.9%) appeared biologically younger. The residual index BA-CA averaged out at 0 ± 12.87 years, while the range in biological age spanned from 31 years below to 30 years above the chronological age. Gender didn't influence this BA-CA difference (t-test: p= 0.171) (Table 1).

Finally, the predictive accuracy was assessed using median absolute error and the age correlation, as outlined in Methods 3.3. The MedAE is set to 9 years, which signifies a median discrepancy of 9 years between miRNA-3Age and chronological age (Table 3). To put it into perspective, this implies that while half of the biological age predictions deviate by a margin of less than nine years, the remaining half exceed this threshold.

Shifting attention to the age correlation, Figure 6 shows a subtle yet positive association between biological and chronological age, as evidenced by a Spearman coefficient ρ of 0.13 (p= 0.0017). The yellow trendline further highlights a predominantly linear relationship, with a slight curvature approaching the very end of the age spectrum. Notably, the visualization highlights the pronounced standard deviations at both the beginning and end of the age spectrum (grey area).

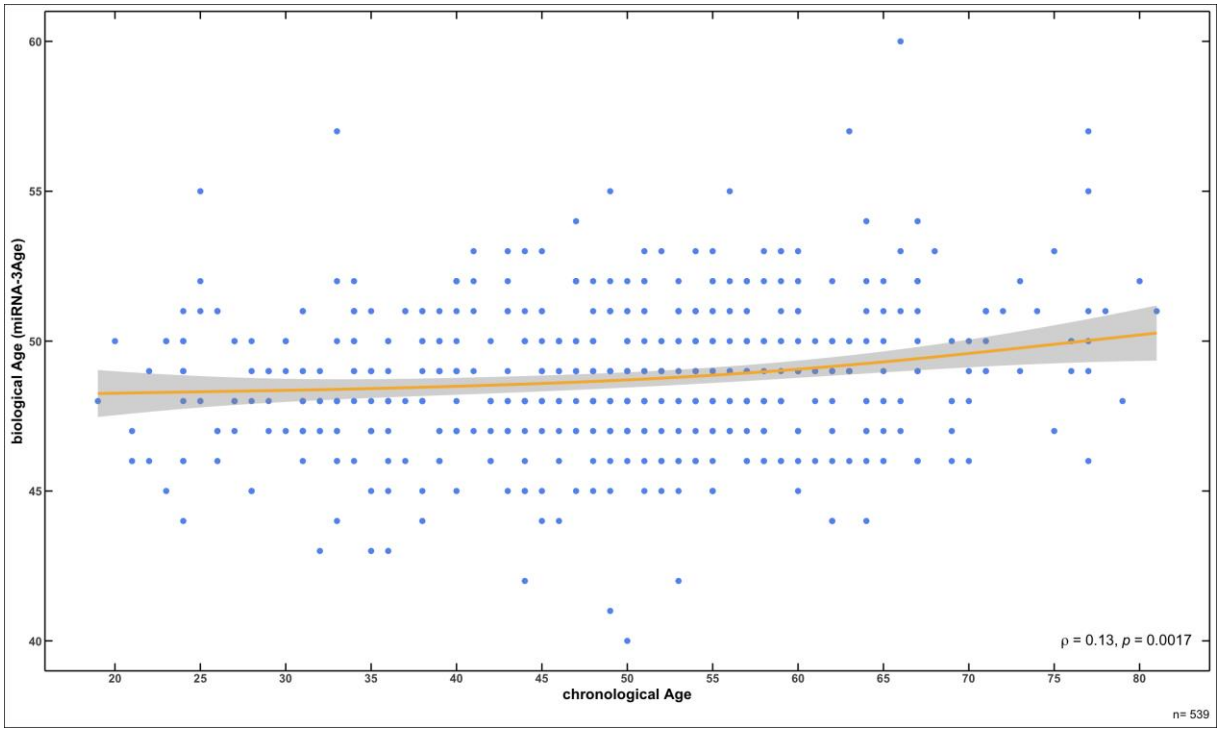

Figure 6: Scatter plot between chronological age and biological age (miR-3Age). The yellow trend line highlights the type of relationship, with the gray zone indicating the standard deviation.

Upon dividing the Figure into quadrants, one can observe that individuals in the upper left-hand quadrant possess a markedly elevated biological age compared to their lower

chronological age. Within this quadrant, two participants, highlighted in pink, stand out prominently. The first of these participants has a chronological age of 33 years in contrast with a biological age of 57 years, leading to a notable 24-year disparity. Conversely, the second participant showcases an even more pronounced difference of 30 years, with a chronological age of 25, against a biological age of 55 (Figure 7).

On the opposite spectrum, located in the lower right quadrant, are individuals with a biological age considerably younger than their actual chronological age. Highlighted in pink, one participant is noticeable with a pronounced 31-year age gap (BA: 46 years vs. CA: 77 years). As a rule, the closer a person is to the center, the closer their predicted biological age is to their actual age.

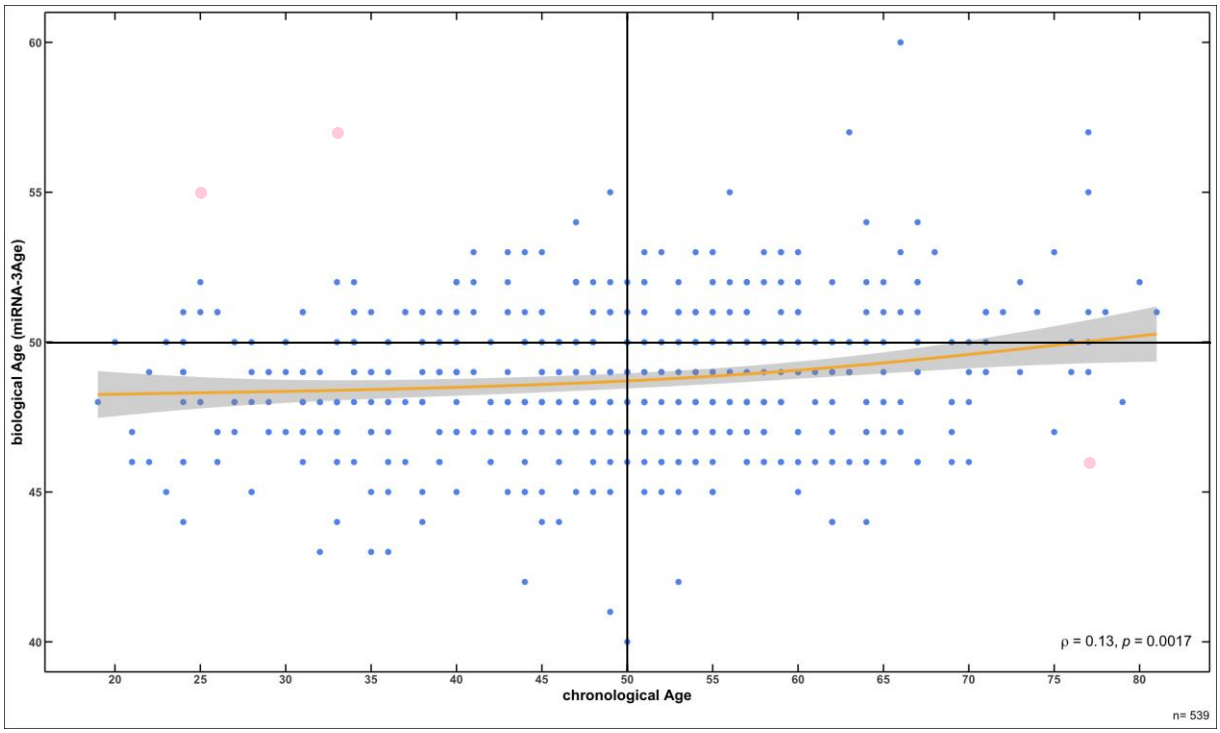

Figure 7: Scatter plot between chronological age and biological age (miR-3Age) divided into quadrants, with three participants highlighted in pink. The yellow trend line highlights the type of relationship, with the gray zone indicating the standard deviation.

4.4 Influence of Lifestyle and Dietary Characteristics on miRNA-3Age

A logistic regression model explored potential associations between lifestyle, dietary habits, and age acceleration (Methods 3.4). The results are presented in Tables 4, 5, and 6 in OR and 95% CI (p-value <0.05).

4.4.1 The Use of miRNA-3Age: Characteristics that Decrease Biological Aging

The baseline characteristics significantly associated with a higher chance of being biologically younger included daily alcohol consumption, moderate exercises (1-2 times per week), moderate fish consumption, daily whole grain consumption, and daily green tea intake.

For example, daily drinkers are 2.85 times more likely to be biologically younger than those who drink alcohol occasionally or are non-drinkers (OR= 2.85 [1.63, 5.26], p= 0.0004). Conversely, this suggests that daily alcohol intake is significantly associated with increased odds of being biologically younger. Interestingly, only daily alcohol intake showed considerably higher odds; all other groups showed no significant association. On the counterpart, it should be noted that heavy drinkers face a 99.99% reduction in their chances of being biologically younger despite being non-significant (OR= 0.000002 [NA], p= 0.980). Moreover, it is worth mentioning that both daily drinkers (73 participants) and heavy drinkers (one participant) are significantly less than those who don't drink alcohol at all (224 participants) and those with a weekly intake (221 participants).

Similarly, individuals exercising 1-2 times per week showed a significant 69% increased chance of being biologically younger than those who do not exercise regularly (OR= 1.69 [1.04, 2.79], p= 0.036). However, participants who exercise more than two times per week could not significantly increase their odds compared to those with no exercise routine (OR= 1.56 [0.98, 2.46], p= 0.059). To put it differently, more exercise does not inevitably result in decreased biological aging.

Moreover, participants with a moderate fish intake are 2.30 times more likely to have a lower biological age than their chronological age than non-frequent fish eaters (OR= 2.30 [1.54, 3.46], p= 0.00005). On the contrary, high weekly fish intake did not significantly affect biological aging (OR= 2.49 [0.79, 8.64], p= 0.126). It is worth

mentioning that the group with the highest fish intake consists of a smaller number of participants, with only 13 individuals. In contrast, the other categories contain 133 (seldom to never) and 384 (moderate) participants.

Furthermore, people with a daily whole grain intake are 1.80 times more likely to be biologically younger (OR= 1.80 [1.05, 3.09], p= 0.032). Interestingly, compared to other dietary and lifestyle factors mentioned above, a moderate whole grain intake was insufficient to significantly affect biological aging (OR= 1.01 [0.64, 1.59], p= 0.953). Only a daily whole grain consumption can increase the chances of being biologically younger.

Shifting the focus to beverages, participants who frequently consume green tea have a 54% higher chance of having a lower biological than chronological age than those who do not drink green tea regularly (OR= 1.54 [1.06, 2.25], p= 0.024). Interestingly, frequent coffee consumption did not affect the odds of being biologically younger whatsoever (OR= 1.44 [0.97, 2.16], p= 0.074).

4.4.2 The Use of miRNA-3Age: Characteristics that Increase Biological Aging

The baseline lifestyle and dietary characteristics that seemed to impede the likelihood of being considered biologically younger included smoking, regular fast-food intake, and elevated stress levels.

For instance, those who smoke face a stark 60% reduction in their chances of being biologically younger than non-smokers (OR= 0.4 [0.24, 0.66], p= 0.0004).

Likewise, individuals who regularly consume fast food see a similar 60% dip in the odds relative to those who rarely, if ever, indulge in such meals (OR= 0.4 [0.26, 0.62], p= 0.00005).

On the topic of stress, data reveals that those with high-stress levels have their chances of being biologically younger slashed by 42% compared to those with low to moderate stress levels (OR= 0.58 [0.40, 0.85], p= 0.005). Notably, individuals experiencing even higher stress levels have a 56% decrease in their chances compared to those with low to moderate

stress levels (OR= 0.44 [0.24, 0.80], p= 0.008). This pattern underscores the undeniable relationship that as stress levels increase from "high" to "really high", the chances of being labeled "biologically younger" further decrease.

4.4.3 The Use of miRNA-3Age: Characteristics that do not Influence Biological Aging

Interestingly, not all characteristics showed a significant association with biological aging. Dietary characteristics that were not significantly associated with lower odds of being biologically younger consist of fruit and vegetable intake (moderate intake: OR= 1.24 [0.84, 1.83], p= 0.268; high intake: OR= 0.68 [0.42, 1.09], p= 0.106), milk and milk products (seldom, every other day: OR= 1.43 [0.83, 2.47], p= 0.199; daily intake: OR= 1.44 [0.83, 2.51], p= 0.193), meat intake (moderate intake: OR= 1.01 [0.69, 1.49], p= 0.940; high intake: OR= 0.49 [0.22, 1.01], p= 0.055), sweets (several times per week: OR= 0.83 [0.57, 1.20], p= 0.317), liquid intake (1-2 liters: OR= 1.52 [0.72, 3.28], p= 0.268; > 2 liters: OR= 1.34 [0.94, 1.91], p= 0.109), and as already mentioned above coffee intake.

Switching the focus to lifestyle characteristics, one can see that when physical activity levels increase, the likelihood of being biologically younger increases by 83 % and 87 % (1 time per week: OR= 0.97 [0.32, 3.00], p= 0.951; 2-5 times per week: OR= 1.83 [0.68, 5.19], p= 0.235; 6-7 times per week: OR= 1.87 [0.69, 5.35], p= 0.223), however, the results were insignificant.

Furthermore, the tests could show that increasing the BMI by one unit [$\frac{kg}{m^2}$] does not significantly raise the chances of having a lower biological age (OR= 1.04 [0.99, 1.08], p=0.051).

Table 4: Association between BA-CA and baseline lifestyle factors.

Lifestyle Factors	**Cases**	**OR [95% CI]**	**p-value**
Alcohol			
seldom to never	224 (43.9%)	1.00	
weekly	221 (42.6%)	1.28 [0.73, 1.23]	0.200
daily	73 (14.1%)	2.85 [1.63, 5.26]	**0.0004**
heavy	1 (0.002%)	0.000002 [NA]	0.980
Smoking			
No	445 (85.2%)	1.00	
Yes	77 (14.8%)	0.4 [0.24, 0.66]	**0.0004**
Stress			
low to moderate	289 (55.9%)	1.00	
high	174 (33.9%)	0.58 [0.40, 0.85]	**0.005**
really high	52 (10.2%)	0.44 [0.24, 0.80]	**0.008**
Physical Activity			
seldom to never	17 (3.3%)	1.00	
1 time per week	57 (11%)	0.97 [0.32, 3.00]	0.951
2-5 times per week	251 (48.4%)	1.83 [0.68, 5.19]	0.235
6-7 times per week	194 (37.3%)	1.87 [0.69, 5.35]	0.223
Exercise			
seldom to never	106 (20.6%)	1.00	
1-2 times per week	161 (31.3%)	1.69 [1.04, 2.79]	**0.036**
> 2 times per week	247 (48.1%)	1.56 [0.98, 2.46]	0.059
BMI, $\frac{kg}{m^2}$	539 (100%)	1.04 [0.99, 1.08]	0.051

Note: Biologically older was set as the reference category along with the first category of the variable. So, every following OR demonstrates the odds of being biologically younger than the first category. Significant p-values are shown in boldface.

Table 5: Association between BA-CA and baseline dietary characteristics (Part 1).

Dietary Intake	Cases	OR [95% CI]	p-value
Fruit and Vegetable			
seldom to never	242 (46.1%)	1.00	
moderate	187 (35.6%)	1.24 [0.84, 1.83]	0.268
high	96 (18.3%)	0.68 [0.42, 1.09]	0.106
Milk and Milkproducts			
never	67 (12.8%)	1.00	
seldom, every other day	243 (46.5%)	1.43 [0.83, 2.47]	0.199
daily	213 (40.7%)	1.44 [0.83, 2.51]	0.193
Fish			
seldom to never	133 (25.1%)	1.00	
moderate	384 (72.5%)	2.30 [1.54, 3.46]	**0.00005**
high	13 (2.4%)	2.49 [0.79, 8.64]	0.126
Meat			
seldom to never	153 (29%)	1.00	
moderate	338 (64%)	1.01 [0.69, 1.49]	0.940
high	37 (7%)	0.49 [0.22, 1.01]	0.055
Whole Grain			
seldom to never	101 (19.3%)	1.00	
moderate	299 (57.3%)	1.01 [0.64, 1.59]	0.953
daily	122 (23.4%)	1.80 [1.05, 3.09]	**0.032**

Note: Biologically older was set as the reference category along with the first category of the variable. So, every following OR demonstrates the odds of being biologically younger than the first category. Significant p-values are shown in boldface.

Table 6: Association between BA-CA and baseline dietary characteristics (Part 2).

Dietary Intake	Cases	OR [95% CI]	p-value
Fast Food			
seldom to never	414 (79.8%)	1.00	
moderate to high	105 (20.2%)	0.40 [0.26, 0.62]	**0.00005**
Sweets			
seldom to never	341 (67.8%)	1.00	
several times per week	163 (32.2%)	0.83 [0.57, 1.20]	0.317
Liquids			
< 1 liter	33 (6.2%)	1.00	
1-2 liters	275 (52%)	1.52 [0.72, 3.28]	0.268
> 2 liters	221 (41.8%)	1.34 [0.94, 1.91]	0.109
Coffee			
No	137 (23.9%)	1.00	
Yes	402 (76.1%)	1.44 [0.97, 2.16]	0.074
Green Tea			
No	374 (69.4%)	1.00	
Yes	165 (30.6%)	1.54 [1.06, 2.25]	**0.024**

Note: Biologically older was set as the reference category along with the first category of the variable. So, every following OR demonstrates the odds of being biologically younger than the first category. Significant p-values are shown in boldface.

5. Discussion

5.1 Aging Marker

For over half a century, researchers have aimed to quantify biological age, proposing it as an alternative for measuring aging. Their primary goal has been to devise a formula incorporating biomarkers, like miRNAs, to estimate biological age, with chronological age as the output variable in their models (Salih et al., 2023). Numerous studies have identified that many miRNAs change their expression levels significantly with age, positioning them as promising indicators of biological aging (Noren Hooten et al., 2010). The present study intended to combine age-dependent miRNAs in an age estimator (miRNA-3Age), validate it, and finally use it to determine the biological age of a cohort.

5.1.1 Spearman Rank Correlation Test and miRNA Trends

Before developing miRNA-3Age, a Spearman rank test was utilized to assess the correlation of each individual miRNA with chronological age. This step was crucial to mitigate the potential confounding effects of combining multiple miRNAs in a single linear model. The choice of Spearman rank correlation, a non-parametric measure, was necessitated by the non-normal distribution of the age variable. However, it's essential to recognize the limitations of this test, particularly its lower sensitivity compared to other statistical methods like linear regression (Schlag, 2008). While Spearman's test can identify the strength and direction of a monotonic relationship, it may not capture more nuanced interactions or adequately account for the effects of multiple variables (Hauke & Kossowski, 2011). Therefore, the results obtained from the Spearman rank serve only as an initial overview and should be viewed cautiously.

More and more miRNA expression studies suggest that many age-associated miRNAs decrease in abundance as age progresses. The present analysis only corroborated this trend for miR-24, showing a decrease in expression (increase in ΔCt value) with age (ρ= 0.04, p= 0.340, R^2= 0.001). Nevertheless, this finding aligns with numerous previous studies that reported a negative correlation between miR-24 levels and chronological age (L.-H. Chen et al., 2010; ElSharawy et al., 2012; Huan et al., 2018; Khee et al., 2014; Noren Hooten et al., 2010).

While only miR-24 verified that most miRNAs are downregulated with age, a broad range of evidence could confirm the upregulation of miR-21 with increasing age. For instance, research on miR-21 as a marker of inflamm-aging highlighted an increase in its expression with age (Olivieri et al., 2017), reflecting the outcomes presented in this thesis

(ρ= -0.12, p= 0.011, R^2= 0.020). Subsequent studies further affirmed the upregulation of miR-21 with advancing age (Mensà et al., 2020).

Shifting the focus to miR-155, several studies have reported contradictory results regarding the expression. While on the one hand, this investigation indicated a minor upregulation with age (ρ= -0.07, p= 0.130, R^2= 0.006), going in line with research about the miRNA expression profile of older women (Sredni et al., 2011), Hooten et al. (2010) and Wang et al. (2011), on the other hand, present conflicting evidence, showcasing the intricate relationship between miR-155 and aging.

Several elements could account for the disparities in the expression analysis. Firstly, population samples across different studies might vary in age distribution, health conditions, lifestyle factors, or ethnic backgrounds, all potentially influencing miRNA's expression values. Secondly, methodological differences can introduce variability, especially in miRNA extraction and measurement techniques. Moreover, recurring uncertainty across various studies remains regarding the housekeeping gene used to determine ΔCt value, highlighting the need for clarity. Given that the choice of housekeeping gene can profoundly impact results (Garcia-Bardon & Thal, 2016), this underscores the challenges in drawing direct comparisons between studies. Lastly, the Spearman test's non-significant correlation for miR-155 with age calls for prudence (p= 0.130). It underscores the necessity of rigorous validation and potentially signals the multifaceted interactions underpinning miR-155 expression and aging complexity that warrant more profound, more granular research.

5.1.2 Building miRNA-3Age

The background of age estimation includes several methods, each with its approach to modeling the passage of time within biological systems.

Techniques such as multiple linear regression, Hochschild's method, principal component analysis, and support vector regression offer diverse perspectives. Alternatively, the analytical framework is expanded by non-regression methods like deep learning and decision trees (Salih et al., 2023). Elastic net regression has become the method of preference over time for aging clocks, especially in studies with extensive biomarkers (Horvath & Raj, 2018; Huan et al., 2018). This specific approach combines the strengths of Lasso and ridge regression, promotes parsimony of the model, and compensates for the influence of co-expressed biomarkers (Huan et al., 2018), which is particularly advantageous when the number of predictors exceeds the

number of observations (Horvath & Raj, 2018). The penalized regression model, by design, penalizes the inclusion of excessive variables, effectively shrinking less contributory variables' coefficients towards zero. This approach is instrumental in identifying a streamlined set of variables, which is advantageous in constructing a robust, optimal-performing model (Horvath & Raj, 2018).

In the context of miRNA-3Age, the choice fell on a multiple linear regression model. This choice is substantiated by the significant interrelations among all three miRNAs of interest, suggesting that a linear model could effectively capture the insights offered by their collective dynamics (miR-21 vs. miR-24: $\rho = 0.2$, $p = 2.5E^{-5}$; miR-21 vs. miR-155: $\rho = 0.12$, $p = 0.005$; miR-24 vs. miR-155: $\rho = 0.5$, $p < 2.2E^{-16}$). Additionally, evidence shows that miRNAs are ideal for controlling processes and pathways at a network level. This indicates that several miRNAs share the same target and should, therefore, perform significantly better in a combined model (Olivieri et al., 2013). The linear model interpretability further justified the selection, especially given the study's focus on only three predictors and a sizeable observation count ($n = 539$), making a penalized regression model unnecessary (Horvath & Raj, 2018).

Despite their inherent methodological differences, the linear regression and Spearman rank tests yielded harmonious outcomes, underscoring the findings' reliability and robustness. Specifically, both statistical methods consistently identified negative associations for miR-21 and miR-155 ΔCt values with chronological age, implying an upregulation with ongoing years and a downregulation of miR-24. Moreover, the significant correlation of miR-21 in both tests highlighted its potential as a critical biomarker of aging. The subtle difference in the significance levels of miR-155 (Spearman rank: $p = 0.130$ vs. lm: $p = 0.011$) and miR-24 (Spearman rank: $p = 0.340$ vs. lm: $p = 0.02$), on the other hand, might hint at the augmented sensitivity and power of linear regression when considering multiple variables simultaneously.

Furthermore, the significance of all three miRNAs in the linear model and the higher R^2 confirm, once again, that the chosen miRNAs' expression influences each other. This verifies the assumption that age estimators should not be based on a single biomarker but on a targeted selection (Lin, 2022; Wagner et al., 2016).

5.1.3 Regression Performance Metric and Predictive Accuracy

R^2

The R^2 from the linear model, while modest at 5.1%, contrasted with the lower values corresponding to the Spearman rank, ranging from 0.1% to 2%. This suggested that while both methods align on general trends, the linear model delves deeper, offering more nuanced clinical insights into miRNA expressions' aging correlations. It's important to note that the explained variability by the miRNAs remains relatively minor, hinting at the multifactorial essence of aging where even a modest R^2 can be meaningful, given the complexity of the biological systems at play.

It was hypothesized that a model's fit improves by including more biomarkers. This hypothesis finds support in different studies. For example, a US-based research group could create a similar miRNA age estimator with 80 different miRNAs, achieving an R^2 of 49% (Huan et al., 2018), clearly exceeding the present model. Similarly, Horvath's DNAm aging clock, with close to 400 DNA methylation sites, boasts an R^2 of 97% (Horvath, 2013), supporting that a more extensive set of biomarkers yields more accurate and robust age estimators.

When expanding the present model with another age-associated miRNA, particularly miR-146a, an inflamm-aging miRNA, the R^2 value is more than three times higher than before, with 17.92%, respectively. However, the model is based on a small sample size (n= 27), making the calculated biological age susceptible to different measurement errors, age-related cofounders, and, most importantly, an imperfect statistical prediction. This led to the conclusion that the three miRNA-based model, with its broader dataset (n= 539), would offer more clinically relevant insights in this investigation, setting the basis for subsequent analysis.

Mean Absolute Error and Median Absolute Error

Two analytical metrics were considered in the first place to assess the performance of the age estimators: The Mean Absolute Error (MAE) and the Median Absolute Error (MedAE). In this analysis, the derived MAE stood at 10.4 years, indicating that the model's predictions, on average, diverged from actual ages by about 10.4 years. Conversely, the MedAE, employed to measure the predictive accuracy of the biological age, was nine years, denoting that half of the prediction errors were contained within nine years. The minor difference between MAE and MedAE could arise due to outliers or the distribution of errors.

Surveying the scientific literature, several studies were found with analogous metrics. A research group based in the USA crafted a microbiome-based aging clock from microbial gene expressions extracted from stool samples, yielding an MAE of 9.21 years (Gopu et al., 2020). Moreover, Galkin et al. (2020) metagenomic aging clock reported a slightly higher MAE at 10.6 years.

Interestingly, to the present knowledge, Horvath's multi-tissue DNAm aging clock achieved the lowest error found in literature, with a MedAE of a mere 3.6 years. However, when narrowing the focus to, for example, the breast tissue-based model, the MedAE rose to 8.9 years. It is suspected that such an abnormality in the breast tissue is caused by hormonal influences and increased cell proliferation in this type of tissue (Horvath, 2013). In the present study, capillary blood was the primary sample for determining miRNA ΔCt levels. The diversity of its cell types and susceptibility to heightened cell proliferation, like breast tissue, could account for the higher MAE and MedAE values. In addition, Riccardo E. Marioni et al. (2015) confirmed age-related differences in blood composition. These differences could be a potential undetected cofounder as to why the present study reports significantly higher margins of error than the multi-tissue DNAm aging clock.

Moreover, Bell et al. (2019) illustrated the inverse relationship between sample size and error size (Figure 8), stating that for a sample size of less than 1000, akin to the present study, a root mean square error of approximately eight is commonplace. This statement could also justify why the present miRN-3Age has a high error of around ten years. The experts concluded that enlargement in training sample size generally augments the precision in chronological age measurement (Bell et al., 2019), so further research with a larger sample size is mandatory to lower the error.

Biological age refers to an individual's development or physiological condition that can indicate their lifespan or health relative to their chronological age (Rivero-Segura et al., 2020). Given the variability in human physiology, any model predicting biological age will be expected to have a certain degree of error (Salih et al., 2023). Furthermore, it is believed that the age prediction model with the most negligible error in estimating biological age is not always the most accurate in assessing the "age gap". Impeccable age prediction does not necessarily yield a meaningful age gap, also known as the biomarker paradox (Salih et al., 2023).

If miRNA-3Age were to measure chronological age perfectly, it would eliminate any variability and thus preclude the possibility of detecting associations with the biological aging process (Bell et al., 2019; Zhang et al., 2019). Therefore, it can be concluded that assessing the effectiveness of an age-estimator model is better done by evaluating its consistency across different datasets rather than by looking at the specific error alone (Salih et al., 2023).

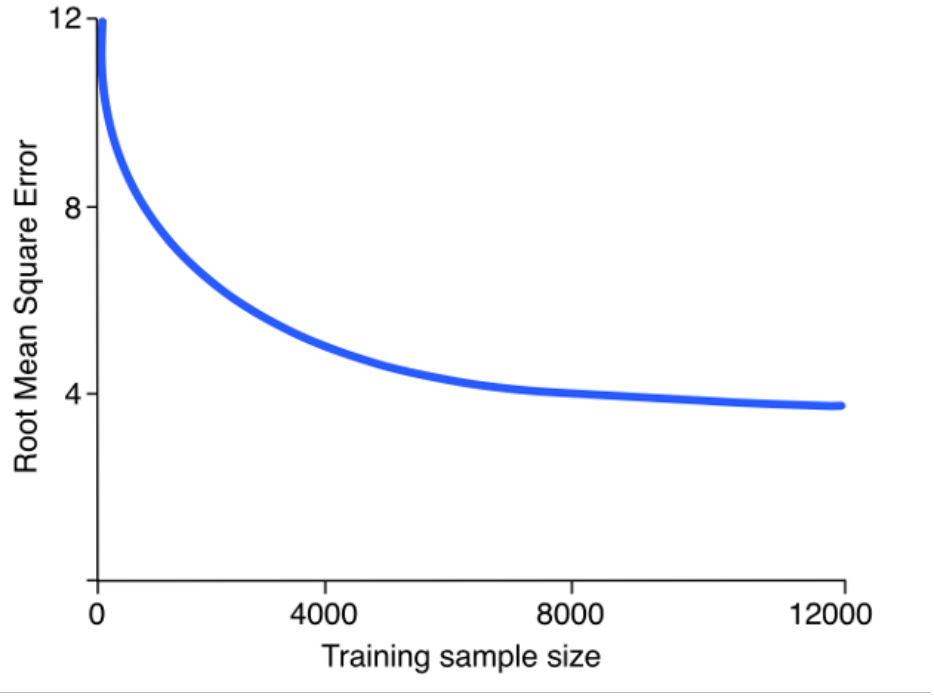

Figure 8: Age estimator error. Average Root Mean Squared Error (RMSE) in relation to Training sample size. y-axis: RMSE of the predicted biological age (Reprinted by Bell et al., 2019, p. 4).

Age Correlation

Experts state that an age estimator can only be termed an "aging clock" if its age correlation exceeds ρ= 0.80 in a broad independent validation dataset encompassing 20 to 100 years (Horvath & Raj, 2018). To put it more precisely, Horvath & Raj (2018) argue that a particular number of participants is crucial to achieving a width of 0.10= 0.98–0.88 of a 95% CI. They point out that the larger the sample size n, the lesser the correlation coefficient ρ needed for an adequate CI. For example, an n of 404 would necessitate an ρ of 0.7, while n= 867 would only require an ρ of 0.5 (Horvath & Raj, 2018).

In assessing predictive accuracy, the relationship between chronological and biological age was analyzed through a Spearman rank test, as illustrated by age correlation, resulting in a correlation coefficient ρ of 0.13 (p= 0.002). Evidence could show that several age estimators outperform the presented age correlation. Notably, Huan et al. 's (2018) miRNA age estimator predicts a biological age that correlates with chronological age by 0.7. Meanwhile, Horvath's aging clock further outperforms Huan et al. 's miRNA-based age estimator and miRNA-3Age by a significant margin with a near-perfect correlation of ρ= 0.9 (Horvath, 2013; Levine et al., 2018).

Considering Horvath's criteria for an aging clock implies that with a sample size of 539, the correlation should be at least 0.67 to earn the title of an aging clock, which needs to be met for the study at hand. Therefore, studies should emphasize enlarging the sample size and incorporating more validated age-associated miRNAs. If these criteria are met, the miRNA-3Age has the potential to excel and thus earn the "aging clock" accolade.

Lastly, various studies have indicated that biological and chronological age exhibit non-linear correlations, predominantly during adolescence and later stages of life (Alisch et al., 2012; Horvath, 2013). Figure 9 underscores that Horvath's aging clock accelerates during adolescence, possibly due to childhood and teenage changes, while maintaining linearity during adulthood (Bell et al., 2019; Horvath, 2013). Additionally, Bell et al. (2019) mentioned an asymptote later in life, where the chronological age increases faster than its biological counterpart. Due to these non-linear correlations, some studies have already adopted a logarithmic-linear transformation specifically for these two groups (Horvath & Raj, 2018). Contrasting these correlations reveals that the miRNA-3Age age estimator remains linear, except for a subtle upward trend at the age spectrum's end, where biological age increases faster than the chronological one. In addition, younger and older people show a considerably larger standard deviation, hinting at a considerable age variation in these two age ranges (Figure 6). These findings indicate that future aging clocks should work on the de-lineralization between chronological and biological age, using diverse functions such as logarithmic, linear, or cubic to represent the entire lifespan best.

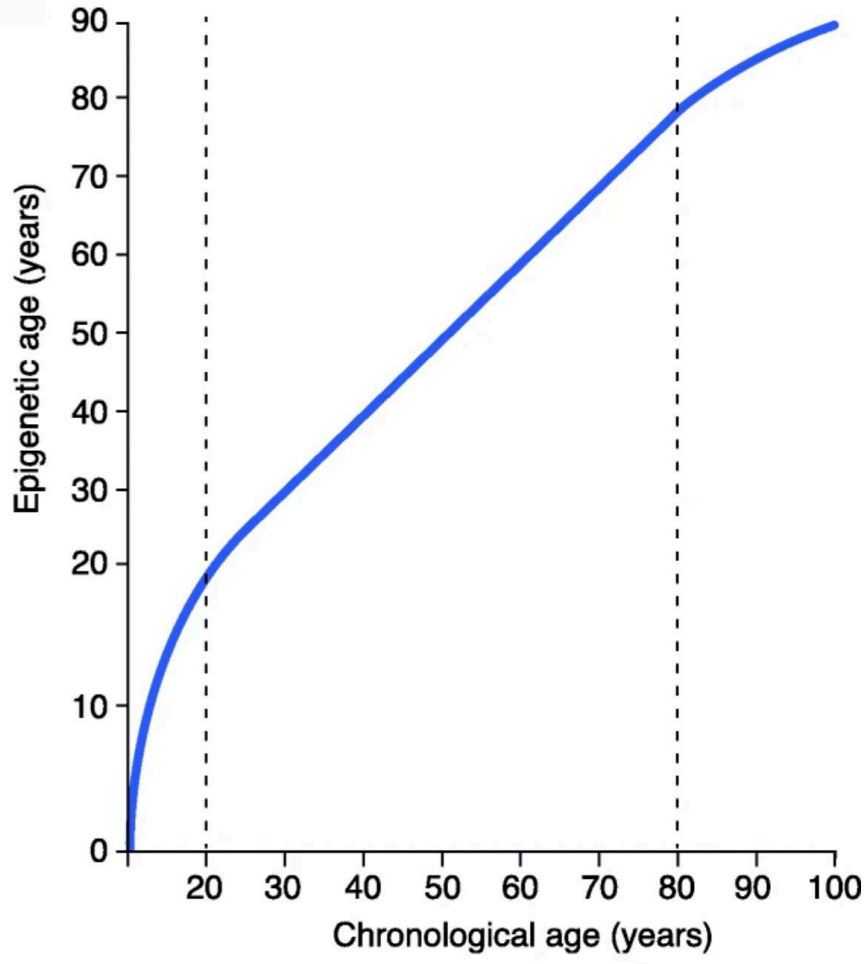

Figure 9: Age correlation. Biological age in relation to chronological age. (Reprinted by Bell et al., 2019, p.4).

5.1.4 Summary and Reflections on Aging Markers

This study embarked on the ambitious task of developing a novel miRNA-based age estimator, drawing on the rich fundamental research of aging and the potential of miRNAs as biomarkers. Intricately analyzing the relationship between miRNA expression and chronological age laid the groundwork for miRNA-3Age, an innovative biological age estimator. The selection of miR-21, miR-24, and miR-155 for this model was predicated on their established roles in aging, inflammation, and cellular senescence.

The findings indicate that the chosen miRNAs show significant, albeit weak, correlations in a combined model with chronological age, underscoring their relevance in aging. While modest, the variance explained by the model is a step toward unraveling the complex biology of aging and the role of miRNAs therein. However, the journey to fully comprehend and accurately estimate biological age is ongoing. This study's relatively low R^2 values suggest a vast

unexplored territory in the relationship between miRNAs and biological aging. This opens possibilities for future research to explore additional miRNAs and integrate other biomarkers. The goal remains to refine and enhance the predictive accuracy of biological age estimators.

5.2 Lifestyle model

The fascination with human life expectancy has captured the attention of both scientists and the public. As a result, there has been a surge in investigations into the different factors that impact the length and quality of life. Twin studies have suggested that genetics may only determine 25% of the human lifespan, indicating that environmental, lifestyle, and dietary factors play a significant role in the remaining 70-80% (Partridge et al., 2018). This realization highlights an urgent public health priority to identify and improve modifiable factors, including lifestyle choices and behavioral patterns, that can effectively prolong individuals' health span and overall well-being (Ng et al., 2020).

At the forefront of this search are some selected biomarkers whose significance is derived from the dynamics of epigenetic changes, such as miRNA biomarkers. Unlike genetic mutations, these changes are theoretically reversible (Huan et al., 2018; Peters et al., 2015), illuminating new possibilities for anti-aging interventions. The potential of age estimators, in general, as a tool is rooted in their capacity to reveal discrepancies between an individual's biological age and chronological age. Identifying such outliers can reveal accelerated or decelerated aging at the biological level, facilitating a more nuanced comprehension of the phenomenon beyond chronological age (Huan et al., 2018).

With the increasing interest in applying epigenetic therapies to delay or even reverse aging-related changes, the present study is the first to illuminate the association between miRNA age and an array of lifestyle, behavioral, and dietary factors. Within the scope of this work, only some selected characteristics will be discussed in detail. Numerous other studies explore the connection between various other biological age estimators and lifestyle, behavioral, and dietary factors. Notable studies expanding this research's horizon include the analysis of physical activity (Quach et al., 2017), exercise (Levine et al., 2018), BMI (Lin, 2022; Quach et al., 2017), and red meat (Levine et al., 2018).

5.2.1 The Impact of Alcohol Consumption on Biological Aging

The relationship between alcohol consumption, smoking, and its impact on health is an area of keen interest. Current findings from the present study, alongside other research by Levin et al. (2018) and Lin (2022), have undeniably illustrated the negative impact of smoking on biological age. However, the effects of alcohol consumption on biological age remain somewhat unclear.

Before delving into the core findings, it's crucial to differentiate between heavy and moderate alcohol consumption. The American National Institute on Alcohol Abuse and Alcoholism (NIAAA) categorizes heavy drinking as men consuming five or more drinks on any day per week or 15+ drinks weekly and women consuming four or more drinks on any day or 8+ drinks weekly. In contrast, moderate drinking involves up to two drinks daily for men and one for women (NIAAA, 2023).

Historically, light to moderate alcohol intake has been linked to certain health benefits. Such consumption has been associated with reduced morbidity, all-cause mortality, and coronary heart disease (CHD) mortality (de Labry et al., 1992; Knott et al., 2015; Thun et al., 1997). The protective nature of light alcohol consumption may be attributed to its anti-inflammatory properties, potentially reducing inflammatory markers like IL-6 and C-reactive protein (CRP) (Quach et al., 2017).

Furthermore, the current study and various other research (Lin, 2022; Quach et al., 2017) have suggested a negative association between moderate alcohol consumption and biological age acceleration. Remarkably, this study's findings indicate that daily alcohol drinkers are 2.85 times more likely to have a lower biological age than their chronological one compared to infrequent drinkers. However, caution is needed when looking at these daily drinkers. The term "daily drinkers" is quite ambiguous; it does not clarify the amount or exact frequency of consumption. This limits the understanding, as there is no indication of whether these individuals meet or exceed the NIAAA's moderation guidelines.
Moreover, it may be essential to distinguish between the type of alcohol, for which the present questionnaire unfortunately does not provide any information. While beer and spirits are associated with a higher biological age, wine, probably due to its high content of polyphenols, does not show such an association (Galkin et al., 2023).

Contrastingly, heavy drinking presents a starkly different picture. Notably, Ng et al. (2020) found that individuals consuming more than three drinks daily exhibit an older biological age. Similarly, individuals with an alcohol use disorder appear 2.2 years biologically older than healthy controls (Luo et al., 2020).

Building on this, the World Health Organization has expressed a clear statement regarding the implications of alcohol consumption on health, particularly highlighting the risks of cancer. The organization underlines that the risk of developing cancer increases proportionally with the quantity of alcohol consumed. They emphasize that light and moderate alcohol consumption is responsible for half of all alcohol-related cancers in Europe. The review states the absence of a clear threshold at which alcohol's carcinogenic effects become active in the human body and stresses that potential protective effects of moderate drinking on type-2 diabetes and cardiovascular diseases might be influenced by the specific comparison groups chosen, the statistical methodologies employed, and possibly other overlooked factors. The organization firmly states that there is no concrete evidence suggesting that the potential benefits of light and moderate drinking on certain diseases can outweigh the cancer risks posed by the same levels of alcohol consumption (WHO, 2023).

It's pivotal to note that the seemingly young biological age of daily drinkers in the study could be influenced more by their overall healthy lifestyle than their alcohol consumption. Here, most daily alcohol consumers led a healthy lifestyle - they were non-smokers, physically active, with a mean BMI under 25, and reported lower stress levels. This healthy lifestyle may, to some extent, offset the potential harms of daily alcohol consumption. Consequently, a healthy lifestyle, as exhibited by the daily drinker subgroup, could be a more significant factor in determining biological age than alcohol consumption in isolation.

While some evidence suggests the potential benefits of light to moderate alcohol consumption on biological age (Lin, 2022; Quach et al., 2017), it is essential to consider the broader health implications of alcohol and the compounding factors that might influence these findings. Even if moderate drinking appears beneficial in some contexts, the potential risks must be considered, as highlighted by the WHO (WHO, 2023).

5.2.2 The Impact of Stress on Biological Aging

Literature indicates that psychological stress is a substantial risk factor for accelerated aging (Epel et al., 2004).

The differentiation between chronic and acute stress is crucial in understanding their impacts on aging. Chronic stress, characterized by its persistence over an extended period, has been repeatedly linked with more severe biological consequences, including telomere shortening. In contrast, acute stress-short–term responses to immediate challenges–might not have the same enduring impact on cellular aging (Zannas, 2016; Zannas et al., 2015).

It's been highlighted that cumulative stress amplifies cellular oxidative stress, subsequently posing threats to human longevity (Epel et al., 2004). Stress-induced oxidative stress results from the chronic activation of the body's stress responses, leading to excess adrenal hormones like glucocorticoids. These hormones can potentiate neuronal damage by disrupting the balance of oxidative and antioxidative molecules within the cell, which may result in telomere shortening, a significant marker of biological age (Epel et al., 2004).

Furthermore, oxidative stress is pivotal in developing cellular senescence through psychological stressors (Galkin et al., 2023). This stress-induced senescence predisposes individuals to a spectrum of age-related diseases, including cardiovascular disorders, immune dysregulation, and neuropsychiatric conditions (Zannas et al., 2015).

The current study could show that individuals subjected to high-stress levels have a significant 42% decline in the probability of exhibiting a biological age younger than their chronological age compared to those with lower stress levels. Further accentuating this trend, those experiencing really high-stress levels faced a marked 56% decline in similar chances. The findings underscore the correlation between increased stress levels and biological aging, supporting the thesis that Zannas et al. (2015) posited that chronic, cumulative lifestyle stress significantly impacts epigenetic aging markers. Furthermore, it has been documented that lifetime stress may be responsible for a discrepancy of up to 3.6 years in biological age among individuals (Zannas, 2016; Zannas et al., 2015). This highlights the importance of in-depth research on the complex connection between stress and biological aging. While this thesis utilized one stress metric from the questionnaire, further research should investigate stress's various types, durations, and triggers to understand its multifaceted effects on the aging process and miRNA expression.

Studies suggest that interventions targeting stress reduction can positively impact epigenetic markers of aging. One such intervention is mindfulness meditation, which has been linked to a decrease in biological age, with consistent practice resulting in a reduction of approximately 0.24 years per year of training (Chaix et al., 2017). The potential of various stress-management techniques to slow down the aging process highlights the importance of taking a multifaceted approach to stress management.

The undeniable association between stress and aging necessitates an integrative approach to intervention. Stress mitigation strategies must be incorporated as a cardinal component of any comprehensive anti-aging interventions to augment dietary and lifestyle modifications.

5.2.3 The Impact of Different Dietary Characteristics on Biological Aging

In recent years, the influence of nutrition on biological aging has become increasingly apparent. The Mediterranean diet (MD), characterized by its composition rich in omega-3 fatty acids, vital nutrients, and dietary fiber from fruits and vegetables, stands at the center of this discussion (Gurău et al., 2018). Gurău et al. (2018) emphasize the Mediterranean diet for its association with longevity and its noteworthy reduction in the risk of all-cause mortality and cardiovascular diseases. Similarly, Quach et al. (2017) identify the components of MD–particularly omega-3 fatty acids from fish and fiber from whole grains–as critical in mitigating risk factors that accelerate biological aging and age-related diseases.

The present investigation has provided further empirical support to these findings, explicitly elucidating the role of whole grains and fish in attenuating systemic inflammation, a key contributor to the aging phenomenon inflamm-aging. Daily whole grain intake was notably associated with a 1.80-fold increase in the likelihood of being biologically younger.
In comparison, moderate fish consumption was linked to an even more significant 2.3-fold increase. These findings align with the anti-inflammatory and metabolic pathways enhanced by omega-3 fatty acids, specifically eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), known for their role in anti-inflammatory cytokine synthesis (Quach et al., 2017). This corroborates the notion that fish intake can counteract various age-related diseases (Quach et al., 2017). However, the lack of additional benefits from higher weekly fish consumption suggested a saturation point, indicating a potential threshold effect or other unexplored factors that modulate this relationship.

Contrastingly, the anticipated correlation between fruit and vegetable intake and younger biological age was not observed in the present cohort. This deviation from the literature, where these foods are often ascribed with properties against age-related chronic diseases, such as CHD, stroke, and type-2 diabetes, mediated by anti-inflammatory and cardiometabolic effects, gives rise to further investigations (Quach et al., 2017). Moreover, this could suggest that the specific miRNAs used in the biological age estimator might not be sensitive to the influences of fruit and vegetables or that these effects need to be more nuanced to be detected by the current methodologies.

In examining dietary patterns that differ from MD, the data clearly shows the disadvantageous effects of regular fast-food consumption, which was associated with a 60% decrease in the likelihood of being biologically younger. This aligns with existing literature that suggests that diets high in glycemic, saturated, and trans-saturated fats and those rich in omega-6 fatty acids are linked to epigenetic aging (Koemel & Skilton, 2022). The health outcomes of poor dietary habits are likely a consequence of heightened inflammatory responses and oxidative damage, in conjunction with a decline in muscle mass and strength, factors which collectively degrade physical capabilities and overall health condition (Fan et al., 2021; McPhee et al., 2016). Additionally, a recently published article indicates that specific components of fast food may contribute to age-related inflammation, disrupted cellular functionality, and lipid metabolism, further substantiating the intricate relationship between diet and biological aging processes (Koemel & Skilton, 2022).

Furthermore, this thesis contributes novel insights into the relationship between green tea consumption and biological age. A consistent green tea intake was linked to a 54% increase in the odds of being biologically younger, a finding that corroborates previous research on the anti-inflammatory and antioxidant properties of tea, particularly its content of the bioactive polyphenol epigallocatechin-3-gallate (EGCG) (Gurău et al., 2018).
This polyphenol is renowned for its anti-senescent capabilities (Gurău et al., 2018; Xiong et al., 2017). It may directly affect the aging biomarkers evaluated in the present age estimator, given that the miRNAs employed are intrinsically linked to senescence processes.
For instance, research has demonstrated EGCG's capacity to inhibit H_2O_2-induced cell-senescence in human fibroblasts while reducing levels of acetylated p53, an essential regulatory protein in cell cycle and apoptosis (Gurău et al., 2018). Supporting this, the work of Koemel & Skilton (2022) reinforces the link between tea consumption and the slowing of biological aging processes. Additionally, animal studies by Niu et al. (2023) have illustrated an increase in

lifespan by eight to twelve weeks in rats with regular intake of green tea, suggesting a broader implication of green tea in reducing systemic inflammation and possibly, in extension, enhancing longevity.

This growing body of evidence indicates a correlation and a possible causal relationship between green tea consumption and younger biological age, mediated by molecular mechanisms that involve the downregulation of inflammatory pathways and the accumulation of cellular defense systems against oxidative stress. Given the dose-dependent effects observed in certain studies, further investigation is warranted into the optimal consumption levels for maximal anti-aging benefits.

5.2.4 Summary and Reflections on Lifestyle Models

The study's outcomes highlight the complex relationship between lifestyle, dietary patterns, and the biological mechanisms of aging, as reflected by miRNA-3Age utilized herein. The three miRNAs selected for constructing this estimator are known for their roles in senescence and inflamm-aging, which are understandably modulated by lifestyle and dietary habits. Upon reflection, the outcomes are not surprising, given that identical health risks and protective determinants are also crucial factors of chronic disabilities and diseases related to aging.

While the miRNA-3Age does reflect the impact of lifestyle and dietary patterns, there is evidence that epigenetic markers such as DNAm Age and DNAm PhenoAge do not show significant changes in response to short-term lifestyle interventions (Quach et al., 2017), suggesting complexity in the epigenetic detection of such changes. Additionally, the effectiveness of anti-aging interventions might be variable across different tissues, as indicated by the disparate effects of hormone replacement therapy on epigenetic aging in buccal epithelial versus blood cells (Levine et al., 2016).

Given the considerable differences in lifestyles and exposure risks in different population groups (Fan et al., 2021), the generalizability of the results may be limited. It is essential to consider these differences when applying the conclusions to broader contexts.

In conclusion, the research provides valuable insights into the relationship between miRNA-3Age and lifestyle factors, adding to a growing body of evidence supporting the potential reversibility of epigenetic age markers. These findings highlight the importance of a healthy lifestyle and pave the way for developing personalized anti-aging strategies.

5.3 Evaluating miRNA-3Age as a Comprehensive Biomarker of Aging

Lastly, it needs to be discussed whether the miRNA-3Age can be considered a true biomarker of aging. As mentioned in the introduction, the American Federation for Aging Research has outlined four criteria for an ideal biomarker of aging: predicting the aging rate, monitoring basic aging processes, being testable repeatedly without harm, and functioning across species (T. E. Johnson, 2006).

Starting with cross-species applicability, miRNA-3Age demonstrates significant potential in meeting the AFARs fourth criterion since gain and loss of function studies in C.elegans and Drosophila melanogaster were the first to establish miRNAs' roles in aging (Boehm & Slack, 2005; Ibáñez-Ventoso & Driscoll, 2009). Subsequent research in animal models further underscored miRNAs' involvement in aging processes (Lettieri-Barbato et al., 2022; Olivieri et al., 2021; Onodera et al., 2017). This thesis extends these findings, noting analogous human miRNA patterns and demonstrating the applicability and effectiveness across species. However, the specificity of miRNA-3Age as an aging estimator, and not only the miRNA expression patterns, requires further validation in animal models, especially concerning its predictive accuracy across different species.

The methodological approach of miRNA-3Age satisfies the third criterion through its analysis of capillary blood samples and exemplifies the noninvasive, repeatable testing criterion. The stability of miRNAs in bodily fluids aligns with this criterion and highlights their promise in clinical settings. This aspect of miRNA-3Age offers a practical and ethical advantage over invasive testing methods.

Furthermore, miRNA-3Age focuses on vital biological mechanisms underlying the aging process, such as cellular senescence, inflammation, and DNA repair, by studying miR-21, miR-24, miR-155. This aligns with the second criterion.

Addressing the first criterion, predicting the rate of aging more accurately than chronological age, presents a more complex challenge. Extensive cohort studies are essential to determine whether miRNA-3Age can predict functional capability and lifespan better than chronological age, absent disease influences. Such studies are crucial for advancing the understanding of miRNA-3Age's potential to serve as a valid biomarker of aging.

In conclusion, while pursuing an ideal biomarker of aging is fraught with complexities, miRNA-3Age presents a promising candidate. Its adherence to AFAR's criteria-particularly in

terms of cross-species validity, non-invasive testing, and the monitoring of fundamental aging processes, positions it as a significant contribution to the field. As early as 2006, Johnson stated that there are probably no biomarkers that fulfill the above criteria (T. E. Johnson, 2006). However, the quest to find a biomarker that completely fulfills these criteria, especially in predicting the rate of aging, remains an ongoing endeavor.

6. Limitations and Future Research

While the development of the miRNA-3Age biological age calculator is a significant step forward in aging research, several limitations highlight areas for future investigation.

Primarily designed for adult blood samples, the calculator's specificity introduces bias when estimating the biological age of adolescents and centenarians and fails to account for the variability in epigenetic changes across different tissues and cell types. Furthermore, the study's dataset predominantly features German and Austrian participants, limiting its generalizability across different demographics. To enhance the miRNA3-Age model's robustness and applicability, future research must employ more extensive, more diverse datasets that include a variety of age groups, populations, ethnicities, genders, tissues, cells, and diseases.

Due to dataset constraints, the linear model relies on a narrow selection of only three miRNAs. Using a limited set of biomarkers can lead to statistical disadvantages, including overfitting and failure to capture the genetic diversity impacting aging. This limitation is particularly evident when contrasted with Horvath's methylation clock, which employs nearly 400 methylation markers for precise age estimation, and Huan et al. 's work, which utilized a broader set of miRNAs, though not based on a systematic literature review (Horvath, 2013; Huan et al., 2018). The incremental improvement by adding one more miRNA (miR-146a) underscores the need for a more comprehensive miRNA selection by conducting extensive literature reviews. Such an expansion would enhance the model's precision and mitigate statistical issues, ensuring a more accurate representation of the genetic diversity influencing aging.

Furthermore, the reliance on self-reported lifestyle data introduces notable biases and inaccuracies that may diminish the model's capacity to represent real lifestyle impacts on aging and potentially produce false negative results. The need for detailed dietary information further complicates the assessment of nutritional status and caloric intake, highlighting the need for more precise and objective data collection methods.

Another critical area is the validation of the findings. The absence of external replication limits the study's validation efforts, contrasting sharply with models like Horvath's DNAm age, which have been validated across numerous independent datasets (Horvath & Raj, 2018). While integrating machine learning techniques to split the dataset for independent testing and training has shown potential, the necessity for external dataset validation remains a paramount goal for future studies to establish the reliability and applicability of the proposed aging models.

Furthermore, the dynamic nature of miRNA expression over time still needs to be explored. Longitudinal studies, tracking individuals across their lifespan, are essential for comprehensively understanding the aging process and its biomarkers.

Addressing these limitations by developing more inclusive, standardized, and validated aging models and adopting advanced data collection methods and longitudinal study designs will significantly enhance future biomarkers of aging.

7. Conclusion

The scientific community has long been intrigued by the intricate interplay between biological and chronological age. The aim is to unravel the underlying mechanisms driving the aging process and identify potential avenues for extending Healthspan. This thesis marks a significant contribution to this endeavor by investigating the role of miRNAs, particularly miR-21, miR-24, and miR-155, in estimating biological age through the innovative miRNA-3Age model. It reinforces the notion that aging is a complex, multifaceted process that cannot be accurately represented by any single (type of) biomarker, which the limited performance can see of miRNA-3Age. As Wagner et al. (2016) aptly noted, the intricacies of biological and molecular aging mechanisms necessitate a comprehensive approach, combining diverse biomarkers with established physical and functional parameters to achieve a more accurate measure of healthy aging.

The findings from this study not only shed light on the dynamics of miRNA expression in relation to chronological age but also pave the way for future investigations to enhance the predictive accuracy of miRNA-based biological age estimators. Refining and extensively validating the miRNA-3Age model is necessary to fully utilize its potential as a novel tool for biological aging assessment. Understanding the dynamics of miRNA age will improve as more studies obtain repeated measures of miRNA expression.

This research has also illuminated the significant influence of lifestyle, behavioral, and socio-environmental factors on biological aging. The correlation between specific lifestyle choices, such as alcohol consumption, physical activity, and dietary habits, and variations in miRNA expression levels unveils modifiable factors that could influence aging and a person's Healthspan. These discoveries provide promising pathways for targeted anti-aging interventions, suggesting the possibility of decelerating biological aging and improving life quality in later years by modifying certain lifestyle factors.

Yet, it remains unknown whether directly influencing age-related miRNA expression will slow down the biological aging process. This thesis emphasizes the need for longitudinal studies and further exploration into how miRNAs interact within a comprehensive multi-omics framework. Such endeavors are crucial for understanding the intricate web of factors that drive aging and pinpointing key domains that might steer the trajectory toward successful or unsuccessful aging.

In sum, this thesis has significantly advanced the understanding of miRNAs as potential biomarkers for biological aging and underscored the impact of lifestyle factors on the aging process. The creation of the miRNA-3Age model signifies progress in quantifying biological age with greater precision. As research moves forward, integrating multi-omics data and deepening insights into the modifiable determinants of aging will be vital in developing strategies to create personalized medicine interventions as we age to ensure that the additional years of life are more extended, healthier, and more vibrant.

8. References

Alisch, R. S., Barwick, B. G., Chopra, P., Myrick, L. K., Satten, G. A., Conneely, K. N., & Warren, S. T. (2012). Age-associated DNA methylation in pediatric populations. *Genome Research*, *22*(4), 623–632. https://doi.org/10.1101/gr.125187.111

Baker, G. T., & Sprott, R. L. (1988). Biomarkers of aging. *Experimental Gerontology*, *23*(4–5), 223–239. https://doi.org/10.1016/0531-5565(88)90025-3

Beard, J. R., Officer, A., de Carvalho, I. A., Sadana, R., Pot, A. M., Michel, J.-P., Lloyd-Sherlock, P., Epping-Jordan, J. E., Peeters, G. M. E. E. (Geeske), Mahanani, W. R., Thiyagarajan, J. A., & Chatterji, S. (2016). The World report on ageing and health: A policy framework for healthy ageing. *The Lancet*, *387*(10033), 2145–2154. https://doi.org/10.1016/S0140-6736(15)00516-4

Bell, C. G., Lowe, R., Adams, P. D., Baccarelli, A. A., Beck, S., Bell, J. T., Christensen, B. C., Gladyshev, V. N., Heijmans, B. T., Horvath, S., Ideker, T., Issa, J.-P. J., Kelsey, K. T., Marioni, R. E., Reik, W., Relton, C. L., Schalkwyk, L. C., Teschendorff, A. E., Wagner, W., … Rakyan, V. K. (2019). DNA methylation aging clocks: Challenges and recommendations. *Genome Biology*, *20*(1), 249. https://doi.org/10.1186/s13059-019-1824-y

Benetos, A., Okuda, K., Lajemi, M., Kimura, M., Thomas, F., Skurnick, J., Labat, C., Bean, K., & Aviv, A. (2001). Telomere length as an indicator of biological aging: The gender effect and relation with pulse pressure and pulse wave velocity. *Hypertension (Dallas, Tex.: 1979)*, *37*(2 Pt 2), 381–385. https://doi.org/10.1161/01.hyp.37.2.381

Boehm, M., & Slack, F. (2005). A developmental timing microRNA and its target regulate life span in C. elegans. *Science (New York, N.Y.)*, *310*(5756), 1954–1957. https://doi.org/10.1126/science.1115596

Bohatá, J., Horváthová, V., Pavlíková, M., & Stibůrková, B. (2021). Circulating microRNA alternations in primary hyperuricemia and gout. *Arthritis Research & Therapy*, *23*(1), 186. https://doi.org/10.1186/s13075-021-02569-w

Bu, H., Baraldo, G., Lepperdinger, G., & Jansen-Dürr, P. (2016). Mir-24 activity propagates stress-induced senescence by down regulating DNA topoisomerase 1. *Experimental Gerontology*, *75*, 48–52. https://doi.org/10.1016/j.exger.2015.12.012

Bu, H., Wedel, S., Cavinato, M., & Jansen-Dürr, P. (2017). MicroRNA Regulation of Oxidative Stress-Induced Cellular Senescence. *Oxidative Medicine and Cellular Longevity*, *2017*, e2398696. https://doi.org/10.1155/2017/2398696

Buck Louis, G. M., Smarr, M. M., & Patel, C. J. (2017). The Exposome Research Paradigm: An Opportunity to Understand the Environmental Basis for Human Health and Disease.

Current environmental health reports, *4*(1), 89–98. https://doi.org/10.1007/s40572-017-0126-3

Caglar, O., & Cayir, A. (2019). Total circulating cell-free miRNA in plasma as a predictive biomarker of the thyroid diseases. *Journal of Cellular Biochemistry*, *120*(6), 9016–9022. https://doi.org/10.1002/jcb.28173

Campisi, J., & Vijg, J. (2009). Does Damage to DNA and Other Macromolecules Play a Role in Aging? If So, How? *The Journals of Gerontology Series A: Biological Sciences and Medical Sciences*, *64A*(2), 175–178. https://doi.org/10.1093/gerona/gln065

Chaix, R., Alvarez-López, M. J., Fagny, M., Lemee, L., Regnault, B., Davidson, R. J., Lutz, A., & Kaliman, P. (2017). Epigenetic clock analysis in long-term meditators. *Psychoneuroendocrinology*, *85*, 210–214. https://doi.org/10.1016/j.psyneuen.2017.08.016

Chalise, H. N., Nath, H., Prithvi, N., & Campus. (2023). Healthy Ageing: A Basic Concept. *Asian Journal of Population Sciences*, *2*, 124–128. https://doi.org/10.3126/ajps.v2i1.51096

Chen, L.-H., Chiou, G.-Y., Chen, Y.-W., Li, H.-Y., & Chiou, S.-H. (2010). microRNA and aging: A novel modulator in regulating the aging network. *Ageing Research Reviews*, *9*, S59–S66. https://doi.org/10.1016/j.arr.2010.08.002

Chen, W.-X., Ren, L.-H., & Shi, R.-H. (2014). Implication of miRNAs for inflammatory bowel disease treatment: Systematic review. *World Journal of Gastrointestinal Pathophysiology*, *5*(2), 63–70. https://doi.org/10.4291/wjgp.v5.i2.63

de Labry, L. O., Glynn, R. J., Levenson, M. R., Hermos, J. A., LoCastro, J. S., & Vokonas, P. S. (1992). Alcohol consumption and mortality in an American male population: Recovering the U-shaped curve--findings from the normative Aging Study. *Journal of Studies on Alcohol*, *53*(1), 25–32. https://doi.org/10.15288/jsa.1992.53.25

Ding, L., Ai, D., Wu, R., Zhang, T., Jing, L., Lu, J., & Zhong, L. (2016). Identification of the differential expression of serum microRNA in type 2 diabetes. *Bioscience, Biotechnology, and Biochemistry*, *80*(3), 461–465. https://doi.org/10.1080/09168451.2015.1107460

Ding, R., Su, D., Zhao, Q., Wang, Y., Wang, J.-Y., Lv, S., & Ji, X. (2023). The role of microRNAs in depression. *Frontiers in Pharmacology*, *14*. https://doi.org/10.3389/fphar.2023.1129186

Eastman, B. D. (1984). Important Statistics Generated by Most Regression Programs. In B. D. Eastman (Hrsg.), *Interpreting Mathematical Economics and Econometrics* (S. 59–94). Palgrave Macmillan UK. https://doi.org/10.1007/978-1-349-17702-8_6

ElSharawy, A., Keller, A., Flachsbart, F., Wendschlag, A., Jacobs, G., Kefer, N., Brefort, T., Leidinger, P., Backes, C., Meese, E., Schreiber, S., Rosenstiel, P., Franke, A., & Nebel, A.

(2012). Genome-wide miRNA signatures of human longevity. *Aging Cell*, *11*(4), 607–616. https://doi.org/10.1111/j.1474-9726.2012.00824.x

Epel, E. S., Blackburn, E. H., Lin, J., Dhabhar, F. S., Adler, N. E., Morrow, J. D., & Cawthon, R. M. (2004). Accelerated telomere shortening in response to life stress. *Proceedings of the National Academy of Sciences of the United States of America*, *101*(49), 17312–17315. https://doi.org/10.1073/pnas.0407162101

Eurostat. (2023). *EU's population projected to drop by 6% by 2100*. https://ec.europa.eu/eurostat/web/products-eurostat-news/-/ddn-20230330-1

Fan, J., Yu, C., Pang, Y., Guo, Y., Pei, P., Sun, Z., Yang, L., Chen, Y., Du, H., Sun, D., Li, Y., Chen, J., Clarke, R., Chen, Z., Lv, J., Li, L., & China Kadoorie Biobank Collaborative Group. (2021). Adherence to Healthy Lifestyle and Attenuation of Biological Aging in Middle-Aged and Older Chinese Adults. *The Journals of Gerontology. Series A, Biological Sciences and Medical Sciences*, *76*(12), 2232–2241. https://doi.org/10.1093/gerona/glab213

Galkin, F., Kovalchuk, O., Koldasbayeva, D., Zhavoronkov, A., & Bischof, E. (2023). Stress, diet, exercise: Common environmental factors and their impact on epigenetic age. *Ageing Research Reviews*, *88*, 101956. https://doi.org/10.1016/j.arr.2023.101956

Garcia-Bardon, A., & Thal, S. C. (2016). Normalization with Corresponding Naïve Tissue Minimizes Bias Caused by Commercial Reverse Transcription Kits on Quantitative Real-Time PCR Results. *PLOS ONE*, *11*(11), e0167209. https://doi.org/10.1371/journal.pone.0167209

Gopu, V., Cai, Y., Krishnan, S., Rajagopal, S., Camacho, F. R., Toma, R., Torres, P. J., Vuyisich, M., Perlina, A., Banavar, G., & Tily, H. (2020). *An accurate aging clock developed from the largest dataset of microbial and human gene expression reveals molecular mechanisms of aging* (S. 2020.09.17.301887). bioRxiv. https://doi.org/10.1101/2020.09.17.301887

Gurău, F., Baldoni, S., Prattichizzo, F., Espinosa, E., Amenta, F., Procopio, A. D., Albertini, M. C., Bonafè, M., & Olivieri, F. (2018). Anti-senescence compounds: A potential nutraceutical approach to healthy aging. *Ageing Research Reviews*, *46*, 14–31. https://doi.org/10.1016/j.arr.2018.05.001

Halper, B., Hofmann, M., Oesen, S., Franzke, B., Stuparits, P., Vidotto, C., Tschan, H., Bachl, N., Strasser, E.-M., Quittan, M., Wagner, K.-H., & Wessner, B. (2015). Influence of age and physical fitness on miRNA-21, TGF-β and its receptors in leukocytes of healthy women. *Exercise Immunology Review*, *21*, 154–163.

Hauke, J., & Kossowski, T. (2011). Comparison of Values of Pearson's and Spearman's Correlation Coefficients on the Same Sets of Data. *QUAGEO*, *30*(2), 87–93. https://doi.org/10.2478/v10117-011-0021-1

Hayflick, L. (1965). THE LIMITED IN VITRO LIFETIME OF HUMAN DIPLOID CELL STRAINS. *Experimental Cell Research*, *37*, 614–636. https://doi.org/10.1016/0014-4827(65)90211-9

Hijmans, J. G., Diehl, K. J., Bammert, T. D., Kavlich, P. J., Lincenberg, G. M., Greiner, J. J., Stauffer, B. L., & DeSouza, C. A. (2018). Association between hypertension and circulating vascular-related microRNAs. Journal of Human Hypertension, 32(6), 440–447. https://doi.org/10.1038/s41371-018-0061-2

Hinkel, R., Ramanujam, D., Kaczmarek, V., Howe, A., Klett, K., Beck, C., Dueck, A., Thum, T., Laugwitz, K.-L., Maegdefessel, L., Weber, C., Kupatt, C., & Engelhardt, S. (2020). AntimiR-21 Prevents Myocardial Dysfunction in a Pig Model of Ischemia/Reperfusion Injury. *Journal of the American College of Cardiology*, *75*(15), 1788–1800. https://doi.org/10.1016/j.jacc.2020.02.041

Horvath, S. (2013). DNA methylation age of human tissues and cell types. *Genome Biology*, *14*(10), R115. https://doi.org/10.1186/gb-2013-14-10-r115

Horvath, S., & Raj, K. (2018). DNA methylation-based biomarkers and the epigenetic clock theory of ageing. *Nature Reviews Genetics*, *19*(6), Article 6. https://doi.org/10.1038/s41576-018-0004-3

Huan, T., Chen, G., Liu, C., Bhattacharya, A., Rong, J., Chen, B. H., Seshadri, S., Tanriverdi, K., Freedman, J. E., Larson, M. G., Murabito, J. M., & Levy, D. (2018). Age-associated microRNA expression in human peripheral blood is associated with all-cause mortality and age-related traits. *Aging Cell*, *17*(1), e12687. https://doi.org/10.1111/acel.12687

Huang, Y., Tang, S., Ji-Yan, C., Huang, C., Li, J., Cai, A.-P., & Feng, Y.-Q. (2017). Circulating miR-92a expression level in patients with essential hypertension: A potential marker of atherosclerosis. *Journal of Human Hypertension*, *31*(3), 200–205. https://doi.org/10.1038/jhh.2016.66

Ibáñez-Ventoso, C., & Driscoll, M. (2009). MicroRNAs in C. elegans Aging: Molecular Insurance for Robustness? *Current Genomics*, *10*(3), 144–153. https://doi.org/10.2174/138920209788185243

Johnson, A. A., Shokhirev, M. N., Wyss-Coray, T., & Lehallier, B. (2020). Systematic review and analysis of human proteomics aging studies unveils a novel proteomic aging clock and identifies key processes that change with age. *Ageing Research Reviews*, *60*, 101070. https://doi.org/10.1016/j.arr.2020.101070

Johnson, T. E. (2006). Recent results: Biomarkers of aging. *Experimental Gerontology*, *41*(12), 1243–1246. https://doi.org/10.1016/j.exger.2006.09.006

Khee, S. G. S., Yusof, Y. A. M., & Makpol, S. (2014). Expression of senescence-associated microRNAs and target genes in cellular aging and modulation by tocotrienol-rich fraction. *Oxidative Medicine and Cellular Longevity*, *2014*, 725929. https://doi.org/10.1155/2014/725929

Kim, S., Kim, H., Lee, E.-S., Lim, C., & Lee, J. (2022). Risk score-embedded deep learning for biological age estimation: Development and validation. *Information Sciences*, *586*, 628–643. https://doi.org/10.1016/j.ins.2021.12.015

Knott, C. S., Coombs, N., Stamatakis, E., & Biddulph, J. P. (2015). All cause mortality and the case for age specific alcohol consumption guidelines: Pooled analyses of up to 10 population based cohorts. *BMJ*, *350*, h384. https://doi.org/10.1136/bmj.h384

Kochunov, P., Charlesworth, J., Winkler, A., Hong, L. E., Nichols, T. E., Curran, J. E., Sprooten, E., Jahanshad, N., Thompson, P. M., Johnson, M. P., Kent, J. W., Landman, B. A., Mitchell, B., Cole, S. A., Dyer, T. D., Moses, E. K., Goring, H. H. H., Almasy, L., Duggirala, R., … Blangero, J. (2013). Transcriptomics of cortical gray matter thickness decline during normal aging. *NeuroImage*, *82*, 273–283. https://doi.org/10.1016/j.neuroimage.2013.05.066

Koemel, N. A., & Skilton, M. R. (2022). Epigenetic Aging in Early Life: Role of Maternal and Early Childhood Nutrition. *Current Nutrition Reports*, *11*(2), 318–328. https://doi.org/10.1007/s13668-022-00402-7

Lettieri-Barbato, D., Aquilano, K., Punziano, C., Minopoli, G., & Faraonio, R. (2022). MicroRNAs, Long Non-Coding RNAs, and Circular RNAs in the Redox Control of Cell Senescence. *Antioxidants (Basel, Switzerland)*, *11*(3), 480. https://doi.org/10.3390/antiox11030480

Levine, M. E. (2013). Modeling the rate of senescence: Can estimated biological age predict mortality more accurately than chronological age? *The Journals of Gerontology. Series A, Biological Sciences and Medical Sciences*, *68*(6), 667–674. https://doi.org/10.1093/gerona/gls233

Levine, M. E., Lu, A. T., Chen, B. H., Hernandez, D. G., Singleton, A. B., Ferrucci, L., Bandinelli, S., Salfati, E., Manson, J. E., Quach, A., Kusters, C. D. J., Kuh, D., Wong, A., Teschendorff, A. E., Widschwendter, M., Ritz, B. R., Absher, D., Assimes, T. L., & Horvath, S. (2016). Menopause accelerates biological aging. *Proceedings of the National Academy of Sciences of the United States of America*, *113*(33), 9327–9332. https://doi.org/10.1073/pnas.1604558113

Levine, M. E., Lu, A. T., Quach, A., Chen, B. H., Assimes, T. L., Bandinelli, S., Hou, L., Baccarelli, A. A., Stewart, J. D., Li, Y., Whitsel, E. A., Wilson, J. G., Reiner, A. P., Aviv, A., Lohman, K., Liu, Y., Ferrucci, L., & Horvath, S. (2018). An epigenetic biomarker of aging for lifespan and healthspan. *Aging (Albany NY)*, *10*(4), 573–591. https://doi.org/10.18632/aging.101414

Lin, W.-Y. (2022). Lifestyle Factors and Genetic Variants on 2 Biological Age Measures: Evidence From 94 443 Taiwan Biobank Participants. *The Journals of Gerontology. Series A, Biological Sciences and Medical Sciences*, *77*(6), 1189–1198. https://doi.org/10.1093/gerona/glab251

López-Otín, C., Blasco, M. A., Partridge, L., Serrano, M., & Kroemer, G. (2013). The Hallmarks of Aging. *Cell*, *153*(6), 1194–1217. https://doi.org/10.1016/j.cell.2013.05.039

Luo, A., Jung, J., Longley, M., Rosoff, D. B., Charlet, K., Muench, C., Lee, J., Hodgkinson, C. A., Goldman, D., Horvath, S., Kaminsky, Z. A., & Lohoff, F. W. (2020). Epigenetic aging is accelerated in alcohol use disorder and regulated by genetic variation in APOL2. *Neuropsychopharmacology*, *45*(2), Article 2. https://doi.org/10.1038/s41386-019-0500-y

Machida, T., Tomofuji, T., Ekuni, D., Maruyama, T., Yoneda, T., Kawabata, Y., Mizuno, H., Miyai, H., Kunitomo, M., & Morita, M. (2015). MicroRNAs in Salivary Exosome as Potential Biomarkers of Aging. *International Journal of Molecular Sciences*, *16*(9), 21294–21309. https://doi.org/10.3390/ijms160921294

Marioni, R. E., Shah, S., McRae, A. F., Chen, B. H., Colicino, E., Harris, S. E., Gibson, J., Henders, A. K., Redmond, P., Cox, S. R., Pattie, A., Corley, J., Murphy, L., Martin, N. G., Montgomery, G. W., Feinberg, A. P., Fallin, M. D., Multhaup, M. L., Jaffe, A. E., … Deary, I. J. (2015). DNA methylation age of blood predicts all-cause mortality in later life. Genome Biology, 16(1), 25. https://doi.org/10.1186/s13059-015-0584-6

McPhee, J. S., French, D. P., Jackson, D., Nazroo, J., Pendleton, N., & Degens, H. (2016). Physical activity in older age: Perspectives for healthy ageing and frailty. *Biogerontology*, *17*(3), 567–580. https://doi.org/10.1007/s10522-016-9641-0

Mensà, E., Guescini, M., Giuliani, A., Bacalini, M. G., Ramini, D., Corleone, G., Ferracin, M., Fulgenzi, G., Graciotti, L., Prattichizzo, F., Sorci, L., Battistelli, M., Monsurrò, V., Bonfigli, A. R., Cardelli, M., Recchioni, R., Marcheselli, F., Latini, S., Maggio, S., … Olivieri, F. (2020). Small extracellular vesicles deliver miR-21 and miR-217 as pro-senescence effectors to endothelial cells. *Journal of Extracellular Vesicles*, *9*(1), 1725285. https://doi.org/10.1080/20013078.2020.1725285

Munk, R., Panda, A. C., Grammatikakis, I., Gorospe, M., & Abdelmohsen, K. (2017). Senescence-Associated MicroRNAs. *International Review of Cell and Molecular Biology*, *334*, 177–205. https://doi.org/10.1016/bs.ircmb.2017.03.008

Nakatsu, R. T. (2021). An Evaluation of Four Resampling Methods Used in Machine Learning Classification. *IEEE Intelligent Systems*, *36*(3), 51–57. https://doi.org/10.1109/MIS.2020.2978066

Ng, T. P., Zhong, X., Gao, Q., Gwee, X., Chua, D. Q. L., & Larbi, A. (2020). Socio-Environmental, Lifestyle, Behavioural, and Psychological Determinants of Biological Ageing:

The Singapore Longitudinal Ageing Study. *Gerontology*, *66*(6), 603–613. https://doi.org/10.1159/000511211

NIAAA, N. I. on A. A. and A. (2023, Oktober 1). *Drinking Levels Defined | National Institute on Alcohol Abuse and Alcoholism (NIAAA)*. https://www.niaaa.nih.gov/alcohol-health/overview-alcohol-consumption/moderate-binge-drinking

Niu, Y., Na, L., Feng, R., Gong, L., Zhao, Y., Li, Q., Li, Y., & Sun, C. (2013). The phytochemical, EGCG, extends lifespan by reducing liver and kidney function damage and improving age-associated inflammation and oxidative stress in healthy rats. Aging Cell, 12(6), 1041–1049. https://doi.org/10.1111/acel.12133

Noren Hooten, N., Abdelmohsen, K., Gorospe, M., Ejiogu, N., Zonderman, A. B., & Evans, M. K. (2010). microRNA expression patterns reveal differential expression of target genes with age. *PloS One*, *5*(5), e10724. https://doi.org/10.1371/journal.pone.0010724

Oberdoerffer, P., & Sinclair, D. A. (2007). The role of nuclear architecture in genomic instability and ageing. *Nature Reviews. Molecular Cell Biology*, *8*(9), 692–702. https://doi.org/10.1038/nrm2238

Olivieri, F., Capri, M., Bonafè, M., Morsiani, C., Jung, H. J., Spazzafumo, L., Viña, J., & Suh, Y. (2017). Circulating miRNAs and miRNA shuttles as biomarkers: Perspective trajectories of healthy and unhealthy aging. *Mechanisms of ageing and development*, *165*(Pt B), 162–170. https://doi.org/10.1016/j.mad.2016.12.004

Olivieri, F., Prattichizzo, F., Giuliani, A., Matacchione, G., Rippo, M. R., Sabbatinelli, J., & Bonafè, M. (2021). miR-21 and miR-146a: The microRNAs of inflammaging and age-related diseases. *Ageing Research Reviews*, *70*, 101374. https://doi.org/10.1016/j.arr.2021.101374

Olivieri, F., Rippo, M. R., Procopio, A. D., & Fazioli, F. (2013). Circulating inflamma-miRs in aging and age-related diseases. *Frontiers in Genetics*, *4*. https://www.frontiersin.org/articles/10.3389/fgene.2013.00121

Onodera, Y., Teramura, T., Takehara, T., Obora, K., Mori, T., & Fukuda, K. (2017). miR-155 induces ROS generation through downregulation of antioxidation-related genes in mesenchymal stem cells. *Aging Cell*, *16*(6), 1369–1380. https://doi.org/10.1111/acel.12680

Partridge, L., Deelen, J., & Slagboom, P. E. (2018). Facing up to the global challenges of ageing. *Nature*, *561*(7721), 45–56. https://doi.org/10.1038/s41586-018-0457-8

Peters, M. J., Joehanes, R., Pilling, L. C., Schurmann, C., Conneely, K. N., Powell, J., Reinmaa, E., Sutphin, G. L., Zhernakova, A., Schramm, K., Wilson, Y. A., Kobes, S., Tukiainen, T., NABEC/UKBEC Consortium, Ramos, Y. F., Göring, H. H. H., Fornage, M., Liu, Y., Gharib, S. A., … Johnson, A. D. (2015). The transcriptional landscape of age in human peripheral blood. *Nature Communications*, *6*, 8570.

https://doi.org/10.1038/ncomms9570

Quach, A., Levine, M. E., Tanaka, T., Lu, A. T., Chen, B. H., Ferrucci, L., Ritz, B., Bandinelli, S., Neuhouser, M. L., Beasley, J. M., Snetselaar, L., Wallace, R. B., Tsao, P. S., Absher, D., Assimes, T. L., Stewart, J. D., Li, Y., Hou, L., Baccarelli, A. A., … Horvath, S. (2017). Epigenetic clock analysis of diet, exercise, education, and lifestyle factors. *Aging*, *9*(2), 419–446. https://doi.org/10.18632/aging.101168

Rivero-Segura, N. A., Bello-Chavolla, O. Y., Barrera-Vázquez, O. S., Gutierrez-Robledo, L. M., & Gomez-Verjan, J. C. (2020). Promising biomarkers of human aging: In search of a multi-omics panel to understand the aging process from a multidimensional perspective. *Ageing Research Reviews*, *64*, 101164. https://doi.org/10.1016/j.arr.2020.101164

Rudnicka, E., Napierała, P., Podfigurna, A., Męczekalski, B., Smolarczyk, R., & Grymowicz, M. (2020). The World Health Organization (WHO) approach to healthy ageing. *Maturitas*, *139*, 6–11. https://doi.org/10.1016/j.maturitas.2020.05.018

Salih, A., Nichols, T., Szabo, L., Petersen, S. E., & Raisi-Estabragh, Z. (2023). Conceptual Overview of Biological Age Estimation. *Aging and Disease*, *14*(3), 583–588. https://doi.org/10.14336/AD.2022.1107

Santos, A. S., Cunha Neto, E., Fukui, R. T., Ferreira, L. R. P., & Silva, M. E. R. (2019). Increased Expression of Circulating microRNA 101-3p in Type 1 Diabetes Patients: New Insights Into miRNA-Regulated Pathophysiological Pathways for Type 1 Diabetes. *Frontiers in Immunology*, *10*, 1637. https://doi.org/10.3389/fimmu.2019.01637

Schlag, K. H. (2008). Exact Tests for Correlation and for the Slope in Simple Linear Regressions Without Making Assumptions. *SSRN Electronic Journal*. https://doi.org/10.2139/ssrn.1162125

Sheedy, F. J. (2015). Turning 21: Induction of miR-21 as a Key Switch in the Inflammatory Response. *Frontiers in Immunology*, *6*. https://doi.org/10.3389/fimmu.2015.00019

Sredni, S. T., Gadd, S., Jafari, N., & Huang, C.-C. (2011). A Parallel Study of mRNA and microRNA Profiling of Peripheral Blood in Young Adult Women. *Frontiers in Genetics*, *2*, 49. https://doi.org/10.3389/fgene.2011.00049

Tacke, F., Spehlmann, M. E., Vucur, M., Benz, F., Luedde, M., Cardenas, D. V., Roy, S., Loosen, S., Hippe, H.-J., Frey, N., Trautwein, C., Koch, A., Roderburg, C., & Luedde, T. (2019). miR-155 Predicts Long-Term Mortality in Critically Ill Patients Younger than 65 Years. *Mediators of Inflammation*, *2019*, 6714080. https://doi.org/10.1155/2019/6714080

Thun, M. J., Peto, R., Lopez, A. D., Monaco, J. H., Henley, S. J., Heath, C. W., & Doll, R. (1997). Alcohol consumption and mortality among middle-aged and elderly U.S. adults. *The New England Journal of Medicine*, *337*(24), 1705–1714.

https://doi.org/10.1056/NEJM199712113372401

Wagner, K.-H., Cameron-Smith, D., Wessner, B., & Franzke, B. (2016). Biomarkers of Aging: From Function to Molecular Biology. *Nutrients*, *8*(6), 338. https://doi.org/10.3390/nu8060338

Wang, X., Li, D., Liu, F., Cui, Y., & Li, X. (2020). Dietary citrus and/or its extracts intake contributed to weight control: Evidence from a systematic review and meta-analysis of 13 randomized clinical trials. *Phytotherapy Research: PTR*, *34*(8), 2006–2022. https://doi.org/10.1002/ptr.6673

WHO, W. H. O. (2023, Januar 4). *No level of alcohol consumption is safe for our health*. https://www.who.int/europe/news/item/04-01-2023-no-level-of-alcohol-consumption-is-safe-for-our-health

Xiao, Y., Li, B., & Liu, J. (2018). miRNA-27a regulates arthritis via PPARγ in vivo and in vitro. *Molecular Medicine Reports*, *17*(4), 5454–5462. https://doi.org/10.3892/mmr.2018.8531

Xiong, L.-G., Chen, Y.-J., Tong, J.-W., Gong, Y.-S., Huang, J.-A., & Liu, Z.-H. (2017). Epigallocatechin-3-gallate promotes healthy lifespan through mitohormesis during early-to-mid adulthood in Caenorhabditis elegans. *Redox Biology*, *14*, 305–315. https://doi.org/10.1016/j.redox.2017.09.019

Zannas, A. S. (2016). Editorial Perspective: Psychological stress and epigenetic aging - what can we learn and how can we prevent? *Journal of Child Psychology and Psychiatry, and Allied Disciplines*, *57*(6), 674–675. https://doi.org/10.1111/jcpp.12535

Zannas, A. S., Arloth, J., Carrillo-Roa, T., Iurato, S., Röh, S., Ressler, K. J., Nemeroff, C. B., Smith, A. K., Bradley, B., Heim, C., Menke, A., Lange, J. F., Brückl, T., Ising, M., Wray, N. R., Erhardt, A., Binder, E. B., & Mehta, D. (2015). Lifetime stress accelerates epigenetic aging in an urban, African American cohort: Relevance of glucocorticoid signaling. *Genome Biology*, *16*, 266. https://doi.org/10.1186/s13059-015-0828-5

Zhang, Q., Vallerga, C. L., Walker, R. M., Lin, T., Henders, A. K., Montgomery, G. W., He, J., Fan, D., Fowdar, J., Kennedy, M., Pitcher, T., Pearson, J., Halliday, G., Kwok, J. B., Hickie, I., Lewis, S., Anderson, T., Silburn, P. A., Mellick, G. D., … Visscher, P. M. (2019). Improved precision of epigenetic clock estimates across tissues and its implication for biological ageing. *Genome Medicine*, *11*(1), 54. https://doi.org/10.1186/s13073-019-0667-1

Zhu, X., Wu, L., Mo, X., Xia, W., Guo, Y., Wang, M., Zeng, K., Wu, J., Qiu, Y., Lin, X., Lu, X., Deng, F., & Lei, S. (2019). Identification of PBMC-expressed miRNAs for rheumatoid arthritis. *Epigenetics*, *15*(4), 386–397. https://doi.org/10.1080/15592294.2019.1676613