T.C.
CUKUROVA UNIVERSITY
INSTITUTE OF ADDICTION AND FORENSIC SCIENCES
DEPARTMENT OF FORENSIC SCIENCE

# A BIOMARKER FOR AGE ESTIMATION IN FORENSIC MEDICINE: ANALYSIS of AGE AND SEX-DEPENDENT miR-766-3p EXPRESSION LEVELS IN SALIVA

**ZENAB BINTOU NSOUNMFON**

**DEPARTMENT OF FORENSIC SCIENCES MASTER'S PROGRAM**

**MASTER'S THESIS**

**ADVISOR**

Prof. Dr. Mehmet Bertan YILMAZ

**ADANA-2024**

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This thesis has been prepared by Cukurova University Scientific Research Projects Unit. It was supported as the project numbered TYL Thesis No

ADANA-2024

**ACCEPTANCE AND APPROVAL**

Department of Forensic Sciences

carried out within the framework of the master's Program with a Thesis. The study titled

**" A BIOMARKER FOR AGE ESTIMATION IN FORENSIC MEDICINE: ANALYSIS OF AGE and SEX-DEPENDENT MiR-766-3p EXPRESSION LEVELS IN SALIVA"**

was accepted by the following jury as the Final Thesis Defense.

Date: 05/ 07 / 2024

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## ACKNOWLEDGEMENTS

I would like to thank God Almighty, for his faithful guidance, protection and grace upon me during this research project.

I extend my sincerest gratitude to my supervisor Prof. Dr. Mehmet Bertan Yilmaz, who made this work possible. His guidance and disposition have been instrumental in the execution of this study. My warmest thanks to Dr. Nermin Seda Ilgaz for her assistance throughout the research process. My thanks to my laboratory mates, Buket Kalfa and Mehmet Önsan Kütukoglu for their support, open-mindedness and camaraderie.

To late Prof.Dr.Mete Korkut Gülmen , Founding Director of Addiction and Forensic Science Institute, I extend my gratitude for his endeavors towards the adhesion and integration of international students to the institute. I am grateful to Dr. Ayşe Serin, vice Director, of the institute for her support from the start to the completion of my master's program. My regards to Prof.Dr. Hülya Yükseloğlu for her availability and support.

To my family and friends, I extend my profound gratitude for your unconditional support, love, patience and prayers. Thank you, Baba for being my strength

**~ ZENAB BINTOU NSOUNMFON**

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**ABBREVIATIONS**

| | |
|---|---|
| SIRT6: | Sirtuin 6 |
| miRNA: | Micro ribonucleic acid |
| RNA: | Ribonucleic acid |
| DNA: | Deoxyribonucleic acid |
| IT: | Information Technology |
| PCR: | Polymerase Chain Reaction |
| mRNA: | Messenger RNA |
| MRI: | Magnetic Resonance Imaging |
| NGS: | Next Generation Sequencing |
| DVI: | Disaster Victim Identification |
| NAD+: | Nicotinamide adenine dinucleotide |
| ADP: | Adenosine diphosphate |
| RISC: | RNA-induced silencing complex |
| miRDB: | MicroRNA database |
| SEPT6: | Septin6 |
| SALIgAE® test: | Saliva Identification Test Kit - Abacus diagnostics |
| RSID™: | Rapid Stain Identification of Human Saliva |
| ml: | Milliliter |
| DNases: | Deoxyribonuclease |
| RNAases: | Ribonuclease |
| RPM: | Rounds per Minute |
| µl: | Microliter |
| RT: | Reverse Transcriptase |
| cDNA: | Complementary DNA |
| rnu6: | U6 small nuclear 1 |
| DTT: | Dithiothreitol |
| dNTPs: | Deoxynucleotide Triphosphates |

| | |
|---|---|
| qPCR: | Quantitative Polymerase Chain Reaction |
| RT-PCR: | Real Time Polymerase Chain Reaction |
| BMI: | Body Mass Index |

## ÖZET

**Adli Tıpta Yaş Tahminine Yönelik Bir Biyobelirteç: MiR-766- Tükürükte Yaşa ve Cinsiyete Bağlı Ekspresyon Düzeylerinin Analizi**

**Rutin adli vakalarda ilgili kişinin yaşını belirlemek soruşturma için çok önemli olabilir, ancak bu işlem hâlâ kolay değildir. Adli kapsamın ötesinde, yaş tahmin araçları göç, antropoloji ve afet mağdurlarını tanımlama programları da dahil olmak üzere diğer birçok alan için de faydalı olabilir. Etkili bir yaş tahmin modeli oluşturmak amacıyla yaşlanmanın karmaşık temellerini anlamak bize çeşitli ipuçları verebilir. Başlangıçta histon deasetilasyonundaki rolleriyle bilinen sirtuinler (SIRT), yaşlanma ve yaşa bağlı hastalıklardaki rolleri nedeniyle giderek artan ilginin merkezi olmuştur. SIRT6 yaşlanmayla birlikte bir azalma gösterir. "SIRT6 ifadesini hangi faktörler etkiler?" sorusunun cevabı yaşlanmayla ilgili karmaşık ilişkileri açıklamaya çalışırken uygulanacak olan yaş tahmin modellerinin tasarlanmasında da faydalı olacaktır. Gen susturmadaki rolleriyle bilinen mikroRNA'lar bu sınıfta yer alan bir faktördür. MiR-766-3p, bir geri bildirim düzenleyici döngü aracılığıyla SIRT6'yı hedefler. Farklı yaş gruplarındaki tükürük örneklerinde miR-766-3p'nin ekspresyon modellerinin analizi, miRNA-yaşla ilgili devrelere ilişkin daha fazla bilgi verebilir ve yaş tahmin araçları için aday bir biyobelirteç olarak potansiyelini belirleyebilir. Sonuçlarımız kadın ve erkeklerin ifade kalıplarında farklılık olduğunu göstermektedir. Kadınlarda yukarı düzenleme görülürken, erkeklerde yaşla birlikte azalma görülüyor.**

## SUMMARY

**A Biomarker for Age Estimation in Forensic Medicine: Analysis of Age and Sex-Dependent Expression Levels of MiR-766-3p in Saliva**

Determining a person of interest's age in routine forensic cases can be a pivotal lead to an investigation, however, this task remains challenging. Beyond the forensic scope, age prediction tools could be an asset to several other fields including immigration, anthropology and disaster victim identification programs. Several leads have been explored to understand the complex underpinnings of ageing to create an effective prediction model. Initially known for their role in histone deacetylation, sirtuins (*SIRT*s) have received growing attention, regarding their role in ageing and age-related diseases as *SIRT6* shows a downregulation with ageing. The question of "What factors affect *SIRT6* expression?" would, therefore, be valid, when trying to elucidate the complex network involved in ageing and when designing age prediction models. MicroRNAs known for their role in gene silencing are one such factor. *MiR-766-3p* targets the *SIRT6* via a feedback regulatory loop. The analysis of the expression patterns of *miR-766-3p* in the saliva samples of different age groups may give more insight into miRNA-age-related circuitry and determine its potential as a candidate biomarker for age prediction tools. Our results show a difference in the expression patterns of women and men. Where women show an upregulation, men show a downregulation with age.



**Keywords**: Aging, Senescence, Biomarker, Saliva, SIRT6, MiR-766-3p, Chronological age estimation, Forensic Sciences, RNA extraction, RT-PCR; Real-Time,

Polymerase Chain Reaction.

# CHAPTER I

## 1. Introduction

Forensic science applies physical and natural science methodologies to civil and criminal law matters. In forensic science, crimes such as murder and rape can be investigated and prosecuted as well as civil wrongs such as willful air or water pollution. Forensic science comprises various disciplines with a common set of challenges, ensuring reliable results, and communicating accurate and understandable findings to non-experts and jury amongst other things. Forensic scientists must collect and examine data from the crime scene to assist courts in their decision-making [Upadhyay et al. (1)].

### 1.1. Significance of crime scene

The place of occurrence of a crime has all the physical evidence and is of utmost importance. Forensic scientists meticulously collect and analyze the evidence to establish a link to the crime. The nature of the samples determines the type of scientific technique used, hence the various forensic sub-disciplines. The primary branches include forensic biology and DNA, chemistry or instrumental analysis, physics or pattern analysis, crime scene or death investigation, and information technology multimedia. Each discipline branches into an array of subdisciplines [Verma et al. (2)].

### 1.2. Forensic Biology and DNA

Forensic biology involves the analysis of biological material collected from crime scenes for identification and individualization purposes. The extraction and purification of DNA from biological evidence involves the use of different scientific protocols such as polymerase chain reaction (PCR), DNA sequencing etc. Biological evidence comprises blood, saliva, tissue, hair, bone, and other bodily fluids. Once analyzed the generated DNA profiles are compared against forensic databases to identify individuals or determine the absence or presence of relatedness amongst individuals [Bukyya et al. (3)].

### 1.3. Biomarkers

Biomarkers can be categorized in various ways. They could be classified into two broad groups which are biomarkers of exposure or biomarkers of disease. In molecular biology and genetics, they are usually classified as type 0 or natural history markers, type 1, also known as drug activity markers and type 2 or surrogate-end points markers. Another classification divides them into monitoring, diagnostic, pharmacodynamic, predictive, prognostic or risk biomarkers. Their applications range from general applications such as scientific concept advancement to specific uses in clinical practices or research perspectives such as treatment tracking or drug development. Biomarkers can be detected using various methods ranging from blood tests to magnetic resonance imaging (MRI), each of which may affect the downstream usability of the biomarkers [Tandon (5)].

### 1.4. Forensic biomarkers

Biomarkers can be applied in many ways in forensic science to provide vital investigation leads. Biomarkers can be used to prove or disprove a person's involvement in a crime. They can be used to identify an individual and place them at a crime scene. Furthermore, they are used in estimating postmortem intervals, wound age, and certain traits such as sex or age.

### 1.5. Age Determination in Forensic Sciences

The accurate determination of a suspect or victim's age can be a vital investigation lead. However, in practice, this remains a challenging task. Several prediction models have been suggested throughout the years. With the advent of next-genome sequencing (NGS) and bioinformatic tools, novel biomarkers are investigated more extensively to find the most reliable and practical ones. Some of the most investigated biomarkers include telomere shortening, DNA methylation (DNAm) patterns, mRNA, miRNA, and protein expression levels [Wang et al. (6)].

# CHAPTER II

## 2. Literature Review

The complex pathways and processes involved in ageing, have been extensively studied. There is a lot left to elucidate, however, research carried out so far gives more insight into the interplay between markers and genes and the role they play in ageing. One such interaction has been demonstrated in earlier studies between *micro-RNA 766-3p* and one of its target genes sirtuin6, a well-known age-sex-related gene. Since sirtuin6's implication in age is established, investigating factors that affect its expression such as *miR-766-3p* could be of significant relevance to age prediction studies.

### 2.1. Ageing and senescence:

Ageing is mainly characterized by a steady decline in function and is a predominant risk factor for many diseases, including neurodegenerative disorders, cardiovascular diseases, cancer, and diabetes. Senescence is one of the main hallmarks of ageing and is defined as growth arrest in response to cellular stress and damage [Di Micco et al. (10)], [Zhang et al. (7)]. The cell, which nonetheless retains its metabolic activity, can damage adjacent cells through the release of toxins. Over time, the accumulation of senescent cells constitutes a risk factor for the diseases mentioned above [Ruchi and Parmjit (8)]. A person's age is often evaluated by chronological age, which refers to the lapse between birth and a specific point in time. Although biological age can be implied from chronological age, age-dependent biological changes occur at different rates among individuals. Therefore, forensic age estimation usually refers to chronological rather than biological age. In the last decade, more research has been geared towards finding more reliable chronological age prediction models that can be applied within the forensic medicine scope and beyond [Refn et al. (9)].

### 2.2. Applications of age determination:

Forensic medicine and sciences encompass many disciplines and heavily rely on technology to carry out investigations [ Ramazan et al. (10)]. A key parameter in forensic investigation is age determination [Ellingham and Adserias-Garriga (11)], especially in cases where evidence collected from the crime scene do not match any of the suspects' DNA profiles from the crime DNA database. Narrowing the age could help infer age-related physiological features, such as baldness, that could further investigative leads [Refn et al. (9)]. Immigration-related cases equally rely on applying age prediction techniques to differentiate between legal age thresholds. In such cases, an individual's age must be correctly determined to apply the appropriate legal measures [Naue (12)], [Hagen et al. (13)]. Age estimation is also employed in anthropological investigations. Typically, the identification of human skeletal remains relies on key features like the person's age at the time of death. Furthermore, age predictions from biological samples can be used in disaster victim identification (DVI) as a screening tool to come up with a potential missing persons' list. Lastly, direct-to-consumer companies involved in investigative genetic genealogy may use the genetic information in their possession to search for a potential perpetrator's distant relative via DNA profiles from crime scenes. Predicting a person's age could help determine what generation should be investigated to find the perpetrator among distant relatives. [Freire- (Aradas et al. (14)], [Bjelopavlovic et al. (15)].

### 2.3. Past and current forensic age estimation methods:

In the past, forensic age estimations relied on osteological, stomatological, and radiographical techniques [Ashifa (16)]. However, with the advent of innovative techniques, there has been a significant shift towards more molecular-based approaches, marking a notable evolution in forensic science [Ambroa-Conde et al. (17)], [Aliferi et al. (18)]. Methodologies that examine skeletal or dental evidence have limited applicability, considering the odds of finding biological material with morphological age features at crime scenes. Furthermore, in asylum cases, for instance, ethical issues may arise regarding the adverse effects of X-radiation when used on minors. In contrast, molecular methodologies offer distinct advantages as they can, in theory, analyze trace amounts of biological material more commonly found at crime scenes.

Over the years, molecular approaches involving DNA, RNA, and protein-based assays have been proposed. Scientists, however, focus on methodologies involving blood DNA analyses since blood is the most common material at crime scenes, and DNA remains relatively stable even in dried samples. First proposed in 2014 by Weidner et al, DNA-methylation-based assays are one of the most prevalent age-prediction models, mainly because DNA methylation is one of the most studied and well-understood DNA markers. However, the high DNA input amount and the bi-sulfate conversion efficiency challenge the robustness of these assays. These models rely on high throughput techniques, require bisulfate conversions and PCR amplification. Bisufate treatments highly degrade DNA that is only available in trace amounts [Aliferi et al. (18)], [Thangaraj et al. (20)], [Maulani and Auerkari (21)]. These setbacks thereby prompt further research to find reliable biomarkers from different tissues that may apply to routine forensic cases. The research described in this thesis aimed to analyze the expression levels of *micro-RNA 766-3p* mediated by age-dependent *SIRT6* genes in saliva samples from different age groups to determine the potential of *micro-RNA 766-3p* as a candidate biomarker for age-prediction models applicable in forensic cases.

**2.4. SIRTUIN 6:**

In mammals, sirtuins are a family of 7(*SIRT 1-7*) nicotinamide dinucleotides (NAD+)-dependent histone deacetylases with remarkable properties in disease prevention and delay of cellular senescence through complex cellular processes. *SIRT6* mainly, is an ADP-ribosyl transferase deacetylase found in chromatin, involved in lifespan extension, DNA repair, genome stability, and telomere maintenance [Lee et al. (23), Zhao et al. (24)]. The expression of *SIRT6* is upregulated in younger subjects while downregulated in older ones [Owczarz et al. (25), Dzidek (26)]. Given the known importance and correlation between age and *SIRT6*, it would be noteworthy to investigate the factors that affect the expression of *SIRT6* directly or indirectly to gain more insight into the complex networks modulating ageing, to identify reliable age prediction markers. One such factor is micro RNAs (miRNAs), crucial in post-transcriptional regulation.

**2.5. Micro RNA 766-3p:**

MiRNAs are small non-coding RNAs of approximately 18-24 nucleotides. They play a role as post-transcriptional modulators of gene expression and are less prone to environmental decay than longer strands of RNA. [Hayakawa et al. (27)], [li and Yu (28)], [Ergin and Çentinkaya (29)]. They are processed in the nucleus and the cytoplasm by DROSHA and DICER endonucleases, respectively. The resulting mature miRNA transcripts are then formed and loaded into the RNA-induced silencing complex (RISC) to carry out the specific biological functions [ Li and Yu (18)], [Ergin and Çentinkaya (29)]. Generally, the complex is involved in negative post-transcriptional regulations, such as the downregulation of genes [Rani and Sengar (30)], [Michlewski et al. (31)], [Leitão and Enguita(32)]. Online prediction repositories can be used to predict the type and number of miRNAs targeting a specific gene. The miRDB (2020) database used in this study showed that *hsa-miR-766-3p* (previously known as *miR-766*) had the highest target score value (89) for *SIRT6*. In earlier studies, Armit et al. discussed *miR-766-3P*-mediated post-transcriptional regulations of *SIRT6* genes in human induced pluripotent stem cells, thus elucidating a possible feedback regulatory loop. *MiR-766-3p* are intronic miRNAs transcribed as part of a larger transcript, including that of *SEPTIN6* mRNA (previously known as *SEPT6*) [Gupta et al. (33)]. In older subjects, *SEPTIN6* and *miR-766-3p* are upregulated and acetylation increases in *SEPTIN6* upstream regions. Conversely, in younger individuals, histone residues in promoter regions of targeted genes are deacetylated by *SIRT6*. This indicates there is a loop mechanism between the *SIRT6* gene and *miR-766-3p*. [Dzidek (16)], [Fiorentino et al. (34)]. *SIRT6* plays an essential role in the expression of *miR-766-3p* by modulating acetylation at *SEPTIN6* promoter regions. The high expression levels of *SIRT6* in younger subjects increase the deacetylation of *SEPTIN6* promoter regions and several others, leading to *miR-766-3p* downregulation. However, in older subjects, *SIRT6* expression levels are lower, leading to increased acetylation at the 9th lysine residue of the histone H3 protein (H3K9) and elevated *miR-766-3p* levels. The increased micro-RNAses bind to the 3'untranslated *SIRT6* region resulting in the downregulation of *SIRT6* expression [Chang et al. (35)].

**2.6. Saliva:**

Gene expression levels are often analyzed from blood samples since it is the most common biological evidence at crime scenes. Saliva is nonetheless very common, and in suicide cases, it is more common to encounter saliva than blood. Usually found in nibble marks, saliva can also be found in pool or stained forms. Saliva identification may be challenging due to its transparency. However, this key feature is saliva's main advantage over blood. In an attempt to cover their tracks, it is common for criminals to dispose of any visible evidence, such as blood. Saliva is a clear liquid, so the likelihood of it being tampered with is low. Other perks include non-invasive evidence collection, easy access, and safer handling of material [Chatterjee (36)], [Gandhi et al. (37)]. There is also a lower contamination risk for saliva, especially for transmittable diseases like hepatitis [Upadhyay et al. (1)].

**2.7. Saliva detection methods.**

Saliva collection is often based on two techniques depending on the surface from which it is collected. The single-swab method involves rolling a wet sterile cotton swab over the collection site. The double swabbing technique consists of an initial wet swab, followed by a dry swab. The first technique is used on non-absorbent surfaces such as glasses and plastic evidence. The second technique is used for absorbent surfaces such as cloth, skin, or half-eaten food items. After collection, preliminary and advanced screening can be carried out. These tests can be destructive or non-destructive.

Preliminary or presumptive tests are based on salivary-specific antibody activity, salivary fluorescent activity, and salivary alpha-amylase activity. Salivary amylase, however, can also be found in other body fluids such as sweat and breast milk hence, making these tests non-specific. Presumptive tests include polilight, phadebas, SALIgAE and immunochromatographic strip tests. Polilight shows a specific-coloured light that cannot be read on a bright background and mimics fluorescence emitted from other body fluids. However, this method is low-cost and portable. Phadebas and SALIgAE rely on the alpha-amylase activity of saliva. They are simple to conduct but are neither time efficient nor economical and cross-reactivity is common.

Immunochromatographic kits are based on monoclonal antibody detection. They are the most sensitive but typically give false negative results when used for other body fluids.

Advanced screenings confirm the identity of the saliva on the substrate for further downstream processes. They include immunological techniques, microscopy techniques, RNA profiling, DNA methylation, spectroscopy techniques and microbial detection. RNA profiling is costly, time-consuming and highly unstable. With single processing, DNA methylation protocol can be used to identify individuals and body fluids. Amongst these methodologies, next-generation (NexGen) sequencing is a better approach but remains costly, and unavailable in many places. Spectroscopy techniques on the other hand are robust and non-destructive. However, the multiple spectra of a single body fluid remain its biggest demerit. Microbial profiling falls under forensic microbiology and is a quite recent advancement. It is expensive and requires skills. Nonetheless, higher microbial survival rate in the environment than that of protein and nucleic acids constitute a main advantage. Detection of saliva provides quantitative and qualitative information. After identification, saliva becomes a major DNA source further processed for DNA profiling. Destructive methods such as phadebas, SALIgAe, RSID, immunological tests, RNA profiling, DNA methylation, and spectrometry, consume the sample reducing the amount of DNA left for downstream analyses [Upadhyay et al. (1)].

The significance of determining chronological age in forensic medicine underscores the need to investigate reliable biomarkers for age prediction models. This research aimed to investigate the regulation of age-related *SIRT6* gene by *miR-766-3p*, thus determining the potential of the latter as a candidate biomarker for chronological age prediction. In addition to that, highlighting *the* advantages of using saliva as forensic biological material.

# CHAPTER III

## 3. Materials and Methods

The following materials and methodologies were used when carrying this study.

### 3.1. Materials

- A total of 40 healthy volunteer participants including 20 males and 20 females
- All study participants signed a consent form approved by the University.
- The mean age of the volunteers was 4-9, 14-19, 24-29, 30+ age groups.
- NO history of malignancy, autoimmune disorders, metabolic disorders,
- NO history of infectious diseases was reported in the past 6 months.

### 3.2. Salivary sample collection

- Participants were asked not to eat or drink for at least an hour before sample collection.
- Participants were asked to rinse their mouth before saliva collection.
- Unstimulated whole saliva was collected in 50mL sterile DNase- and RNase-free tubes via the passive drooling method.
- All samples were frozen on dry ice for up to 2 hours before RNA preparation.

### 3.3. Sample pre-processing

- Frozen salivary samples were thawed at room temperature.
- A water bath was not used to expedite thawing, as it may have degraded the RNA and compromised quality.
- Samples were divided into 1mL aliquots in 1.7mL sterile DNase- and RNase-free Denville Posi-Click Tubes
- Centrifuged at 13400 RPM and 4°C for 20 minutes.

### 3.4. RNA extraction protocol

In this study, we used the protocol-High-Quality and High-Yield RNA Extraction Method from Whole Human Saliva-Biomarker Insights Volume 15: 1–9 DOI: 10.1177/1177271920929705)

All procedures were performed in an RNase-free environment assigned for RNA isolation using the modified TRIzol.

### 3.5. Modified TRIzol and RNA Isolation Protocol

- 1 ml of TRIzol reagent was added to each pellet, pipetted several times, and vortexed for 20 seconds to homogenize.
- Samples were incubated at room temperature for 5 minutes.
- 200µL of chloroform was added to each tube and vortexed for 20 seconds.
- Samples were incubated at room temperature for 3 to 5 minutes.
- The samples were then centrifuged at 13400 RPM for 20 minutes at 4°C.
- 700µL of the upper aqueous layer was transferred from each sample into a new 1.7-mL tube.
- These chloroform steps were repeated twice, and for each consecutive time, less amount was used.
- 600µL first, and 500µL of the upper aqueous layer the second time were carefully transferred to the new tubes.
- 500µL of cold isopropyl alcohol was added to each tube, and vortexed for a few seconds.
- The tubes were incubated at −20°C for at least 30 minutes to assure precipitation of RNA.
- The samples were then centrifuged for 20 minutes at 1°C at 13400 RPM.
- The supernatant was removed.
- The pellet was washed with 1mL of cold 80% molecular-grade ethanol and centrifuged at 13400 RPM for 5 minutes at 1°C.
- This step was repeated. Samples were then briefly centrifuged and excess ethanol was removed using a pipette.

- The pellet was air-dried at room temperature for at least 5 minutes.
- Finally, samples were resuspended in 20µL of DNase- and RNase-free water.

### 3.6. FIRE Script RT cDNA Synthesis KIT

**Table 3.1.** Reverse Transcriptase Set-up

| **Component** | **Volume** |
|---|---|
| Template RNA | 5 µl |
| gene specific primer (rnu6) | 1 µl |
| gene specific primer (mir766) | 1 µl |
| 10x RT Reaction Buffer with DTT | 2 µl |
| dNTP MIX (20 mM of each) | 0.5 µl |
| FIREScript RT | 1 µl |
| dH2O | 9,5 µl |
| Total | 20 µl |
| | |

**Table 3.2.** Reverse cDNA Transcriptase Thermo-cycler Protocol

| **Step** | **Temperature** | **Time** |
|---|---|---|
| Primer annealing | 25$^{o}$C | 5-10 min |
| Reverse transcription | 37-60°C | 15-30 min |
| Enzyme inactivation | 85°C | 5 min |

### 3.7. Real-time PCR amplification and detection

ID3EAL miRNA qPCR ASSAY

Important: keep all reagents on ice (or at 4ºC)at all times during set-up

- Gently thaw cDNA, IDEAL miRNA qPCR Master Mix (2x)
- And IDEAL miRNA qPCR assays (10x) on ice. Mix by vortexing and spin down by centrifugation.
- Dilute cDNA from step 5 by 1: 10 in nuclease-free water. Pipette diluted cDNA (important to keep PCR plate cool on the cold block before loading qPCR master mix!)
- Assemble qPCR reaction according to Table 3.3.

**Table 3.3**. Real-Time PCR (RT-PCR) Reaction Setup (per reaction)

| Reagent | Volume/µl (96 well) | Volume/µl (384 well) |
|---|---|---|
| IDEAL qPCR Master Mix (2x) | 10 | 5 |
| Nuclease-free Water | 3 | 1 |
| IDEAL qPCR Assays (10x) | 2 | 1 |
| Diluted cDNA | 5 | 3 |
| Total Volume | 20 | 10 |

- Centriguge PCR briefly (30s at 200g).
- Perform real-time PCR amplification with the following parameters.

**Table 3.4**. Real-time PCR thermocycling protocol

| Cycle | Temperature | Time | Notes |
|---|---|---|---|
| 1x | 95 ºC | 10min | Polymerase Activation |
| | 40 ºC | 5min | |
| 40x | 95 ºC | 10s | Denaturation |
| | 60 ºC | 30s | Annealing/extension (acquire fluorescence reading at end of step.) |

# CHAPTER IV

## 4. Results

The results of this study were summarized into three bar charts representing the general findings of women and men, women only and men only respectively.

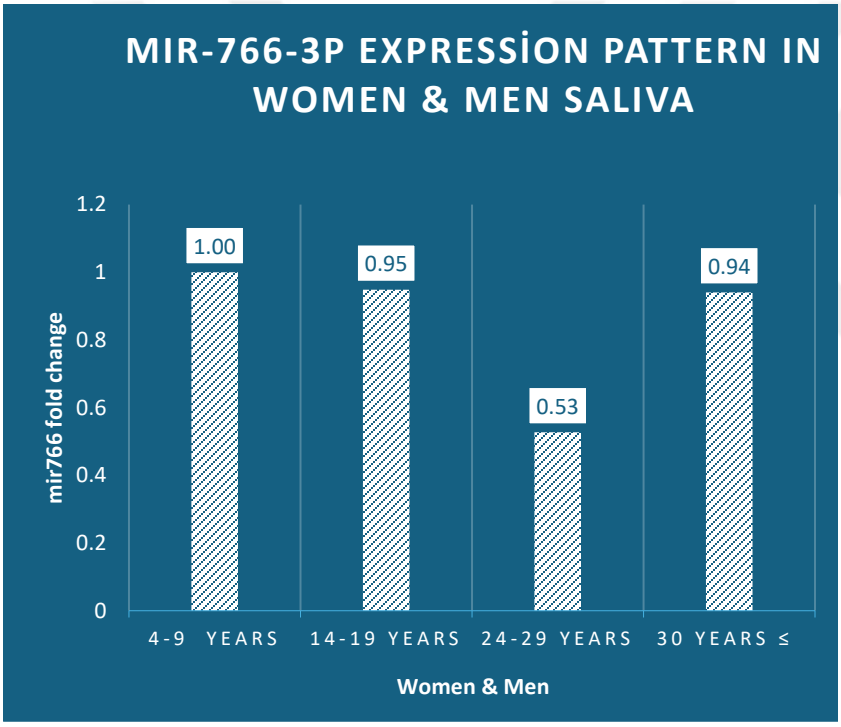


**Figure 4.A. Expression Pattern in Women and Men**

### 4.1. General results (Women & Men)

With the 4-9 age group as the control group, results show a slight decrease in expression levels of *miR-766-3p* in age groups 14-19 and ≥30 to approximately 0.95 and 0.94 respectively. For the age group 24-29, a 1-fold decrease is observed.

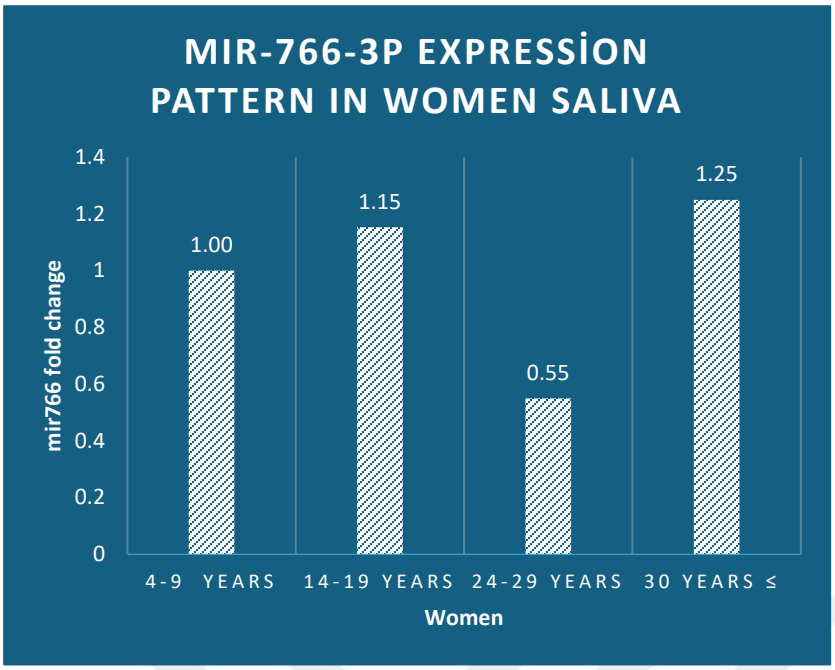


**Figure 4.B. Expression Pattern in Women**

### 4.2. Women results

With the 4-9 age group as control, results show an increase in expression to approximately 1.15 and 1.25 for age groups 14-19 and ≥30 respectively. However, approximately, 1-fold decrease is observed for the age group 24-29.

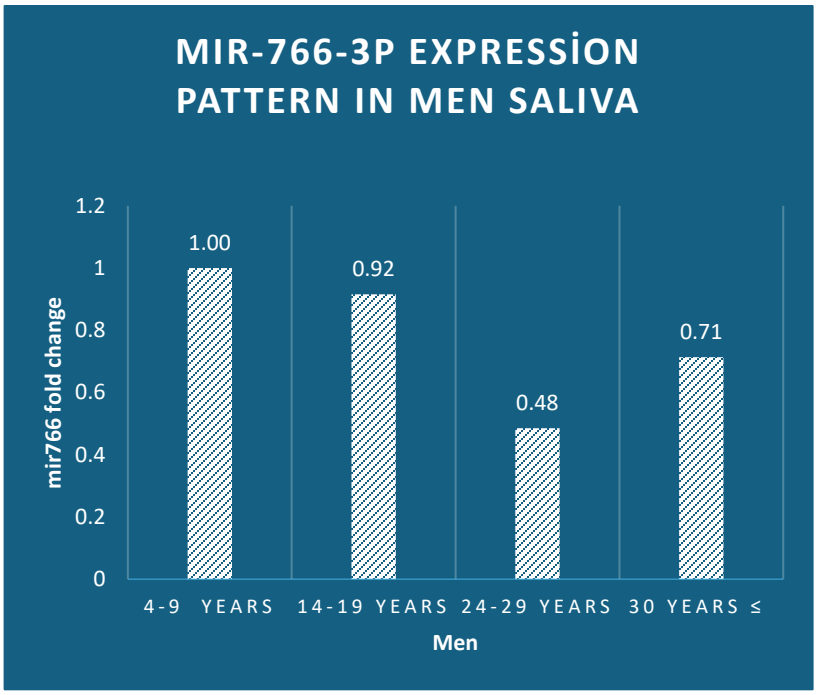


**Figure 4.C. Expression Pattern in Men**

### 4.3. Men results

With the 4-9 age group as control, results show a slight decrease in expression to 0.92 and 0.71 approximately for groups 14-19 and for ≥30 respectively. For the age group 24-29, a 1-fold decrease is observed.

## CHAPTER V

### 5. Discussion

Several studies have shown the pivotal role of *SIRT6* in modulation pathways and gene expression relevant to ageing. The expression levels of *SIRT6* tend to be higher in younger subjects while they decrease in older subjects. A lot more research is needed to elucidate the molecular mechanisms involved. Nonetheless, recognizing the vital role of microRNA in post-transcriptional regulation gives more insight. Target genes and microRNA interactions are complex, involving positive and negative feedback mechanisms. *SIRT6/miR-766-3p* feedback regulation was first discussed by Sharma et al. [38], thus prompting further investigations to determine the potential of microRNAs as candidate biomarkers for age prediction protocols.

In younger subjects, higher *SIRT6* expression ensures deacetylation at several promoter regions including, the *SEPT6/miR-766-3p* promoter. This results in a decrease in the expression of the micro-RNA. In older subjects, however, lower *SIRT6* expression levels likely result in higher acetylation and *miR-766-3p levels*, which leads to a further decrease in expression levels of *SIRT6*.

In our study, however, the results show a few discrepancies from the expected patterns. The regulatory effects of several factors could explain the discrepancies observed in miRNA expression levels. These factors include.

- **Sex-specific differences**

Despite the increasing number of studies about the influence of sex on miRNA expression, the molecular and functional consequences are mostly unexplored. However, salivary gland-specific

sexual dimorphism has been observed in several studies via transcriptomic analyses. A better understanding of molecular pathways may identify new miRNA and their gene targets [Mukaibo et al. (39)], [Szakats et al. (40)]. From a sex perspective, the modulation of miRNA expression is influenced by the regulation of miRNA transcription and processing by estrogen and the presence of microRNA on the X-chromosome. MiRNA expression is sex-specific, leading to health biases in both sexes. This underscores the importance of considering biological sex when analyzing miRNA patterns [Veryaskina et al. (41)].

- **Tissue-specificity considerations**

MiRNA expression relies upon important determinants such as chromosomal location and genomic distribution of the gene. Sensitivity being a major factor in tissue-specific analyses, further research to explore the expression thresholds and specificity of *miR-766-3p* to salivary tissue may shed light on expression variations observed.

In their study, Ludwig et al [42]. measured the miRNA profiles for 61 tissues from several organs. More than 1000 unique miRNAs were expressed in each tissue of which 143, were common to all studied tissues. This underscores the potential of miRNA as a biomarker and helps elucidate one more factor responsible for the heterogeneity in miRNA patterns. [Veryaskina et al. (41)].

- **Molecular mechanism**

MicroRNA expressions can be regulated at different levels. At the transcriptional and post-transcriptional levels, a series of factors may intervene to change the expression. Regulations at the transcriptional level include changes in the host gene expression levels and hypermethylation of host promoter or miRNA genes. While post-transcriptional regulation affects the processing and stability of miRNA. The presence of genetic variants or aberrations at any of these levels adds to the molecular complexity since these variants may impact functioning and therefore expression [Machowska et al. (43)]. Furthermore, endogenous and exogenous compounds such as hormones and xenobiotics respectively, may alter the expression of microRNA. This multilevel complex regulation should be reviewed taking into consideration internal factors such as cell type, physiological state, and several other external factors. The

layers of complexity and the interplay between these factors certainly add to the challenge of deciphering the causes of miRNA expression variations [Syeda et al. (44)], [Mohanty et al (45)].

- **Normalization approaches/strategies**

The lack of standardization in pre-analytical as well as post-analytical procedures remains a limitation in the use of circular miRNA as biomarkers. Other than the known challenges of low starting sample, sensitivity and specificity, other technical variables specific to the experimental phase such as collection and storage protocols, miRNA isolation and transcription efficiency, greatly impact the results. Furthermore, analytical output is strongly influenced by normalization methods. Several studies have shown how different normalization methods strongly influence the experimental outcome and therefore could be leading factors in expression variations. [Faraldi et al. (46)]. Besides technical factors, the interpretation of qPCR data is biased by the choice of reference genes; one with the least variation in expression between the compared groups and a stable expression despite isolation and storage protocols is ideal [Veryaskina et al. (41)].

- **Dietary and lifestyle modulators**

Increasing evidence establishes that miRNA expression may be influenced by nutritional and lifestyle factors [Pointner et al. (47)]. In several studies, miRNA responses to various food interventions are measured. Most have varying results which may be accounted for by, the amount of food, nutrient composition, and length of the intervention period. Nonetheless, most studies acknowledge the modulation of miRNA expression by a variety of dietary interventions and the increase in intensity of microRNA response in metabolically healthy individuals. More research is needed to identify diet-related miRNA profiles for instance food items involved in the modulation of microRNA expression within the same tissue in a similar population [Chodur and Steinberg (48)].

In a lot of studies, physical activity and body mass index (BMI) are confounding variables of dietary factors when categorizing participants into clusters. Several compare expression levels amongst sedentary, acutely active and chronically active subjects. Some discrepancies can be noticed across the studies, probably introduced by experimental parameters such as type,

duration, intensity of exercise etc. However, it should be noted that some relevant miRNAs correlate with physical activity parameters whether upregulated or downregulated. Despite the heterogeneity in the studies, a change in expression is noted across most studies, underscoring the association between exercise and miRNA expression.

Similar studies have been conducted for smoking, Wang et al (6) note the transient dysregulation of some miRNA expressions in smokers after they stopped smoking while other miRNAs persist in altered expression profiles [Panico et al. (50)]. Lifestyle encompasses a variety of parameters including stress, drug consumption etc., all of which must be investigated to determine any correlation with gene expression.

- **MiRNA sources:**

A few studies report that miRNA can be passed from one species to another. This epigenetic cross-kingdom/ species regulation may be introduced by dietary sources such as crop plants, eggs, and milk [Sandau et al. (51)]. Considering the different sources of miRNA (plant, animal, herb and pathogen-derived miRNA), and the potential of some of these exogenous miRNAs to be absorbed and consequently interact with the gene expression of the host, a full understanding of such interplay is necessary to assimilate the full scope of miRNA expression modulators [DeLucas et al. (52)].

- **Longitudinal Studies**

Intra-individual longitudinal examination of miRNA expression levels and stability are important in determining their quality as a biomarker which ideally should be sensitive, reliable, specific and stable. In healthy individuals, measures over time should be predictable so that any perturbances would enhance the diagnostic value. It is important to determine factors impacting miRNA levels or variance in an individual over time [Sandau et al. (51)].

- **Comparative studies**

Understanding the evolutionary dynamics of microRNA; particularly elucidating their role in the evolution of regulatory networks and how they shape gene expression, hence phenotypic divergence among species is very important. Functional requirements for miRNA are less

demanding than those of protein-coding genes. MiRNAs require only a short sequence to bind to their target mRNA, thus miRNA target sites can easily be lost or acquired in the transcriptome. Furthermore, miRNA can evolve from different unstructured transcripts and can easily diversify if there are changes in their precursor sequences. This flexibility in their regulatory pathways allowed the adoption of miRNAs through evolution in extant animals. However, identifying specific evolutionary miRNA target combinations remains a challenge. Also, the impact of lowly expressed, non-conserved and lineage-specific miRNAs should be studied more to better understand miRNA circuitry and the molecular underpinnings of expression heterogeneity [Wang et al. (49)].

**5.1. Conclusion**

More evidence suggests the pivotal role of miRNA in the regulation of gene expression. In our study, we tried to understand how *miR-766-3p* mediates *SIRT6* expression in different age groups to evaluate the potential of *miRNA-766-3p* as a biomarker for forensic age prediction tools. The rationale behind our study is based on three key points: the downregulation of S*IRT6* with age, *miR-766-3p* having the highest target score for *SIRT6* and the advantages of using saliva as a forensic biomaterial in forensic cases. In younger subjects, high levels of *SIRT6* assure deacetylation at *SEPT6/miR-766-3p* promoter amongst others, which causes a decrease in the expression of *miR-766-3p*. In older subjects, however, low *SIRT6* levels favor hyperacetylation at the promoters, increasing *miR-766-3p* expression levels which further decrease *SIRT6* levels. This regulatory loop mechanism, in other words, translates to miRNA levels being inversely proportional to age. However, our results show a different expression pattern, which could be attributed to several factors. These factors include sex and tissue-specific considerations, normalization methods, dietary and lifestyle modulators, miRNA sources, and intra-individual and evolutionary dynamics of microRNA. All these factors regulate microRNA expressions to different extents.

Our study highlights the importance of chronological age prediction models in the field of forensic science and beyond. Emphasis is made on the advantages of using saliva samples in routine cases. Also, we touch base on the importance of a reliable biomarker in a context where

there seem to be several promising ones, at nascent stages or with more limitations than advantages. We highlight the interplay of *SIRT6* and *miR-766-3p* regarding ageing, offering a foundation for further research in this light. More holistic research would give more insight, considering the layers of complexity and the number of factors involved.

In conclusion, our findings suggest *miR-766-3p* as a potential biomarker, for age estimation, however, more research is needed to dissect the miRNA circuits involved in gene expression regulation and determine possible modulators of miRNAs. This will help understand the heterogeneity in expression patterns across studies and facilitate the determination of reliable markers.

Based on the findings of this research, the following recommendations should be considered:

The incidence of developmental stages on the expression patterns of microRNA may be noteworthy. For instance, wider age brackets that categorize the participants into major developmental stages such as childhood, adolescence, young adult etc. may have different expression patterns from the ones in our findings

Furthermore, in our study all ages were not equally represented or included therefore, it may be noteworthy to analyze samples with equal representation and inclusion of candidates from all ages to determine if these factors have any significant incidence on the overall results.

## 6.References


1. **Upadhyay MK**, **Shrivastava P, Verma K, Bhawana J**. Recent Advancements in Identification and Detection of Saliva as Forensic Evidence: A Review. *Egyptian Journal of Forensic Sciences* (Electronic Journal), **2023**; 13(17). Access: https://doi.org/10.1186/s41935-023-00336-3

2. **Verma S, Parvez A, Ashutosh K**. Forensic science application: An effective tool of criminal investigations. *IP Int J Forensic Med Toxicol Sci* (Electronic Journal), **2022**; 7(2):60-65. Access: https://www.ijfmts.com/article-details/17000

3. **Bukyya JL, Tejasvi MLA, Avinash A, P CH, Talwade P, Afroz MM, Pokala A, Neela PK, Shyamilee TK, Srisha V**. DNA Profiling in Forensic Science: A Review. *Glob Med Genet* (Electronic Journal), **2021**; 8(4):135-143. Access: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC8635824/

4. **Hartmann D.** Biomarker. *Encyclopedia Britannica*(Electronic Journal),**2024**. Access: https://www.britannica.com/science/biomarker

5. **Tandon R.** What is a Biomarker? A Simple Explanation of Their Definitions, Types, and Applications. *Bitesizebio*(Electronic Journal), **2024.** Access: https://bitesizebio.com/26559/biomarkers-explained/

6. **Wang J, Wang C, Wei Y, Zhao Y, Wang C, Lu C, Feng J, Li S and Cong B**. Circular RNA as a Potential Biomarker for Forensic Age Prediction. *Front. Genet* (Electronic Journal), **2022**; 13. Access: https://www.frontiersin.org/journals/genetics/articles/10.3389/fgene.2022.825443/full

7. **Zhang L, Pitcher LE, Yousefzadeh MJ, Niedernhofer LJ, Robbins PD, Zhu Y**. Cellular senescence: a key therapeutic target in aging and diseases. *J Clin Invest* (Electronic Journal), **2022**; 132(15). Access: https://doi.org/10.1172/JCI158450

8. **Ruchi k, Parmjit J**. Mechanisms of cellular senescence: cell cycle arrest and senescence associated secretory phenotype. *Frontiers* (Electronic Journal), **2021**; 9. Access: https://doi.org/10.3389/fcell.2021.645593

9. **Refn MR, Kampmann ML, Morling N, Tfelt-Hansen J, Børsting C, Pereira V**. Prediction of chronological age and its applications in forensic casework: methods, current practices, and future perspectives, *Forensic Sciences Research* (Electronic Journal), **2023**; 8(2):85–97. Access: https://doi.org/10.1093/fsr/owad021

10. **Ramazan A, Burak T, Şerif YM, Canberk AH, Necdet S**. Omics era in forensic medicine: towards a new age.*Turkish Journal of Medical Sciences* (Electronic Journal), **2020**; 50(5):39. Access: https://doi.org/10.3906/sag-1912-197

11. **Ellingham S, Adserias-Garriga J**. Chapter 1 - Complexities and considerations of human age estimation. *Academic Press* (Electronic Journal), **2019**; 1-15 Access: https://doi.org/10.1016/B978-0-12-814491-6.00001-7

12. **Naue J**. Getting the Chronological Age Out of DNA: Using Insights of Age-dependent DNA Methylation for Forensic DNA Applications. *Genes and Genomics* (Electronic Journal), **2023**; 45(10):1239–1261. Access: https://doi.org/10.1007/s13258-023-01392-8

13. **Hagen M, Schmidt S, Schulz R, Vieth V, Ottow C, Olze A, Pfeiffer H, Schmelina A**. Forensic age assessment of living adolescents and young adults at the Institute of Legal Medicine, Münster, from 2009 to 2018. *Int J Legal Med* (Electronic Journal), **2020**; **134**(2):745–751. Access: https://doi.org/10.1007/s00414-019-02239-2

14. **Freire-Aradas A, Pośpiech E, Aliferi A, Girón-Santamaría L, Mosquera-Miguel A, Pisarek A, Ambroa-Conde A, Phillips C, Casares de Cal MA, Gómez-Tato A, Spólnicka M, Woźniak A, Álvarez-Dios J, Ballard D, Court DS, Branicki W, Carracedo Á, Lareu MV**. A Comparison of Forensic Age Prediction Models Using Data from Four DNA Methylation Technologies. *Front Genet* (Electronic Journal), **2020**; 11(932). Access: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7466768/

15. **Bjelopavlovic M, Reder SR, Fritzen I, Brockmann MA, Hardt J, Petrowski K**. Forensic Age Estimation: A Multifactorial Approach in a Retrospective Population Study. *Diagnostics* (Electric Journal), **2023;** 13(12):20-29. Acess: https://doi.org/10.3390/diagnostics13122029https://doi.org/10.3390/diagnostics13122029

16. **Nisha A, Kumar PM, Shriraam U**. Estimation of Age Using Third Molar Development: A Radiological Cross-Sectional Study. *The American Journal of Forensic Medicine and Pathology* (Electronic Journal), **2020**; 41(2):115-118. Access: https://doi:10.1097/PAF.0000000000000540

17. **Ambroa-Conde A, Girón-Santamaría L, Mosquera-Miguel A, Phillips C, Casares de Cal MA, Gómez-Tato A, Álvarez-Dios J, de la Puente M, J Ruiz-Ramírez J, Lareu MV, Freire-Aradas A**. Epigenetic age estimation in saliva and in buccal cells. *Forensic Science International: Genetics* (Electronic Journal), **2022**; 61. Access: https://doi.org/10.1016/j.fsigen.2022.102770

18. **Aliferi A, Sundaram S, Ballard D, Freire-Aradas A, Phillips C, Lareu MV, Court DS**. Combining current knowledge on DNA methylation-based age estimation towards the development of a superior forensic DNA intelligence tool. *Forensic Science International: Genetics* (Electronic Journal), **2022**; 57. Access: https://doi.org/10.1016/j.fsigen.2021.102637

19. **Weidner C, Lin Q, Koch C, Eisele L, Beier F, Ziegler P, Bauerschlag DO, Jöckel K-H, Erbel R, Mühleisen TW, Zenke M, Brümmendorf TH, Wagner W** . Aging of blood can be tracked by DNA methylation changes at just three CpG sites. *GenomeBiology.com* **2014**; 15(2):R24. Access: https://doi.org/10.1186/gb-2014-15-2-r24.

20. **Thangaraj K, Ramesh S**. Age Estimation Using DNA Extracted from Human Saliva as a Diagnostic Measure in Forensic Odontology. *J Res Med Dent Sci* (Electronic Journal), **2021;** 9(2):132-139. Access: https://www.jrmds.in/articles/age-estimation-using-dna-extracted-from-human-saliva-as-a-diagnostic-measure-in-forensic-odontology-63361.html

21. **Maulani C, Auerkari EI**. Age estimation using DNA methylation technique in forensics: a systematic review. *Egypt J Forensic Sci* (Electronic Journal), **2020**; 10(38). Access: https://doi.org/10.1186/s41935-020-00214-2

22. **Di Micco R, Krizhanovsky V, Baker D, D' Adda di Fagagna F**. Cellular senescence in ageing: from mechanisms to therapeutic opportunities. *Nat Rev Mol Cell Biol* (Electronic Journal), **2021**; 22: 75–95. Access: https://doi.org/10.1038/s41580-020-00314-w

23. **Lee SH, Lee JH, Lee HY, Min KJ**. Sirtuin signaling in cellular senescence and aging. *BMB Rep* (Electronic Journal), **2019;** 52(1):24-34. Access: https://doi.org/10.5483/BMBRep.2019.52.1.290

24. **Zhao L, Cao J, Hu K, He X, Yun D, Tong T, Han L**. Sirtuins and their Biological Relevance in Aging and Age-Related Diseases. *Aging Dis* (Electronic Journal), **2020**; 11(4):927-945. Access: www.ncbi.nlm.nih.gov/pmc/articles/PMC7390530/

25. **Owczarz M, Połosak J, Domaszewska-Szostek A, Kołodziej P, Kuryłowicz A, Puzianowska-Kuźnicka M**. Age-related epigenetic drift deregulates SIRT6 expression and affects its downstream genes in human peripheral blood mononuclear cells. *Epigenetics* (Electronic Journal), **2020;** 15(12):1336-1347. Access: https://doi.org/10.1080/15592294.2020.1780081

26. **Dzidek A, Czerwińska-Ledwig O, Żychowska M, Pilch W, Piotrowska A**. The Role of Increased Expression of Sirtuin 6 in the Prevention of Premature Aging Pathomechanisms. *Int. J. Mol. Sci.* (Electronic Journal), **2023**; *24* (11):9655. Access: https://doi.org/10.3390/ijms24119655

27. **Hayakawa K, et al Kawasaki M, Hirai T, Yoshida Y, Tsushima H, Fujishiro M, Ikeda K, Morimoto S, Takamori K, Sekigawa I.** MicroRNA-766-3p Contributes to Anti-Inflammatory Responses through the Indirect Inhibition of NF-κB Signaling. *Int J Mol Sci*. (Electronic Journal), **2019**; 20(4):809. Access: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6413049/

28. **Li M, Yu B.** Recent advances in the regulation of plant miRNA biogenesis. *RNA Biology* (Electronic Journal), **2021**; 18(12): 2087–2096. Access: https://doi.org/10.1080/15476286.2021.1899491

29. **Ergin K, Çetinkaya R**. Regulation of MicroRNAs. *Methods Mol Biol*. (Electronic Journal), **2022**; 2257:1-32. Access: https://link.springer.com/protocol/10.1007/978-1-0716-1170-8_1

30. **Rani V, and Sengar RS**. Biogenesis and Mechanisms of microRNA-mediated Gene Regulation. *Biotechnology and Bioengineering* (Electronic Journal), **2022**; 119(03)685–692. Access: https://doi.org/10.1002/bit.28029

31. **Michlewski G,Javier FC**. Post-transcriptional Control of miRNA Biogenesis. *RNA* (Electronic Journal), **2019**; (1)1–16. Access: http://www.rnajournal.org/cgi/doi/10.1261/rna.068692.118

32. **Leitão AL,Francisco JE**. A Structural View of miRNA Biogenesis and Function. *Non-coding RNA* (Electronic Journal), **2022**; 8(1):10. Access: https://doi.org/10.3390/ncrna8010010

33. **Gupta J, Kareem Al-Hetty HRA, Aswood MS, Turki Jalil A, Azeez MD, Aminov Z, Alsaikhan F, Ramírez-Coronel AA, Ramaiah P, Farhood B**. The key role of microRNA-766 in cancer development. *Front Oncol* (Electronic Journal), **2023**; 13. Access: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC10185842/

34. **Fiorentino F, Favale G, Altucci L, Mai A**. The Two-Faced Role of SIRT6 in Cancer. *Cancers* (Electronic Journal), **2021**; 13(05):11-56 Access: https://doi.org/10.3390/cancers13051156

35. **Chang AR, Ferrer CM, Mostoslavsky R**. SIRT6, a Mammalian Deacylase with Multitasking Abilities. *Physiological Reviews* (Electronic Journal), **2019**; 100(1):145-169 Access: https://doi.org/10.1152/physrev.00030.2018

36. **Chatterjee S**. Saliva as a forensic tool. *J Forensic Dent Sci* (Electronic Journal), **2019**; 11(1):1-4. Access: www.ncbi.nlm.nih.gov/pmc/articles/PMC6822307/

37. **Gandhi V, O'Brien MH, Yadav S**. High-Quality and High-Yield RNA Extraction Method From Whole Human Saliva. *Biomark Insights* (Electronic Journal), **2020**; 15. Access: https://pubmed.ncbi.nlm.nih.gov/32550766/

38. **Sharma A, Diecke S, Zhang WY, Lan F, He C, Mordwinkin NM, Chua KF, Wu JC**. The Role of SIRT6 Protein in Aging and Reprogramming of Human Induced Pluripotent Stem Cells. *Journal of Biological Chemistry/the Journal of Biological Chemistry* (Electronic Journal), **2013**; 288(25):18439–47. Access: https://doi.org/10.1074/jbc.m112.405928.

39. **Mukaibo T, Gao X, Yang N-Y, Oei MS, Nakamoto T, Melvin JE**. Sexual dimorphisms in the transcriptomes of murine salivary glands. *FEBS Open Bio* (Electronic Journal), **2019**;9(5):947–58. Access: https://doi.org/10.1002/2211-5463.12625.

40. **Szakats S, McAtamney A, Cross H, Wilson MJ**. Sex-biased gene and microRNA expression in the developing mouse brain is associated with neurodevelopmental functions and neurological phenotypes. *Biology of Sex Differences*(Electronic Journal), **2023**; 14. Access: https://bsd.biomedcentral.com/articles/10.1186/s13293-023-00538-3

41. **Veryaskina YA, Titov SE, Zhimulev IF**. Reference Genes for qPCR-Based miRNA Expression Profiling in 14 Human Tissues. *Medical Principles and Practice* (Electronic Journal),**2022**; 31(4):322–32. Access: https://doi.org/10.1159/000524283.

42. **Ludwig N, Leidinger P, Becker K, Backes C, Fehlmann T, Pallasch C, Rheinhamer S, Meder B, Stähler C, Meese E, Keller A**. Distribution of miRNA expression across human tissues. *Nucleic Acids Research* (Electronic Journal), **2016**; 44(8):3865–77. Access: https://doi.org/10.1093/nar/gkw116.

43. **Machowska M, Galka-Marciniak P, Kozlowski P**. Consequences of genetic variants in miRNA genes. *Computational and Structural Biotechnology Journal* (Electronic Journal),**2022**; 20:6443–57. Access: https://doi.org/10.1016/j.csbj.2022.11.036.

44. **Syeda ZA, Langden SSS, Munkhzul C, Lee M, Song SJ**. Regulatory Mechanism of MicroRNA Expression in Cancer. *International Journal of Molecular Sciences* (Electronic Journal), **2020**; 21(5):1723. Access: https://doi.org/10.3390/ijms21051723.

45. **Mohanty JN, Sahoo S, Routray SP, Bhuyan R**. Does the diverse source of miRNAs affect human health? An approach towards diagnosis and therapeutic management. *Gene Reports* (Electronic Journal),**2022**; 28:101656. Access: https://doi.org/10.1016/j.genrep.2022.101656.https://www.sciencedirect.com/science/article/pii/S2452014422001649

46. **Faraldi M, Gomarasca M, Sansoni V, Perego S, Banfi G, Lombardi G**. Normalization strategies differently affect circulating miRNA profile associated with the training status. *Scientific Reports* (Electronic Journal),**2019**; 9(1). Access: https://doi.org/10.1038/s41598-019-38505-x.https://www.nature.com/articles/s41598-019-38505-x#Sec8

47. **Pointner A, Krammer UDB, Tomeva E, Magnet U, Hippe B, Jacob U, Haslberger AG**. Lifestyle-Driven Variations in Nutrimiromic MicroRNA Expression Patterns across and beyond Genders. *Life* (Electronic Journal )**2024**; 14(3):390. Access: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC10971939/

48. **Chodur GM, Steinberg FM**. Human microRNAs modulated by diet: A scoping review. *Advances in Nutrition* (Electronic Journal),**2024**; :100241. Access: https://doi.org/10.1016/j.advnut.2024.100241.

49. **Wang Y, Tang X, Lu J**. Convergent and divergent evolution of microRNA-mediated regulation in metazoans. *Biological Reviews/Biological Reviews of the Cambridge Philosophical Society* (Electronic Journal), **2023**; 99(2):525–45. Access: https://doi.org/10.1111/brv.13033.https://onlinelibrary.wiley.com/doi/10.1111/brv.13033

50. **Panico A, Tumolo MR, Leo CG, De Donno A, Grassi T, Bagordo F, Serio F, Idolo A, De Misa R, Mincarone P, Sabina S**. The influence of lifestyle factors on miRNA expression and signal pathways: a review. *Epigenomics* (Electronic Journals) **2021**; 13(2):145–64. Access: https://doi.org/10.2217/epi-2020-0289.

51. **Sandau US, Wiedrick JT, McFarland TJ, Galasko DR, Fanning Z, Quinn JF, Saugstad JA**. Analysis of the longitudinal stability of human plasma miRNAs and implications for disease biomarkers. *Scientific Reports* (Electronic Journal),**2024**; 14(1). Access: https://doi.org/10.1038/s41598-024-52681-5.

52. **DeLucas M, Sánchez J, Palou A, Serra F**. The Impact of Diet on miRNA Regulation and Its Implications for Health: *A Systematic Review. Nutrients* (Electronic Journal), **2024**; 16(6):770. Access: https://doi.org/10.3390/nu16060770.

53. From a sex perspective https://www.ncbi.nlm.nih.gov/pmc/articles/PMC10971939/