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

ANALYSIS OF AGE- AND SEX-RELATED SIRT6 GENE EXPRESSION LEVELS IN SALIVA

GIFT ONYINYE OKAFOR

DEPARTMENT OF FORENSIC SCIENCES MASTER'S PROGRAM

MASTER'S THESIS

ADVISOR

Prof. Dr. Mehmet Bertan YILMAZ

ADANA-2023

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

ADANA-2023

ACCEPTANCE AND APPROVAL

Department of Forensic Sciences

carried out within the framework of the Master's Program with Thesis The study titled

" ANALYSIS OF AGE- AND SEX-RELATED SIRT6 GENE EXPRESSION LEVELS IN SALIVA"

was accepted by the following jury as First Graduate Term.

Date: 02/ 10 / 2023

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THANKS

I would like to express my deepest gratitude to those who provided unwavering support throughout the execution of this study and the process of writing my thesis. My advisor, Prof. Dr. Mehmet Bertan Yilmaz, has been instrumental in providing guidance and information without which I could not have achieved anything. I extend my heartfelt thanks to Dr Nermin Seda Ilgaz. I am especially grateful for her continuous assistance and patience during laboratory work and thesis writing. I extend my heartfelt thanks to Mehmet Önsan Kütükoglu for always coming to my aid and showing endless patience and understanding.

I am indebted to Late Prof. Dr. Mete Korkut Gülmen, the formal Head of the Department of Forensic Medicine, for sharing valuable experiences and knowledge throughout/ in my educational journey. I am also thankful to the faculty members who supported me during my studies Prof. Dr. Ayse Serin, Kenan Kaya and Lect. See. Dr. Pınar Efeoğlu.

I want to express my sincere appreciation to my mother Anthonia Uche Duru-Okafor, my father Basil Nnaemeka Duru-Okafor, my sister Vivian Ifunanya Okafor, and all my brothers, and Friends for their unwavering financial and moral support throughout my academic years.

~GIFT ONYINYE OKAFOR

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ICONS AND ABBREVIATIONS

ALU: Alternative locus units

BER: Base excision repair

cDNA: complementary DNA

CN: Cyanide

CNV: Copy number variations

DNA: Deoxyribonucleic acid

DNases: Deoxyribonuclease

FBI: Federal Bureau of Investigation

GAPDH: glyceraldehyde-3-phosphate dehydrogenase

IGF-1: Insulin-like growth factor 1

MEF: Mouse embryonic fibroblast

miRNAs: microRNAs

ml: milliliter

mRNA: Messenger RNA

NAD+: Nicotinamide adenine dinucleotide

NADH: Nicotinamide adenine dinucleotide (NAD) + hydrogen (H)

NER: Nucleotide excision repair

PARP1: Poly (ADP-ribose) polymerase 1

PCR: Polymerase Chain Reaction

PI3K: Phosphatidylinositol 3-kinase

PL500 model: High intensive used in detecting stains in fluid Polilight® wavelength

RNA: Ribonucleic acid

RNAases: Ribonuclease

RSID™: Rapid Stain Identification of Human Saliva

RT-PCR: Real Time Polymerase Chain Reaction

SALIgAE® test: Saliva Identification Test Kit - Abacus diagnostics

SASP: Senescence-associated secretory phenotype

SCN: Thiocyanate

SIR2: Silent information regulator 2

SIRT6: Sirtuin 6

SNPs: Single nucleotide polymorphisms

STRs: Short tandem repeat

TCA: Tricarboxylic acid

THC: Tetrahydrocannabinol

THCA: Tetrahydrocannabinol acid

TNF: Tumor necrosis factor

tRNA: Transfer RNA

VNTRs: Variable number of tandem repeats

µl: Microliter

ÖZET

Tükürükte Yaş ve Cinsiyete Bağlı SIRT6 Geni İfadesi Düzeylerinin Analizi

Hücre yaşlanması, DNA replikasyonu sırasında telomer kısalmasına bağlı olarak ortaya çıkan ve çeşitli stres etkenleri tarafından tetiklenebilen bir süreçtir. Yaşlanan hücreler bölünmeyi bırakır ancak canlı kalır, metabolik olarak aktiftir ve birçok molekül salgılayabilir. Yaşlanmanın bazı işaretlerinden bazıları hücre büyümesi, artan tanecikleri lik, SA-β-galaktosidaz aktivitesinin indüksiyonu ve sikline bağımlı kinaz inhibitörleri p16 ve p21'deki artıştır. Hücre yaşlanması ile ilişkilendirilen yeni oyunculardan biri de Sirtuins Tir. Sirtuins (SIRT'ler), mono-ADP ribose transferaz veya NAD + bağımlı deasetilaz aktivitesine sahip bir protein sınıfı oluşturur. SIRT'ler, genlerin ekspresyonunu ve hücre metabolizmasını epigenetik olarak düzenleyen ve homeostazisin korunmasında önemli bir rol oynayan NADiye bağımlı histon deasetilaz lardır. Sirinlerin, oksidatif veya genotoksik stres gibi çeşitli streslere karşı hücre tepkisi sırasında anahtar rol oynadığı ve hücre metabolizması için çok önemli olduğu rapor edilmiştir. SIRT6, genomları ve telomerleri stabilize eden kromatinle ilişkili bir proteindir. SIRT6, genom transkripsiyon, telomer bütünlüğü, DNA onarımı ve metabolik homeostazinin kritik bir düzenleyicisidir. SIRT6 ve telomeraz arasında stabil ve pozitif bir doğrusal korelasyon ortaya çıktı. Böylece SIRT6 hücrelerin erken yaşlanmasını önler ve eksikliği replikatif yaşlanmayı hızlandırır. Biyolojik numunelerin yaş tahmini için güvenilir bir biyobelirteç bulmak, adli DNA fenotipleme sinde önemli görevlerden biridir. Bu tezde tükürükteki yaş ve cinsiyete bağlı SIRT6 geninin ekspresyon düzeyleri analiz edilecektir.

Genel olarak. Bulgularımıza göre referans olarak 4-9 yaş grubuyla karşılaştırıldığında 14-19 yaş grubunda sirtuin 6 (sirtuin 6) ekspresyonunda yaklaşık 2 kat azalma gözlemledik. 24-29 yaş grubunda ise yaklaşık 1,75 kat artış görülürken, 30 yaş ve üzeri grupta ise 2 kat düşüş yaşandı. Bu sonuçlar SIRT6 ifadesinde yaşa ve cinsiyete bağlı varyasyonların olasılığını göstermektedir. Bu tutarsızlıkların sonuçlarını ve bunların hem sağlık hem de yaşlanma süreci üzerindeki potansiyel etkilerini tam olarak kavramak için ek araştırmalar gereklidir.

Anahtar Kelimeler: Yaşlanma, Biyobelirteç, Tükürük, SIRT6, Telomeraz, Histone deacetylase, Adli yaş tahmini, Adli epigenetik, RNA ekstraksiyonu, RT-PCR; Gerçek Zamanlı Polimeraz Zincir Reaksiyonu

ABSTRACT

Analysis of Age- And Sex-Related SIRT6 Gene Expression Levels in Saliva

Cell senescence is a process that occurs due to telomere shortening during DNA replication and can be induced by various stressors. Senescent cells stop dividing but remain viable, metabolically active and able to secrete many molecules. Some of the hallmarks of aging are cell enlargement, increased granularity, induction of SA-β-galactosidase activity, and an increase in cyclin-dependent kinase inhibitors, p16, and p21. One of the new players associated with cell aging is Sirtuins. Sirtuins (SIRTs) form a class of proteins with mono-ADP ribosyltransferase or NAD+ dependent deacetylase activity. SIRTs are NAD-dependent histone deacetylases that epigenetically regulate the expression of genes and cell metabolism and play an important role in maintaining homeostasis. Sirtuins have been reported to play a key role during cell response to various stresses such as oxidative or genotoxic stress and are crucial for cell metabolism. SIRT6 is a chromatin-associated protein that stabilizes genomes and telomeres. SIRT6 is a critical regulator of genome transcription, telomere integrity, DNA repair and metabolic homeostasis. A stable and positive linear correlation of SIRT6 and telomerase emerged. Thus, SIRT6 prevents premature aging of cells, and its deficiency accelerates replicative senescence. Finding a reliable biomarker for age estimation of biological samples is one of the important tasks in forensic DNA phenotyping. In this thesis, age and sex related SIRT6 gene expression levels in saliva will be analyzed.

Generally, based on our findings, while comparing to the 4-9 age group as the reference, we observed a roughly 2-fold decline in sirtuin 6 (sirt 6) expression among the 14-19 age group. Conversely, in the 24-29 age group, there was an approximately 1.75-fold increase, followed by another 2-fold decrease in the 30years and older group. These results indicate the possibility of age and gender-related variations in SIRT6 expression. Additional research is necessary to fully grasp the consequences of these discrepancies and their potential impacts on both health and the aging process.

Keywords: Aging, Biomarker, Saliva, SIRT6, Telomerase, Histone deacetylase, Forensic age estimation, Forensic epigenetics, RNA extraction, RT-PCR; Real-Time Polymerase Chain Reaction.

1. ENTRANCE

What is forensic science? Forensic science is a fault-finding component of the criminal justice system. Forensic investigators assess, question, and examine evidence from crime scenes and elsewhere to augment or build up accurate results that can facilitate the interrogation of crimes. [1] It prosecutes perpetrators of crimes or exonerates a faultless person from crime or suspicion. Standard forensic science laboratory practices include forensic molecular biology (DNA), forensic chemistry, trace evidence examination (hairs and fibers, paints and polymers, glass, soil, etc.), latent fingerprint examination, handwriting examination, firearms and tool marks analysis, explosives & fire assessments, digital evidence and forensic toxicology. Most forensic disciplines experienced outside the forensic laboratories include forensic entomology, forensic pathology, forensic psychiatry, forensic nursing, and forensic engineering. Medical experts in this discipline are at the corner office of private organizations or universities. [1]

The National Institute of Justice comprehends quality information valued by the forensic science communities. And some stakeholders are engaged in the criminal justice system with high interests in forensic science. The FBI's innovations, programs, and other forensic governmental Agencies modeled supportive criminal justice DNA data profiles. Software used to run these databases contains the DNA profiles of criminals/ suspects contributed by federal, state, and local participating forensic laboratories. [1]

Role of Forensic Science

The aim and role of forensic science, it assesses crime scenes to determine what evidence should be collected and how. Take photographs of the crime scene and evidence, make sketches of the crime scene, document, and jot down observations and outcomes, such as the position and location of evidence. Forensic science is not only in the examination and the perpetration of crimes such as drug trafficking, sexual assault (rape), and murder but also in matters where crime has not been obliged. For instance, someone is charged: with a civil wrong (tort), such as deliberate contamination of air and water, inducing industrial injuries. All sciences can be the same, even the traditional sciences. All science can influence solving a crime and evaluating civil harm. The main difference

is that forensic scientists apply the methods, approaches, and strategies of well-established sciences to legal situations. [2]

Application of forensic science

There are various applications of forensic science, including Forensic anthropology, Criminalistics, Forensic engineering, Jurisprudence, Forensics, odontology, Forensic pathology, Forensic entomology, Psychiatry and behavioral science, Questioned-document analysis, Toxicology, Forensic Nursing, and Forensic Genetics. This review shall briefly discuss three applications of forensic science investigation. [2]

Forensic anthropology: deals with skeletal biology, which includes bone and bone system structures. It identifies their characteristics like gender, age, race, and socioeconomic status. At a crime scene, its analysis is to determine the identity of the deceased person and the cause of death. It identifies the part of the body a bone came from. If there is damage to some of the bones, the anthropologist determines what type of trauma is caused by the injury. [2]

Criminalistics: the execution of scientific techniques to the realization, collection, pinpointing, and differentiation of physical evidence actualized by unlawful or illegal civil activity. The interpretation of incidents is by assessing the physical evidence and the entire crime scene. Criminalists or forensic scientists examine proofs like body fluids to dictate if DNA in the fluids aligns with blood recovered at a crime scene (DNA fingerprinting). [2]

Forensic engineering: it uses the concepts of mechanical, chemical, civil, and electrical engineering as tools in the reconstruction of crimes and accidents and the determination of their cause. A critical part of this work is the reconstruction of traffic accidents. To establish the cause of the resulting accident, forensic engineers utilize evidence such as skid marks, car damages, car positions after the accident, environmental conditions, road, and influence. Estimate injuries to drivers, passengers, and pedestrians; and witness accounts. [[2]

Essential Forensic Tools: 13 Technologies For Modern Forensic Sciences include the following; Phenom Desktop Sem(determining elemental composition), Alternative Light Photography(detect any damages inflicted to a body), Digital Surveillance For Gaming Equipment(The XFT Device will give authorities access to hidden files on Xbox hard drives), Facial Reconstruction(identification of unidentified remains), DNA Sequencing (ancestry or phenotype prediction, DNA profiling), Automated Fingerprint Identification (compare fingerprints), Link Analysis Software(uncover criminal networks), Drug Testing (identify unknown substances), LA-ICP-MS(it identify weapon/ direction of bullet), Fire Technology(establish the origin & cause of the fire), 3D Scanner(a 360 photographic scanner), High-Powered Microscopes(polarized light tracing evidence) and High-Speed Ballistics Photography (scan bullets fired from a gun or bullets penetrating their targets.) [3]

Biomarker

A macronutrient originates in blood, body fluids like semen, saliva, or tissues that declare a pattern of processes for a normal, abnormal, and disease condition as a marker for a particular phenotype. The concept is a reliable signature discovery technique that yields valuable insight into cell biology and the analysis of human disease. Hence it is called signature discovery. A molecular marker is applied to learn how well the body responds to treatments for a disease or condition. These signature molecules, such as hormones, proteins, and genes (DNA), are maximized as biomarkers since it uncovers something about human health. The reformation of Biomarkers is likely retrieved from cancer tissue or other cells in the body in response to cancer. They originated in the blood, stool, urine, tumor tissue, or tissues or bodily fluids. [4]

Learning basic facts about DNA, RNA, and proteins helps understand the importance of biomarkers in cancer and age estimation in males and females. Deoxyribonucleic acid (DNA) is a molecule within the cell that possesses genetic materials and passes them on from generation to generation. Ribonucleic acid (RNA) contains vital information copied from DNA. The body cells construct several distinct types of RNA molecules necessary for synthesizing or producing protein molecules. For instance, messenger RNA (mRNA) molecules serve as templates for protein synthesis from amino acid building blocks and transfer RNA (tRNA) molecules that carry the

residue of the amino acid residues to the ribosome. The proteins synthesize in an organelle called the ribosome. Translation occurs when tRNA “reads” the mRNA template. Proteins assist the body in functioning correctly and are the fundamentals of body structures such as skin and hair. Biomarkers like the sirt6 genes are vital in the analysis of age estimation in forensic science. [5]

Application of Biomarkers used in Age Estimation in Forensic Science

Age assessment is an essential or crucial part of forensic investigations, an actual need to accurately predict the age of an anonymous donor or from a stain at a crime scene can expedite police efforts to narrow down their potential suspects and solve crimes. Molecular biologist or biochemistry-based aspects map out the role of chemical and biochemical analysis in the age assessment of skeletal remains. In forensic age analysis, assessments such as serology deal with the complex task of gathering information on the type of sample age, origin, or sex from biological fluids found at a crime scene. Two methods forensic serology pivots on are DNA and RNA analysis immunoassay [6][7]. Aging outcomes from various biological processes include epigenetic changes across the genome. Estimations built on DNA methylation (biomarker) raised the interest of many forensic practitioners; it serves as a way to optimize age estimation for distinct forensic reasons [8][9]. In this process, DNA methylation levels apply to resolve epigenetic age as a factor for chronological age in forensic research. Biomarkers are measured to estimate biological age compared to chronological age and include IGF-1 (insulin-like growth factor 1), insulin, glucose, C-reactive protein (a measure of systemic inflammation), triglycerides, and blood pressure. The biomarker that examines the methylation profile as an assessor of biological age will be on the market soon, measuring relevant longevity information and biological age. [10] This chapter revises the biochemical examination of biomarkers and processes depicted by the reviewing of unique race and sex as well as determination of saliva sample age. The Observation includes the difference in the Sirt 6 gene expression levels in saliva for different age groups: the underage, middle age, and elderly age and taking into account the sex-related distribution patterns of the Sirt 6 gene expression levels in saliva.

2. GENERAL INFORMATION

This current body of literature on RNA extraction in forensics summarizes current knowledge and generates recommendations for future research. This comprehensive review has been sourced from peer-reviewed journals; publications of The National Center for Biotechnology Information (NCBI) or the national library of medicine, PubMed Central (PMC); a free archive of biomedical and life sciences journal literature in the U.S. National Institutes of Health's, National Library of Medicine (NIH/NLM). Other sources of information include the ResearchGate https://www.researchgate.net and the ScienceDirect https://www.sciencedirect.com The distinctive value of this thesis is the analysis and eventful remarks of RNA extraction and its roles and finding sirt6 expression from the extracted sample. Other added value of this thesis are Evolutionary studies, pathology, RNA profiling, cancer treatment, MicroRNAs as a New Potential Therapeutic Opportunity in Gastrointestinal Cancer.

Age estimation is an important part of forensic analysis. At present, individual age may be predicted by measuring and analysis of some osteal markers like bones and teeth with its acceptable error [11]. However, this method is only confined to certain cases with the existence of a skeleton. Therefore, it is essential to discover more workable techniques for forensic biological sample age prediction. Recent research indicates that DNA damage and telomere shortening is related to aging, but they have limited value as an indicator of biological age [11]. There is accumulating evidence indicating that aging resembles a developmentally regulated process that is tightly controlled by specific epigenetic modifications rather than a random course [11].

2.1. Saliva

Saliva is a biomolecule often taken for granted, yet one of the most valuable oral fluids. The biochemical is a clear liquid, an extracellular liquid made and secreted by salivary glands in the mouth. This watery fluid in the mouth is a significant part of an active body. And critically important in the maintenance and preservation of oral health. Oral health is the condition/ health of gums, teeth, and the entire oral-facial system that enables us to chew, swallow, smile, and speak. And are undoubtedly conserved by saliva.

[12] It moistens and helps in digestion, safeguards the mouth against infections, and keeps the teeth strong, such as by breaking down food particles netted within the dental crevices, thereby protecting the teeth from decay or bacterial infection. These dietary roles of saliva are complex secretions backed up by enzymes, and it may interest you to appreciate the components of saliva. [12]

Saliva is a very dilute fluid composed of 99% water, with the remaining 1% consisting of organic and inorganic biomolecules. It contains a diversity of electrocyte, magnesium, bicarbonate, phosphate, potassium, sodium, and calcium. [13] The complex mixture also contains mucus which consists of glycoproteins; enzymes such as lipase and amylase help to digest fats and breakdown starches, and some antimicrobial agent such as lysosome helps to digest macromolecules by responding against foreign bodies like bacterias, viruses, and antigen. Most importantly, it contains immunoglobulins, white blood cells, and epithelial cells from which DNA can be extracted and identified. Saliva leukocytes, instead of saliva epithelial cells, contain the major source of DNA. [12][13] DNA extraction from a biological sample such as salvia plays a vital role in human identification and profiling.

2.2. Identification of saliva sample

What makes saliva, saliva? To determine if a biological sample retrieved from a crime scene is saliva, it should undergo a series of tests, this includes the presumptive and the confirmatory test for saliva, assess saliva tests are of two types include presumptive and confirmatory tests: Presumptive test sets up the likelihood that a distinct or particular bodily tissue or fluid is present, whereas a confirmatory test, it pinpoints or confirms a biological material. The screening tests were applied to assess evidence to conclude the viable appearance of controlled substances, hormones, and genetic material, and the two tests are obtainable and used in the salivary samples. [14]

2.2.1. Salivary Amylase and saliva detection tools

α-Amylase is a salivary integral. Its significant function is in starch assimilation; it occurs in diploid iso-enzymatic mode – salivary and pancreatic. However, the RSID™

saliva stream is an out-flow immunochromatographic strip specified to detect or recognize the salivary α-amylase in a patch sample, consequently validating the presence of saliva in a sample. Nonetheless, due to insufficient formulation, the SALIgAE® test was brought in as a screening test for saliva. The old Spotty paper test, the SALIgAE spray, the assessment does not require any incubation or extra apparatus, Polilight® (PL500 model wavelength) is applied to determine contaminated parts that can obstruct saliva discovery using 470–555 nm interference filters. The utilization of Polilight®, Saliva fluorescence is uncertain, and the grading system designed by Casey and Price *et al.* (2004) can be applied: 0 – no fluorescence found, 1 – very poor fluorescence, hardly visible, and 2 – poor fluorescence.[14] Biodegradable starch microspheres for saliva detection. [14]

These microspheres are bound to a blue dye covalently, which are usually released when α-amylase is present in the sample. Nonetheless, this test is inadequate in specificity as it cannot discriminate between α-amylase 1 (present in the saliva) and α-amylase 2 (present in the semen and vaginal secretions). An additional critical disadvantage is the loss of samples for consequent DNA analysis. Mass spectrophotometry is a method that can analyze pure and a mixture of biological entities of various species' origin without destroying any DNA. Therefore making the complete sample available for DNA extraction.[14]

2.2.2. Saliva in Addiction Biology

The discovery of psychotropic drugs and ethanol amounts in the saliva as a track-way operation is significant for road management and traffic safety. Cannabis has recognition and records as the most common or popular narcotic (78%) through saliva testing. The serum samples recorded and established as being positive for tetrahydrocannabinol (THC, 70%). The total sensitivity and specificity in the narcotic examination are in the scope of 91%–98%. Other narcotic factors identified as a metabolite, or the drug alone include amphetamine, cocaine, opiates, and cannabinoids.[14] General tests for THC acid (11-nor-9-carboxy-9-THC) antibodies in urine samples do not test positive in oral fluids. THC is only found in the saliva samples for long hours of smoking cocaine (about ten hours) [Table 2.1]. Therefore, salivary

detection is traceable to contaminants attributable to smoking instead of it been concealed or secreted in the oral cavity by saliva.[14][15]

Table 2.1. Detection of Summary of THC & THCA
Number of cases with detection of tetrahydrocannabinol and tetrahydrocannabinolic acid in serum and oral fluid.

	Number of cases
THC and in serum	97
THC in oral fluid	89
Oral fluid negative	8
THCA in serum only	40
THC in oral fluid	6
Oral fluid negative	34
Serum negative	40
THC in oral fluid	1
Oral fluid negative	39

Thiocyanate (SCN): is commonly found in serum, saliva, and urine in small concentrations. It's a prime metabolic effect of cyanide (CN) metabolism. Large CN quantities can start from smoking tobacco and metabolic interchange to hydrogen CN. Gas spectrometry helps determine SCN levels in the saliva. It represents and is applied as a vital variable in categorizing patients as smokers and non-smokers, in the analysis or interpretation of certain medical conditions, and in forensic drug testing.[14] Saliva as a specimen is not an absolute sample for postmortem-testing for ethanol caused by contamination or unmoistened oral cavity conditions postmortem. Vitreous humor is an

ideal specimen for postmortem ethanol analysis since it does not contain glucose or any bacteria for postmortem metabolism to ethanol. [15][16]

2.2.3. Extracellular Nucleic Acid Detection

Extracellular nucleic acids are cancer biomarkers that can be found in a variety of bodily fluids, such as breast milk, semen, saliva, urine, and plasma, in addition to supernatants from cell cultures.[14] For the purpose of identifying mRNA specific from saliva stains as old as ten weeks, Juusola and Ballantyne used real-time polymerase chain reaction (RT-PCR) and gel electrophoresis techniques. The indicators used for salivary detection are Statherin and histatin 3.[14][17] RNA Salivary detection is an evolving scope in forensic molecular diagnostics. The RNAs in saliva are maintained by a variety of methods, including salivary mucins, adenine and uridine-rich element binding protein, salivary chaperone Hsp70, and apoptotic bodies. Exosomes perform a crucial function in safeguarding the salivary transcriptomes. These specially created vesicles carry intracellular mRNA, protecting salivary mRNA stability from external RNAases.

The detection of mRNA from saliva samples is induced possibly by heat coverage at ≥60°C at the start of the RT-PCR pre-amplification process; heat disrupts hydrogen and nonpolar hydrophobic bonds between RNA molecules and proteins; exosomes are usually from the endosomal membrane compartment within a lipid bilayer structure, when exposure to high heatwave, the lipid bilayer changes to a fluidical or aqueous state, thereby the made mRNAs be released.[9] DNA is an elementary salivary biomarker in forensic science, there is>99% homology in the DNA sequences of two individuals, but there might be numerous of genetic differences in some DNA fragments. Mutations and environmental influences are important external factors responsible for these genetic variations. CNV (Copy number variations) are vast genomic regions that can be unavailable or copied in diverse individuals. Single nucleotide polymorphisms (SNPs) are single base similarities among two organisms of the same species. In humans, SNPs occur at an average of every 1/2000 base pairs. The CNV and SNP constitute the most important sources of genetic variations in the human genome.[14] microRNAs (miRNAs) are used to test different biological matrices present within a sample alongside detecting each miRNA carried out in separate wells. DNA methylation is a procedure based on

tissue-specific methylation layouts. This approach exhibits operator-independent effects and may multiplex along with common STRs (short tandem repeats) setting protocols or procedures without added sample loss. The lost function of proteins can lead to false negatives in the current commercial kits. The primary structure of proteins is highly stable for the sequence identified by mass spectrometry after hundreds of years, making it suitable for forensic application. Other types of protein identification tools include fluorescence spectroscopy and Raman spectroscopy. [14] The human genome is known as repetitions at sequence length scales, number, and dispersion; examples of such repetitions are homo- and di-nucleotide repeats (microsatellites) and alternative locus units (ALU) sequences. There are >1 million ALU sequences in the human genome (300 bp long) that can copy themselves in various parts of the genome, generating mutations. Forensic DNA typing uses different techniques for the identification of such genetic alterations. The variable number of tandem repeats (VNTRs) loci are composed of core units that are 3, 4, or 5 nucleotides long/ the number of repeated segments at each locus also varies. VNTRs identify, by slicing genomic DNA with restriction enzymes such as HaeIII, HinfI, or HindIII, electrophoretic deviation of DNA bands and detecting different fragments or bands utilizing DNA probes. Most recently, VNTR identification turned out to be replaced by STR. DNA typing using STR by matching 13–17 nuclear STR markers of a victim's profile to an antemortem sample of a victim or family members (biological parents). A system of 13 STR markers constitutes the combined DNA Index System used in the USA and Canada, while the European countries have their database. Enomorsely, degraded DNA entails a small quantity of very short DNA template molecules (<150 bp), making conventional STR typing (which uses 150–400 bp) unsuccessful.[14] Multiplex PCR involves using several sets of PCR primers for the reaction allowing higher amplification. STR multiplex systems have high sensitivity (matching probability) compared to the combination of five classical single locus DNA fingerprints. Although multiplexing techniques save time and finances, amplifying optimum PCR conditions (annealing temperature and primer concentration), it is quite a challenge setting the exact reaction conditions in sample mixtures.

2.3. Saliva At The Crime Scene:

Saliva as evidence in the forensic is necessary, a shred of evidence that maintains a piece of biological information about the personal contacts of perpetrators and victims. The appearance of saliva can be validated or indicated by Starch-iodide and Phadebas tests. However, Starch-iodide and Phadebas tests do not confirm if saliva is present in human saliva. These are merely the test for amylase activity regardless of whether that amylase has come from humans or any other source. An intricate, *monoclonal antibody-based test kit* is applied to detect human-specific salivary amylase. With the Rapid Stain Identification Series (RSID) method, if a stain gives a positive reaction, it is confirmed that human saliva is present. Additionally, if the person is a secretor, ABO group antigens can also be ascertained in saliva. A secretor is a person whose body fluids and saliva contain the ABO antigens. Approximately 80% of individuals are known to be secretors. Finally, it is possible to identify the DNA profile of a person using advanced DNA profiling techniques: since saliva may also contain buccal mucosa cells.[18]

2.3.1. Location Of Saliva As An Evidence:

Saliva may be on clothes, cigarette butts, bottles, cups, Handkerchiefs, etc. In incidents like biting during a struggle, hanging, kidnapping, assault, spitting, or tobacco spitting at the crime scene, saliva supplies a lot of evidentiary facts. Saliva plays a significant role in crime investigation due to the antigen being present, thereby grouping tests or DNA profiling can be performed by saliva. Blood groups can be detected by saliva, although sometimes saliva could be contaminated by lipstick or blood. If saliva is present in a dry stain, wet it with the help of normal water and make a cotton swab of it, then preserve it in a tube or bag without contaminating it. If chewing gum, or tobacco spit, are present at the crime scene, conserve it in an airtight bag. [18]

2.4. Forensic Examination Of Saliva

The moment samples are collected and tightly packed, they are sent to the laboratory immediately for analysis. Salivary examinations are different tests. [18]

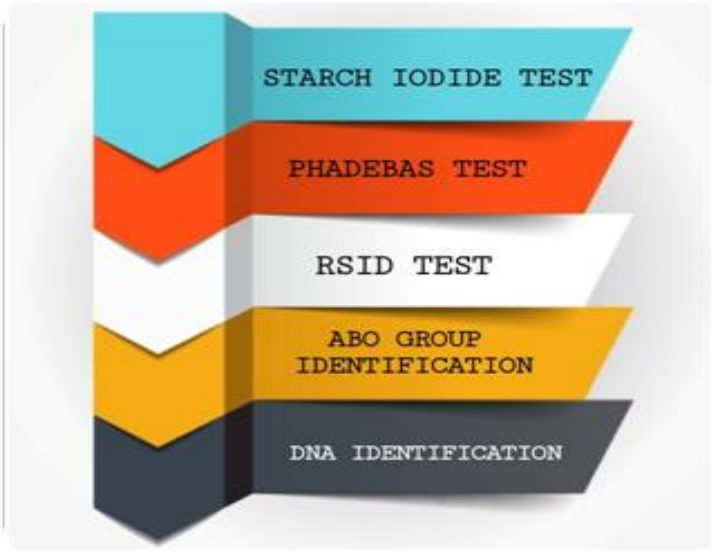

Figure 2.1. Forensic Examinations of Saliva

Amylase

- Unrestricted enzymes (Amylase) form in each animal and plant. They are accountable for the catalysis of the constituents of starch and amylopectin, amylose amylopectin into small, minute complex sugars.
- Amylases consist of two categories: the alpha and beta group: Beta amylase strikes exclusive bonds on the ends of the polyglucan chain, while alpha-amylase initiates bonds around chains. [18]
- Pigs, Primates, elephants, and some rodents possess high amounts of amylase.

Starch- Iodine Test: Reagent Formation

1. 0.5% dissolvable starch solution (50 mg dissolvable starch / 10 ml water (H2O)).
2. Solution: Lugol iodine Protocol
3. 3 tubes were placed in a rack and the following was added:
   a. A 5 mm x 5 mm piece of sample to be tested, was placed in the first tube.
   b. A similar sized unstained control piece was placed in the second tube.
   c. A 5 mm x 5 mm piece of a known saliva stain was placed in the third tube.
4. 3 drops of dissolvable starch solution were added to each tube.
5. The tubes were mixed, corked and incubated for 1 hour at 37ºC.
6. 2 drops of Lugol's iodine solution were added and note the color formation.

7. In the second and fourth tubes, a dark blue starch-iodine synthesis would be noticed. If the dark blue color does not appear, it implies that the starch was hydrolyzed and therefore no longer accessible for synthesis. However, if there were no blue color, it indicates a positive result for amylase activity, demonstrating the presence of saliva. But it is not a confirmation test for saliva. [18]

Note: To make starch dissolve, heat the solution to boiling point. keep the resolution to cool at room temperature. Make use of soluble starch always. Do not apply hydrolyzed starch. Do not use a starch solution over or past 24 hours, Starch solution freshly prepared is mandatory.

Phadebas Test

The efficacy that amylase breaks down starch is tested in this experiment. The Phadebas reagent consists of a dye linked to starch. Saliva breaks down the starch and allows the color to escape from the complex. Thus, the solution takes on a blue hue. This suggests that saliva is present. By measuring the intensity of the generated color at 620 nm wavelength, the test can be applied as a quantitative test. For quantitative data, a standard concentration curve with established colored dye concentrations can be created. [18] The amount of amylase in the sample directly correlates with the level of color.

Phadebas press Test

Six Phadebas tablets are dissolved in 30 milliliters of distilled water to create Phadebas paper. Filter paper sheets made of Whatman grade measure 46 by 57 cm. Using an atomizer, the solution is uniformly sprayed onto one side of the paper, then let to dry. The test object needs to be flat and in close contact with the paper.

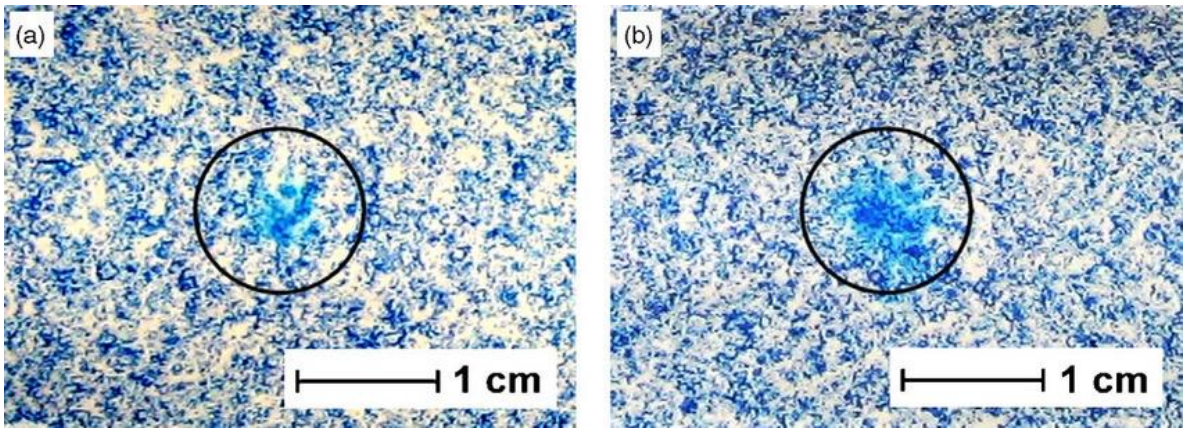

Figure 2.2. Phadebas Press Test

Immunochromatographic Test (RSID)

For testing saliva samples, immunochromatographic kits with commercial development are available. The antigen-antibody interaction principle serves as the method's foundation. The RSID saliva kit is one such kit. A pink line that appears in the RSIDsaliva kit indicates that saliva is present. With this method, saliva as little as 1 μL can be detected.

Amylase Radial Diffusion Test Reagent Preparation:

1. Prepare a pH 6.9, 0.1M phosphate buffer as follows:
   - 62.7 g of anhydrous NaH2PO4
   - 3.9 g of anhydrous Na2HPO4
   - NaCl: 0.2 g Distilled
   - water: 500 ml
2. Gel test plates with (0.1% soluble starch and 2% agarose)
   - pH buffer, 6.9–10.0 ml
   - 1.01g of soluble starch and
   - 0.2g of agarose

Stirring continuously while heating to boiling will ensure that all of the agarose is dissolved. Pour the gel solution into disposable 302" plastic petri dishes after dividing it. Allow the polymer to fully develop. Gels should be kept at 4ºC inverted to prevent dehydration. [18]

3. A remedy for iodine development

- KI 1.65 g,
- I2 2.54 g, and
- 30 ml of distilled water

At 65ºC, dissolve by stirring for 5 minutes. Keep saturated I2 solution in a bottle with a dark cork.

Working solution in distilled water diluted by 1/50.

Sample preparation: by extracting a portion of stained material of about 3 mm^2 with 50 ul of distilled water. When available, prepare an extract from a material that hasn't been discolored in the same way.

Procedure:

1. Use a vacuum pipette to make holes in the gel plate, leaving 1.5 cm between each sample well.
2. Employing a precision pipette, insert the test samples into the sample wells. Each well can accommodate about 4 µl of liquid.
3. Cover the petri dish and keep it in an incubator overnight or at 30ºC for 6 hours
4. To stain the plate, apply a saturated iodine solution diluted 1:50 to the surface. Rinse in water.
5. Areas of amylase activity are indicated by clear circles surrounding the wells. The square root of the amylase concentration determines the clear circle's diameter. [18]

1. Positive controls, first
   - known dilution of fresh liquid saliva (1/200, 1/500 in water)
   - known extraction of dried saliva stain in water (3 mm2 in 50 ul water)
2. Negative regulation: distilled water.
3. Unstained Control: 3 mm2 of untreated material extracted in 50 l of water.

Blood Group Antigens

Saliva samples can provide valid information on the assaulter's blood type, secretor status, and on the presence or absence of salivary amylase and other proteins.

Bite marks are potential sources of salivary transfer. Approximately 80% of humans are secretors of ABO blood group antigens in the saliva. Ninety percent of ABO DNA profiles can be obtained from epithelial cells deposited in saliva by genetic fingerprinting. However, degradation of genetic material with time produces a hindrance in DNA extraction from bite marks residue. [18]

2.4.1. Importance of Saliva as an Evidence

Saliva has recently attracted much attention among researchers, particularly in the branch of forensic sciences. Serological testing and analysis of salivary cellular components are being increasingly applied in crime detection, hormone identification, drug and alcohol abuse, cases of animal bites, and poisoning. Safe handling, along with the easy and noninvasive procedures of saliva collection, has increased its popularity in drug abuse tests. Sex determination and identification of the suspects in scenes of crime, with the help of cytological analysis and DNA profiling of saliva, are finding widespread applications in forensic investigations.

Pros of Saliva as evidence

- It is noninvasive.
- It can be recovered from the skin.
- It can be used for DNA isolation.
- It is used in detection of drug abuse.
- It can be used in bite mark analysis.
- Sex can be determined.

2.5. Saliva's Value as a Source of Evidence

Researchers have recently given saliva a lot of attention, especially those working in the field of forensic sciences. In cases of animal attacks, poisoning, drug and alcohol abuse, hormone identification, and crime detection, serological testing and analysis of salivary cellular components are increasingly used. Saliva collection has become more popular in drug misuse tests due to its safe handling practices as well as its simple and non-invasive processes. In forensic investigations, cytological examination and DNA

profiling of saliva are frequently used to determine the sex of suspects and to identify them at crime scenes.[18]

Benefits of using saliva as evidence

- There is no intrusion.
- It is retrievable from the skin.
- It can be applied to isolate DNA.
- It is employed to identify drug abuse.
- It can be applied to analyze bite marks.
- One can establish gender.

2.6. The SIRT6 Gene

By increasing DNA damage repair, preserving proper chromosome structure controlling energy metabolism, and regulating the senescence-associated secretory phenotype (SASP), the longevity protein SIRT6 can delay the aging of cells, tissues, organs, and the organism as a whole. SIRT6 also prevents immunosenescence, which is a symptom of aging [19]. Additionally, SIRT6 has the ability to control the onset of inflammation. The majority of the available research shows that SIRT6 reduces inflammation by inhibiting the generation of inflammatory cytokines and by encouraging the polarization of immune cells toward an immunosuppressive phenotype. For instance, it has been demonstrated that SIRT6 encourages macrophages to polarize to the M2 state. However, a few studies have shown that SIRT6 has pro-inflammatory action, which is evident in the enhancement of inflammatory immune cell infiltration and survival as well as inflammatory cytokine production. SIRT6 controls the progression of cancer, a condition linked to aging. At various phases of the cancer or in various tumor cell lines, SIRT6 plays conflicting functions as either an oncogene or a tumor suppressor in melanoma, breast, lung, pancreatic, liver, prostate, colon, ovarian, and blood cancers. It only exhibits antitumorigenic effects in glioma and only tumorigenic effects in bladder cancer, nasopharyngeal carcinoma, and osteosarcoma [19].

2.7. Where is SIRT 6 gene found:

Sirtuins, often referred to as silent information regulator (Sir2)-like family deacetylases, are a subset of histone deacetylases. These enzymes are widely expressed and can be found in the cytoplasm, mitochondria, or nucleus of the cell. [20]

2.8. Functions and Characteristics of sirt6 gene

SIRT6 is a nuclear protein that facilitates DNA repair, controls the expression of genes involved in metabolism, and upholds genomic integrity. SIRT6 has been characterized as a tumor suppressor and a pro-liferative regulator through these actions. A multifunctional protein with numerous enzymatic functions is SIRT6. As a class III histone deacetylase with deacetylation activity, SIRT6 is first classified [21]. SIRT6 can control gene expression or protein activity through post-translational modifications (PTMs) since its substrates are not just the acetyl groups on the lysine residues of histone H3 and H4 but also other proteins having acetyl groups on their lysine residues [21]. Second, SIRT6 controls the production of proteins like tumor necrosis factor alpha thanks to its defatty-acylation activity. (TNF-α) Last but not least, SIRT6 is a mono-ADP-ribosylation enzyme that can activate PARP-1, which aids in the repair of DNA damage [21]. The ability of SIRT6 to control a variety of physiological and pathological processes is based on these three enzymatic properties. Here, we quickly review the enzymatic properties of SIRT6, go into depth about the functions of these biochemical and molecular properties in aging, immunity, and cancer, and investigate the functions of SIRT6 in tumor immunology and epigenetic immunity. [21]

2.8.1. SIRT6's Enzymatic Properties

The family of Sir2-like proteins includes SIRT6. Based on their core domain sequences, mammalian sirtuins can be categorized into four groups. Class IV Sirtuins include SIRT6 and SIRT7 (29). Deacetylation, defatty-acylation, and mono-ADP-ribosylation are the three main enzymatic actions of SIRT6. These serve as a crucial foundation for its involvement in the control of physiological and pathological processes in mammals, including aging, immunity, and the onset and progression of cancer. [22]

2.8.2. Activity in Deacetylation

SIRT6 primarily controls the acetylation of lysines on proteins in the nucleus since it is found in the nucleus and only diffuses into the cytoplasm [22]. A NAD+-dependent deacetylase is SIRT6. Since SIRT6 adopts its active conformation via interacting with nucleosomes, nucleosomes are necessary for its deacetylation activity. SIRT6 binds to substrates and catalyzes deacetylation when histones H3 and H4 are bundled as nucleosomes as opposed to lose histones. SIRT6's substrates are histones H3 and H4's numerous lysine acetylation sites. To deacetylate acetylated H3K9 (H3K9ac), for instance, SIRT6 dynamically interacts with chromatin to control telomeric chromatin and prevent end-to-end chromosomal fusion and early aging of cells. By deacetylating H3K9ac and H3K56ac, SIRT6 can be attracted to the Pcsk9 gene's proximal promoter region by the transcription factor forkhead box O3 (FOXO3), preventing gene expression. In mouse oocytes, SIRT6 knockdown results in hyperacetylation of H4K16, which sharply reduces kinetochore-microtubule contacts and considerably raises the prevalence of aneuploidy. The effectiveness of SIRT6's deacetylation on various substrates varies. H3K9, H3K18, and H3K27 can all have their acetyl groups removed by SIRT6, however H3K4ac, H3K14ac, H3K23ac, H3K36ac, and H3K56ac cannot be efficiently deacetylated by SIRT6. The acetylated lysines of additional proteins, including as GCN5 K549ac, PKM2 K433ac, Ku70 K542ac, NAMPT K53ac/369ac, XBP1s K257/297ac, SOD2 K68/122ac, and p53 K382ac, are also substrates of SIRT6 in addition to histones [22].

2.8.3. Defatty-Acylation Activity

SIRT6 has defatty-acylation activity in addition to its deacetylation activity, which has come to be recognized as a mechanism regulating the secretion of numerous proteins. The lysosomal targeting and breakdown of TNF are facilitated by lysine fatty acylation [22]. The crystal structure of SIRT6 revealed a sizable hydrophobic pocket that could hold long-chain fatty acyl groups. By eliminating the fatty acyl modification from TNF-K19 and K20, SIRT6 encourages TNF-secretion (3). Additionally, SIRT6-knockout MEFs secrete a lot of ribosomal proteins, which has been linked to an increase in NIH 3T3 mouse embryonic fibroblast (MEF) proliferation [22]. A SIRT6-knockout mutant in

the same cell line displayed an increase in R-Ras2 lysine fatty acylation, which aided in the localization of R-Ras2 to the plasma membrane and encouraged its interaction with phosphatidylinositol 3-kinase (PI3K), thereby activating the Akt signaling pathway and boosting cell proliferation [22].

2.8.4. SIRT6 and Aging

An essential protein called SIRT6 has anti-aging effects on the body's cells, tissues, organs, and tissues. It prevents aging by promoting DNA damage repair, maintaining the correct telomere structure of chromosomes, controlling the metabolic balance between glucose and NAD+, and controlling SASP [22].

2.8.5. Physiology and Pathology of SIRT6 in Aging

The longevity protein SIRT6 contributes to the preservation of telomere and genomic integrity and slows the aging process. SIRT6-deficient mice had small bodies, lost subcutaneous fat, had deep lymphopenia, lordokyphosis, and severe metabolic abnormalities 2-3 weeks after birth, and eventually perished at about 4 weeks of age, according to a study at the organism level [22]. In SIRT6-deficient monkeys, hyperacetylation of histones near the developmental repressor H19's imprinting regulatory region causes severe prenatal developmental delays and early mortality several hours after delivery [22]. Transgenic mice that overexpress SIRT6 on the other hand live longer than wild-type mice [22]. The anti-aging properties of SIRT6 were also shown at the organ and tissue levels in a study, which also suggested SIRT6 as a possible indicator of ovarian aging. Its expression was favorably linked with the number of primordial follicles, and as people aged, so did the expression of the SIRT6 protein and their ovarian reserves [22]. Heart failure and cardiac hypertrophy symptoms were manifested in SIRT6-deficient mice, and SIRT6 expression was downregulated in failing human hearts [22].

SIRT6 prevents cell senescence, according to investigations done at the cellular level. Cardiomyocyte senescence and heart hypertrophy may be decreased by SIRT6 [22]. Older human dermal fibroblasts become more resistant to the conventional Yamanaka

factor-induced reprogramming with the addition of SIRT6 [22]. Human mesenchymal stem cells lacking SIRT6 showed rapid functional decline that was distinct from conventional cell senescence and was primarily characterized by redox metabolism issues and increased sensitivity to oxidative stress [22]. In contrast, chondrocytes' replicative senescence was reduced by overexpressing SIRT6.

2.8.6. Mechanism of SIRT6 in Anti-Aging

The anti-aging properties of SIRT6 can be explained by cell and molecular biology. First off, SIRT6 is a protein associated with nucleolar chromatin that functions in a variety of DNA damage repair mechanisms. Base excision repair (BER), which is related to SIRT6, has been shown to support DNA damage tolerance in mouse cells, inhibit genomic instability, and support typical DNA recombination [22]. Through mono-ADP-ribosylation activity, SIRT6 activates PARP1 and interacts with two BER enzymes (hMYH and hAPE1) to enhance BER. Nucleotide excision repair (NER) was made possible by SIRT6's recruitment to UV-induced DNA damage sites and deacetylation of damaged DNA binding protein 2 (DDB2) at K35 and K77, which encouraged DDB2's segregation from chromatin [22]. SIRT6 is drawn to DNA double-strand break (DSB) sites in human cells as a result of oxidative stress activating the protein kinase c-Jun N-terminal kinase and phosphorylating SIRT6 at serine 10. In response to oxidative stress, SIRT6 activates PARP1 by mono-ADP-ribosylating its K521 lysine. Genomic instability is prevented through chromatin remodeling, and damaged regions are made easier to repair thanks to SIRT6's recruitment of chromatin recombinant SNF2H to the DNA cleavage site [22].

Second, SIRT6 has the ability to preserve the regular chromosomal structure. For cells to slow down aging, chromosomal and telomere stability is crucial. Similar to the cell abnormalities seen in Werner syndrome, a condition that causes accelerated aging, SIRT6-deficient cells have aberrant telomere structures, such as increased chromosomal fragmentation, disconnected centromeres, and chromosomal gaps [22]. The stability of the genome is greatly influenced by SIRT6's deacetylation activity. To avoid replication-related telomere abnormalities, SIRT6 deacetylates H3K9ac, stabilizes the binding of WRN to telomeric chromatin, and guards against end-to-end chromosomal fusion and

early senescence of cells. To prevent cell senescence, SIRT6 deacetylates H3K9ac at the target gene's promoter through an interaction with the nuclear factor-B (NF-B) RELA subunit. In addition to encouraging H3K18ac deacetylation, SIRT6 inhibits aberrant pericentric transcript accumulation and silences pericentric heterochromatin at centromeres [22]. SIRT6 is crucial for preserving the structure of silent telomeric chromatin and for preserving the silencing of the telomere position effect in human cells. SIRT6, a potent retrotransposon L1 repressor, mono-ADP-ribosylates KRAB-associated protein 1 (KAP1) and encourages the interaction between KAPI and heterochromatin protein 1 alpha (HP1), which helps to pack L1 gene elements into heterochromatin and decrease their expression [22].

Thirdly, SIRT6 slows the aging process by controlling glucose homeostasis and the NAD+ metabolic balance, in addition to maintaining genomic integrity as previously described. In older mice, overexpression of SIRT6 promotes a "young state" of blood glucose and gluconeogenesis. By boosting lipolysis and elevating precursor levels for both the tricarboxylic acid (TCA) cycle and gluconeogenesis, SIRT6 maintains the immature stage of these two cycles. By boosting the expression of genes involved in de novo NAD+ production, SIRT6 also contributes to the maintenance of NAD+ levels. However, SIRT6 is a NAD+-dependent enzyme that uses NAD+ for mono-ADP-ribosylation, deacetylation , and defatty-acylation. NAD+ can slow the aging process by preventing P53 activation. Through mitochondrial malfunction, altering the NAD+/NADH ratio in cell solute may hasten aging. The research by Roichman and the review by Wiley [22] go into great depth on the conclusions mentioned above.

2.8.7. SIRT6 and Immunity

SITR6 controls the inflammatory process and has a variety of functions. It suppresses inflammatory activity by enhancing M2 macrophage polarization, reducing lymphocyte counts, suppressing T cell development, and suppressing innate immune response. However, it also encourages inflammatory responses by encouraging the movement of neutrophils and dendritic cells as well as the release of TNF-. SIRT6 may also control immunometabolism. Below, each of these will be covered in further detail. [22]

2.8.8. SIRT6 and cancer

A tumor's initiation, growth, progression, and metastasis are all influenced by two different factors: on the one hand, frequent DNA damage and mutation will result in canceration, which is a crucial support for the formation of tumors; on the other hand, aging-related chronic inflammation. Immunosenescence moreover promotes cancer cells' ability to evade immune system responses and progress toward malignancy. In order to preserve genomic stability and delay aging, SIRT6 serves a beneficial role, as was previously mentioned. SIRT6 obviously has a role in the control of cancer. Numerous pieces of evidence also show that SIRT6 is intimately connected to the occurrence and progression of cancer [22].

3. MATERIALS AND METHODS

3.1. Materials

- A total of 40 healthy volunteer participants
- Including male & female: 20 male, 20 female
- 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 group
- NO history of malignancy, autoimmune disorders, metabolic disorders, disorders.
- Also, no history of infectious diseases was reported in the past 6 months.

3.2. Salivary sample collection

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

3.3. Sample preprocessing

- Frozen salivary samples were thawed at room temperature
- did not use a water bath to expedite thawing,
- as this may degrade the RNA and compromise 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

- All procedures were performed in an RNase-free environment.
- assigned for RNA isolation using the modified TRIzol
- (Thermo Fisher Scientific) protocol,

3.5. Modified TRIzol and RNA Isolation Protocol

- 1 ml of TRIzol reagent was added to each pellet and pipetted several times,
- Vortexing for 20 seconds to homogenize.
- Incubate the samples at room temperature for 5 minutes
- Then Add 200µL of chloroform to each tube and vortexed for 20 seconds
- Incubate at room temperature for 3 to 5 minutes.
- The samples were then centrifuged at 13400 RPM for 20 minutes at 4°C.
- Transfer 700µL of the upper aqueous layer from each sample into a new 1.7-mL tube.
- These chloroform steps were repeated twice, and for each consecutive time
- less amount, 600µL in first and 500µL of upper aqueous layer in second repetition,
- were carefully transferred to the new tubes
- 500µL of cold isopropyl alcohol was added to each tube, vortexed for a few seconds.
- The tubes 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 again. Samples were then briefly centrifuged and
- Excess ethanol is removed using a pipette.
- The pellet was air dried at room temperature for at least 5 minutes,
- Resuspended in 20µL of DNase- and RNase-free water,
- Incubated in a 55°C water bath for 5 minutes.

- Samples vortexed briefly and used a quick spin to collect samples at the bottom of the tube.

3.6. cDNA Synthesis Procedure

Table 3.1. Remove genomic DNA response (on ice)

Reagent	Usage amount
10×gDNA Eraser Buffer	1.0 μL
gDNA Eraser	1.0 μL
Template RNA	0.5-5 μg
RNaser-Free Water	to 10.0 μL

*Incubate at 42 ° C for 2 min (or room temperature for 5 min) Store at 4 °

Table 3.2. Reverse transcription reaction (on ice)

Reagent	Usage amount
Step 1 reaction solution 10.0 μL	10.0 μL
oligodT（10 μM）	1.0 μL
or pd(N)9（10 μM）	or 1.0 μL
or Gene Specific Primers （10 μM）*1	or 1.0 μL
RNase-Free ddH2O	to 15.0 μL

After heating the mixture at 70 ° C for 5 min, it was quickly placed on ice for cooling. After a brief centrifugation, add the following components:

Table 3.3. Reagents and usage amount

Reagent	Usage amount
Step 1 reaction	solution
5×RT Buffer	4.0 μL
EntiLink™ Reverse Transcriptase* 2	1.0 μL
RNase Inhibitor* 3	1.0 μL

Table 3.4. 3 Reverse transcription program settings

Temperature	Time
25℃* 1	5 min
42℃	30min* 2
85℃	5 min

3.7. Real Time Pcr Protocol

Table 3.5. Reaction System

Composition	96 wells (20μL reaction system)
2 x SYBR Green PCR Master Mix	10μL
PCR Forward Primer (10 μM	0.4μL
PCR Reverse Primer (10 μM)	0.4μL
Template	
*50 x ROX Dye (Optional)	0.4μL
RNase-Free ddH2O	to 20μL

1. a 20μL or 50μL system to ensure the validity and repeatability of the amplification of the gene of interest.
2. Cover or seal the reaction tube/PCR plate and mix gently. It can be centrifuged slightly to ensure that all components are at the bottom of the tube.
3. Place the reaction system in a real-time PCR instrument, collect data and analyze the results. Setup your PCR instrument as shown in the table below. Optimum temperature of the incubation time can be determined by the specific situation.

Table 3.6. Three-step amplification procedure

Stage	Number of cycles	Temperature	Time
Pre-denaturation	1x	95℃	30 sec
Denaturation	35-40x	95℃	5 sec
Annealing		50-60℃	30 sec
extension		72℃	30 sec
Melting curve		Analysis Melting Curve stage)	

After cDNA and 20 ml-PCR reaction system has been synthesized, each 40 samples were divided into two parts and were added 2ul of GAPDH (control gene) and Sirt 6 gene respectively and was inserted into the REAL TIME PCR machine to process for 1hours, using the PCR three-step amplification procedure. To analyze the final result.

4. RESULTS

4.1. General results (Women + Men)

According to our results, when 4-9 years of age group was considered as the control, sirt 6 expression decreased approximately 2 times in the 14-19 age group, increased 1.75 times in the 24-29 age group, and decreased 2 times in the 30 years and older group.

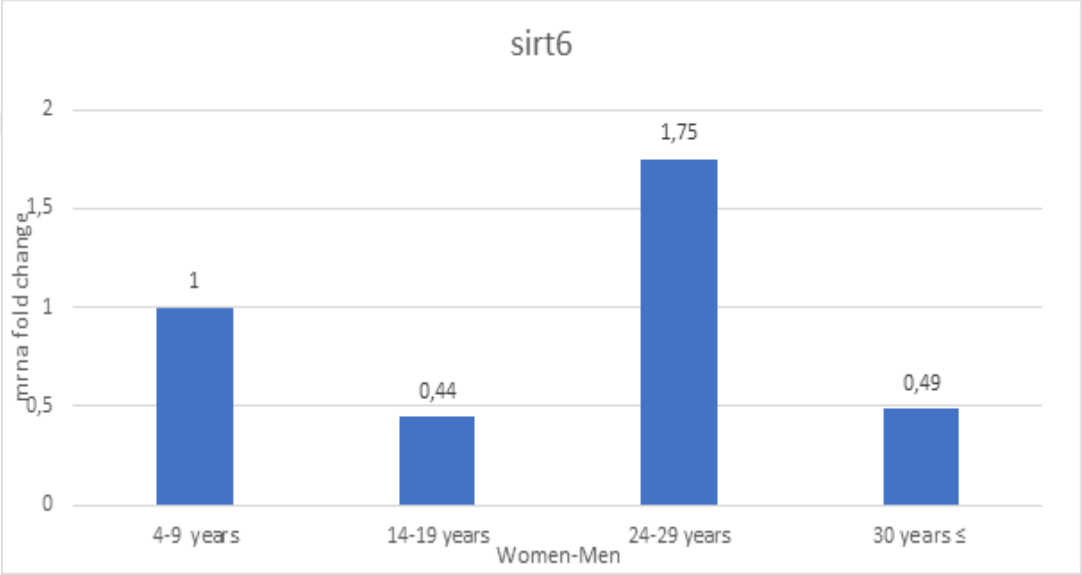

Figure 4.1. General Data Analysis of Women and Men

4.2. Women Findings

In women, when 4-9 years of age group was considered as the control, sirt 6 expression decreased approximately 1,3 times in the 14-19 age group, increased 4,76 times in the 24-29 age group, and increased nearly 1.2 times in the 30 years and older group.

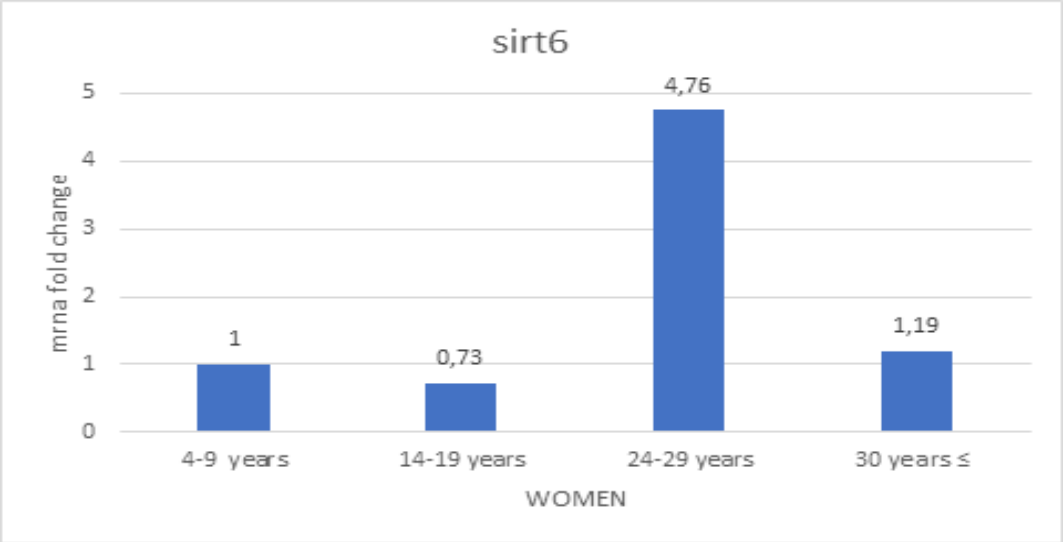

Figure 4.2. Data Analysis of Women

4.3. Men Findings

When 4-9yearS of age group was considered as the control, sirt 6 expression decreased approximately 2 times in the 14-19 age group, decreased 1.2 times in the 24-29 age group, and decreased nearly 7 times in the 30 years and older group.

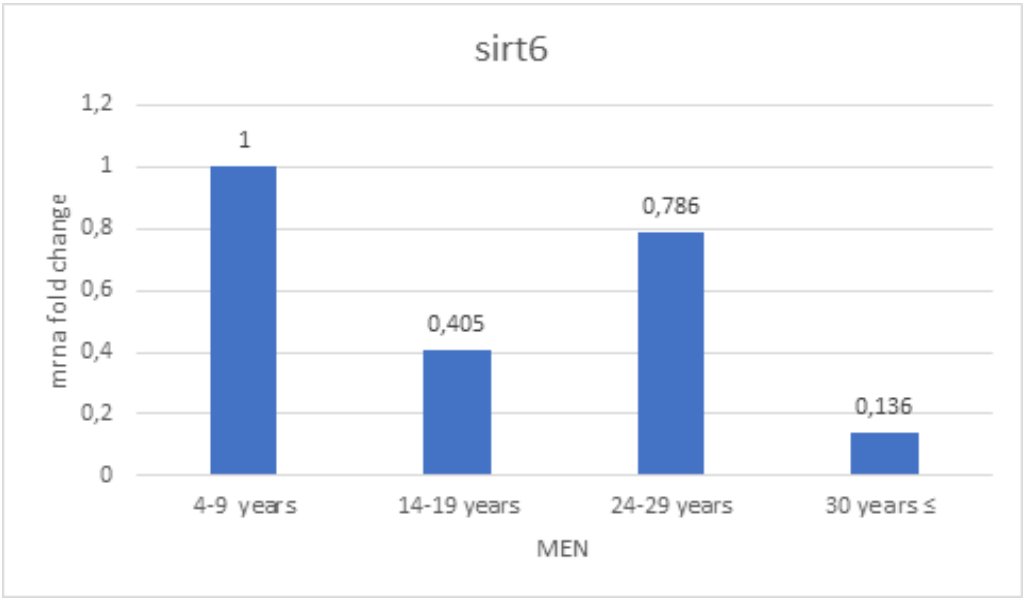

Figure 4.3. Data Analysis of Men

5. ARGUMENTS

Certainly, here are the arguments derived:

1. ***Longitudinal Studies for Age-Related Changes: *** Longitudinal studies should be conducted to track individuals over time and observe how SIRT6 expression changes within the same individuals as they age. This approach will provide valuable insights into individual trajectories of SIRT6 expression and age-related trends.

2. ***Exploring Molecular Mechanisms: *** Research efforts should be directed towards uncovering the molecular mechanisms that regulate SIRT6 expression. Investigating signaling pathways, epigenetic modifications, and interactions with other molecules will contribute to a comprehensive understanding of the reasons behind age-related variations in SIRT6 expression.

3. ***Tissue-Specific Analysis: *** By conducting analyses specific to different tissues or organs, researchers can determine if SIRT6 expression changes differently across various parts of the body. This approach will highlight potential tissue-specific roles that SIRT6 might play in the aging process.

4. ***Genetic Variants and SIRT6 Expression: *** There is a need to explore genetic variations that might be linked to changes in SIRT6 expression as individuals age. Understanding how specific genetic variants contribute to altered SIRT6 levels and their interactions with other factors can provide deeper insights.

5. ***Impact of Lifestyle and Environment: *** Further research is required to examine how lifestyle factors like diet, exercise, stress, and environmental exposures influence SIRT6 expression. Insights gained from these studies can potentially lead to interventions aimed at modulating SIRT6 levels for healthier aging.

6. ***Hormonal Influences on SIRT6: *** The impact of hormonal fluctuations on SIRT6 expression, especially in women, should be investigated. By understanding the correlation between hormonal changes and SIRT6 expression patterns, researchers can uncover gender-specific aspects of SIRT6 regulation.

7. ***Comparative Analysis Across Species: *** Comparative studies that analyze SIRT6 expression across different species can offer evolutionary insights into the role of SIRT6 in the aging process. By identifying age-related trends in various organisms, researchers can better understand the significance of SIRT6 in aging.

8. ***Clinical Implications of Altered SIRT6: *** It's important to explore how changes in SIRT6 expression with age might relate to age-related diseases such as metabolic disorders, neurodegenerative conditions, and cardiovascular diseases. Investigating potential associations can provide a basis for understanding the clinical relevance of SIRT6.

9**. *Effects of Interventions on SIRT6: *** Conducting studies to assess the effects of interventions, such as diet modifications, exercise routines, or pharmaceutical treatments, on SIRT6 expression can offer insights into strategies to modulate SIRT6 levels and potentially impact age-related outcomes.

10. ***Gender-Specific Differences: *** Further examination of gender-specific differences in SIRT6 expression is necessary, taking into account factors such as hormonal fluctuations and genetics. This research can provide a nuanced understanding of how gender influences SIRT6 regulation.

To summarize, the outlined arguments suggest that future research should overcome the limitations of the current study and explore the molecular, genetic, environmental, and clinical aspects of age-related changes in SIRT6 expression. These research directions hold the potential to advance our comprehension of aging processes and offer novel approaches for promoting healthy aging.

6. CONCLUSION

6.1. General Summary

Forensic science is a dynamic and indispensable field within the criminal justice system, encompassing diverse applications and technologies that aid investigators in unraveling mysteries, ensuring justice, and enhancing our understanding of human biology and behavior. Biomarkers, such as those associated with DNA methylation, have emerged as significant indicators for age estimation in forensic investigations. This thesis offers a comprehensive review of RNA extraction in forensic science. It showcases the value of saliva as an essential and often underutilized source of evidence in forensic investigations. The diverse applications of saliva, ranging from identifying blood group antigens to DNA profiling, underscore its significance in modern forensic research. This work serves as a valuable resource for researchers, practitioners, and anyone interested in the evolving landscape of forensic science. This review emphasizes its significance in forensic analysis, discussing its composition, potential as a source of DNA, and its role in human identification. Forensic applications of saliva as evidence take center stage, elucidating its importance in crime scene investigation.

SIRT6 is a longevity protein that exerts a multi-faceted impact on cellular processes. Through enhancing DNA damage repair, maintaining chromosome structure, regulating energy metabolism, and controlling inflammation, SIRT6 significantly contributes to delaying cellular aging and preventing immunosenescence. However, its functions are complex and context dependent.

6.2. Summary of the Methodology

The RNA isolation methodology involved collecting salivary samples from 40 healthy volunteers, evenly distributed between males and females, spanning different age groups. Participants gave informed consent. The process encompassed the following steps:

1. ***Salivary Sample Collection: ***

Participants refrained from eating or drinking for an hour before collection. Unstimulated saliva was gathered using the passive drooling method, frozen on dry ice within two hours.

2. ***RNA Extraction Protocol: ***

RNA isolation was performed under RNase-free conditions. TRIzol reagent was added to each sample, followed by chloroform. After centrifugation, the upper aqueous layer was transferred and subjected to chloroform treatment again. RNA precipitation was achieved with isopropyl alcohol. The RNA pellet was washed, dried, and resuspended.

3. ***cDNA Synthesis and PCR: ***

Subsequently, cDNA was synthesized, and the samples were divided into two sets, each containing GAPDH (control gene) or Sirt 6 gene. Real-time PCR was conducted to amplify the genes, using a three-step amplification procedure.

The protocol aimed to obtain high-quality RNA from salivary samples for subsequent gene expression analysis. This rigorous process ensures the integrity of the RNA molecules, allowing for accurate insights into the genetic markers under investigation.

6.3. Data Analysis of the Result

The present study aimed to investigate the variations in Sirtuin 6 (SIRT6) expression across different age groups in both women and men. The research analyzed data from individuals aged 4 to 9 years as the control group, comparing it with age groups ranging from 14 to 19, 24 to 29, and individuals aged 30 years and older. In the general population, it was observed that SIRT6 expression decreased approximately two-fold in the 14-19 age group compared to the control group. Interestingly, a subsequent increase of 1.75 times in SIRT6 expression was noted in the 24-29 age group, only to return to a decrease of two-fold in the 30 years and older group. Further analysis was conducted separately for women and men. Among women, SIRT6 expression decreased by around 1.3 times in the 14-19 age group, but exhibited a substantial increase of 4.76 times in the 24-29 age group. In the 30 years and older group of women, a moderate increase of nearly

1.2 times in SIRT6 expression was observed. In contrast, among men, SIRT6 expression mirrored the general trend with a two-fold decrease in the 14-19 age group compared to the control group. The 24-29 age group saw a decrease of 1.2 times in SIRT6 expression, while a significant decrease of nearly seven-fold was evident in the 30 years and older group of men.

These findings highlight age-related variations in SIRT6 expression, showing distinct patterns between men and women. The study contributes to the understanding of molecular changes associated with aging, providing valuable insights into potential gender-specific differences in SIRT6 regulation.

In conclusion, this research sheds light on the complex relationship between age, gender, and SIRT6 expression, offering a foundation for further exploration of underlying mechanisms and potential implications for age-related health conditions. These findings suggest that there may be age and gender-specific differences in the expression of SIRT6. Further research would be needed to understand the implications of these variations and their potential effects on health and aging.

The conducted experiment demonstrated a high level of excellence and precision. The participating volunteers, hailing from the specific geographical area of Adana, Turkey, generously provided their samples. These samples were meticulously collected from the dentistry departments of the university and analyzed within the confines of Çukurova University's Cumerlab.

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