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# Correlation of Human Telomerase Reverse Transcriptase Promoter (hTERT) Gene Methylation and Ageing

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**Abstract.** Promoter methylation of the hTERT gene in blood DNA has been proposed to be an epigenetic molecular clock because it is negatively correlated with ageing. Saliva is an alternative source of DNA because it contains buccal cells. Currently, the correlation of hTERT promoter methylation and ageing using saliva is not known. This study aimed to determine: first, the correlation between hTERT promoter methylation and ageing in saliva, as a source of non-invasive DNA sampling, instead of blood was determined. Second, the influence of sex on the methylation of the hTERT promoter in ageing was evaluated. A cross-sectional study design was used, and 119 subjects were recruited, consisting of 25 children (1-5 y.o), 42 teens (17-19 y.o), 16 adults (20-50 y.o), and 36 elderly (60 to 84 y.o). Promoter methylation of the hTERT of extracted DNA was determined using the MSRE (methyl-specific restriction enzyme) method. The relationship between age and the percentage of hTERT methylation was assessed using the Pearson test. The percentage of hTERT methylation in saliva DNA was negatively correlated with age r= -0.4305 (p-value <0.05). Negative correlation was also found in men (r= -0.376) and women (r=-0.43). Negative correlation between hTERT and ageing has been confirmed in saliva as a noninvasive sampling method. The benefit of this research in ethical and social considerations may encourage a greater participation in ageing research, particularly in underprivileged communities, thus democratising access to scientific advancements in longevity studies.

**Keywords**: Ageing; hTERT; Methylation; Saliva.

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## INTRODUCTION

In most living organisms, aging is the intricate and time-dependent degradation of physiological functions (López-Otín et al., 2013). In humans, life expectancy has increased rapidly in recent centuries due to significant improvements in medical care and public health awareness (Crimmins, 2015). These efforts cannot be separated from the study of molecular biology and epigenetics, where various complex factors cause ageing, such as DNA oxidative damage, telomere shortening, mitochondrial and nuclear genome mutations, particularly in DNA repair genes, decreased self-renewing stem cells, and other processes. Improving healthy longevity and reducing the incidence of age-related diseases requires understanding the mechanisms and causes of ageing. It is well-recognized that both genetic and environmental factors contribute to ageing (Farhud, 2022). Epigenetic profiling is an alternative way to explain age-related changes. Furthermore, an increasing amount of data indicates that many ageing-related symptoms are epigenetic in nature.

Epigenetic factors function as a bridge connecting intrinsic and extrinsic signals, influencing the regulation of gene expression without altering the DNA sequence (Foley et al., 2023). The most prevalent epigenetic changes include RNA-based pathways, histone modifications, and DNA methylation (Wang et al., 2022). One of the most researched epigenetic changes in recent years is DNA methylation, which is crucial to understanding because it affects a variety of biological processes, including

differentiation, development, genomic imprinting, and X-chromosome inactivation (Moore et al., 2013). A study revealed that cell survival is affected by demethylation caused by silencing of DNA methyltransferase enzymes (DNMTs: DNMT1, DNMT3A, and DNMT3B) (Moosavi & Ardekani, 2016). Research has indicated that ageing affects the DNA methylation of blood cells (Harris et al., 2020), muscle (Gim et al., 2023), and saliva (Hong et al., 2017). Equally exciting, an increasing amount of research indicates that parents can pass on environmental stimuli-induced variations in DNA methylation to their offspring. Some age-related diseases that involve epigenetic mechanisms are neurodegenerative, periodontitis, cancer, etc (Wulandari & Auerkari, 2018).

An epigenetic process called DNA methylation involves adding a methyl group to the DNA molecule, usually at cytosine bases when CpG dinucleotides are involved. Without changing the DNA sequence, this modification can have an impact on gene expression (Kumar et al., 2018). Telomerase is an enzyme that maintains the length of the telomeres. Its catalytic subunit is encoded by the human telomerase reverse transcriptase (hTERT) gene. Chromosome ends have repetitive nucleotide sequences called telomeres that shield the ends from deterioration and fusion with nearby chromosomes. Telomerase activity is crucial for cellular immortality and is typically active in germ cells, stem cells, and cancer cells, but not in most somatic cells. The hTERT gene promoter contains CpG islands, regions rich in CpG sites where methylation can occur. The methylation status of these CpG islands plays a critical role in the regulation of hTERT expression, such as hypomethylation and hypermethylation. The methylation status of the hTERT promoter is a key factor in determining whether the gene is expressed or silenced, with significant implications for cancer development and potential therapeutic interventions. hTERT influences telomerase activity and cellular ageing. This study aimed to determine the correlation of hTERT promoter methylation and ageing in saliva as a source of noninvasive DNA sampling instead of blood using the MSRE method and evaluate the influence of sex on the methylation of the hTERT promoter in ageing.

The implications of this research for societal development are significant, particularly in the fields of healthcare, aging research, and disease prevention. The confirmation that hTERT promoter methylation in saliva negatively correlates with ageing offers a potential noninvasive biomarker for biological ageing. This can help in the early detection and monitoring of age-related changes without the need for blood samples, making testing more accessible and less invasive. understanding methylation patterns in saliva could contribute to personalised healthcare**,** where ageing-related interventions and treatments can be tailored based on an individual's epigenetic profile.

## METHODS

### Study Design

The study used a cross-sectional approach. The methylation of the promoter of the extracted DNA hTERT was determined using the MSRE (methylspecific restriction enzyme) method. All respondents have completed the informed consent, and this study was approved by the Ethics Committee of the Research Institute of YARSI University (No. 076/KEP-UY/EA.20/IV/2024).

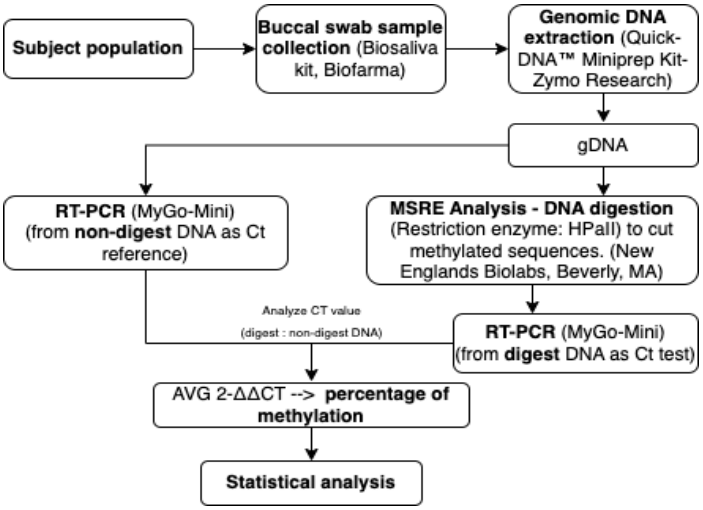


**Figure 1.** The workflow methylation study in ageing using the MSRE method

**Table 1.** Summary data from subjects in the methylation study

| Categories | Sex | | Amount | Age range (years) | Comorbid |
|---|---|---|---|---|---|
| Children | Male<br>Female | 12<br>23 | 25 | 1-5 | - |
| Adolescents | Male<br>Female | 11<br>31 | 42 | 17-19 | - |
| Adults | Male<br>Female | 3<br>13 | 16 | 20-50 | Hypertension, hypercholesterolemia, autoimmune, and gerd |
| Elderly | Male<br>Female | 2<br>34 | 36 | 60-84 | Hypertension, hypercholesterolemia, diabetes, and obesity |

**Buccal Swab Sample Collection**

Subjects were classified into four categories. The subject recruited 119 subjects, consisting of 25 children (1-5 years), 42 adolescents (17-19 years), 16 adults (20-50 years), and 36 elderly (60 to 84 years). There were 28 men and 91 women. All subjects were in good general health (Table 1).

**Genomic DNA Extraction**

gDNA was extracted using the viral nucleic acid extraction Kit II (Geneaid) following the manufacturer's instructions. The final concentration was quantified by UV-VIS spectrophotometer (Tecan Infinite M200 Pro) using a wavelength of 260/280. DNA samples were diluted to 20 ng/µL using EB buffer and stored at −20ºC until use.

**DNA Methylation Analysis and DNA Digestion**

The methylation-sensitive restriction enzyme (MSRE) is used to check the methylation status of cytosine residues in CPG sequences. Methylated DNA cannot be cut by this restriction enzyme, so methylated DNA remains intact. On the contrary, non-methylated DNA cannot be amplified by PCR, because they were cut by MSRE at certain unmethylated cytosine residues. The cutting site of the HPaII restriction enzyme is C/CGG (5'… C↓CGG… 3' and 3'… GGC↓C… 5'). For accurate PCR amplification results, the DNA must be fully digested (Hashimoto et al., 2007). As directed by the manufacturer (New England's Biolabs, Beverly, MA) with a slight modification, genomic DNA (40 ng) was fully digested using restriction enzymes in a total volume of 20 µL. Every restriction enzyme had its reaction, which was conducted separately.

**Real-Time PCR**

To compare the methylation levels in the various groups, we used a real-time PCR

technique. As a result, during the exponential phase of amplification, a precise number of PCR reaction cycles was determined. hTERT: utilized the master mix PCR component below both before and after digestion: 2 µL (20 ng) of each template were pipetted into a 23 µL Go Taq Green Master Mix (SYBR Green Master mix) from Applied Biosystem, USA, and 0.75 µL (10 nmol) of each primer, forward (5′-AGTGTTGCAGGGAGGCACT-3′) and reverse (5′-GCCTAGGCTGTGGGGTAAC-3′) with a size product of 267 bp (de Oliveira Bezerra et al., 2015a). RT-PCR was performed using MyGo Mini under the following conditions: Pre-denaturation: 95°C; ramp 3; hold 120 sec. 3-step amplification 35x: 95°C; ramp 3; hold 120 sec, 60°C; ramp 1,5; hold 30 sec, 72°C; ramp 3; hold 20 sec. Pre-melt hold: default. High-resolution melting: default.

The percentage of methylated DNA (amplifiable template left after HpaII digestion) was measured in relation to the undigested DNA (100%), in accordance with the real-time PCR principle of exponential sample amplification (i.e., a doubling of product at every cycle). The average CT of digested DNA was subtracted from the average CT of undigested DNA to determine the number of cycles (1/2)n, which represents the percentage of methylated sample (Gomes et al., 2006).

**Statistical Analysis**

The Pearson correlation test was used to assess the relationship between age and the percentage of hTERT methylation.

## RESULTS AND DISCUSSION

**Correlation of hTERT promoter methylation and ageing in saliva**

Epigenetic modifications like DNA methylation occur in people at different stages of their lives. The extent to which CpG sites in its promoter region are methylated is directly correlated with its. Based on our results, the percentage of hTERT methylation in saliva DNA was negatively correlated with age at r = -0.4305

(p-value < 0.05). (Table 2, Figure 2).

DNA methylation of the human telomerase reverse transcriptase (hTERT) gene in saliva can be influenced by ageing, and it typically shows a reduction in methylation levels with increasing age (Jung et al., 2019). A methyl group is added to the DNA molecule during DNA methylation, an epigenetic modification that frequently occurs at cytosine-phosphate-guanine (CpG) sites. This modification can regulate gene expression, typically repressing it when present in gene promoter regions. As individuals age, global patterns of DNA methylation change, which can affect various genes, including hTERT. Saliva is increasingly used as a noninvasive source for biomarker studies, including DNA methylation analysis (Nishitani et al., 2018). The ease of collection makes it suitable for ageing studies and monitoring epigenetic changes. The cells in saliva, primarily epithelial cells and leukocytes, can reflect systemic biological processes, including cancer, endometriosis, or ageing. Studies have shown that hTERT promoter methylation levels in saliva can decrease with age; this result is consistent with the previous study (de Oliveira Bezerra et al., 2015; Xiao et al., 2016). This reduction in methylation is believed to upregulate hTERT expression, potentially as a compensatory mechanism to maintain telomere length in the face of ageing-associated telomere attrition. The exact mechanisms behind this reduction are not entirely clear, but they likely involve age-related changes in the regulation of DNA methylation machinery and cellular responses to telomere dynamics (Chakravarti et al., 2021).

In gene regulation, DNA methylation generally acts to suppress gene expression. In young people, higher levels of DNA methylation help regulate the precise expression of genes necessary for development, differentiation, and maintaining cellular functions (Moore et al., 2013; Greenberg & Bourc'his, 2019). Proper gene regulation ensures that genes are turned on or off at the right times, preventing abnormal gene expression that could lead to developmental disorders or diseases. Furthermore, higher levels of methylation in younger individuals contribute to protecting the genome from instability, which could otherwise result in mutations and genomic disorders (Liu et al., 2023). Higher levels of DNA methylation in young people protect against the activation of oncogenes and other harmful genetic elements. This reduces the risk of early-onset diseases, including cancers. It also plays a role in immune function by regulating the expression of immune-related genes and ensuring proper immune responses.

Hypomethylation in elderly individuals can lead to increased genomic instability, activation of transposable elements, and aberrant gene expression (Mutirangura, 2019). Despite global hypomethylation, specific regions, particularly gene promoters and CpG islands, can undergo hypermethylation with age. This may result in the silencing of important genes, including tumour suppressor genes, which could then contribute to age-related diseases such as cancer (Cheng et al., 2016).

The close connection between DNA methylation and ageing has been demonstrated by numerous studies conducted in recent years. According to earlier studies, methylation levels worldwide decline with age. Based on our results, the correlation of hTERT gene methylation with ageing showed that the percentage of hTERT methylation in saliva DNA was negatively correlated with age r= -0.4305 (p-value < 0.05). (Table 2, Figure 1.) according to the results of previous studies, normal diploid fibroblast cultures from mice, hamsters, and humans showed a significant reduction in the genomic 5mC content at the cellular level (He et al., 2021). This finding was also confirmed in other cell types, including lymphocytes (Deng et al., 2018)and T cells (Zhao et al., 2022). At the organismal level, it was reported that in mammals, the rate of demethylation was inversely related to life span, and the rate of total DNA methylation decreased with age (Lu et al., 2023). Furthermore, research has shown that while the 5mC content of DNA does not alter in the liver or lungs, it does alter in the brain, heart, and spleen (Unnikrishnan et al., 2018). Similarly, human blood was found to have less genomic methylation (Heyn et al., 2012). In addition, locus-specific hypermethylation has been associated with ageing. Additionally, research has shown that while ageing-associated DNA hypermethylation occurs at CpG islands and the promoters of bivalent chromatin domains, global loss of methylation typically occurs at repetitive genomic sequences (Rakyan et al., 2010).

The age-related decrease in methylation levels is believed to be due to several factors. The global decrease in methylation could be due to the downregulation of the enzyme Dnmts (Ciccarone et al., 2016), which is responsible for transferring methyl groups from the universal methyl donor to the 5-position cytosine residue in DNA, leading to the transcriptional silencing process. In addition,

decreased methylation can also be influenced by the environment, such as a lack of folic acid availability in elderly subjects (Kok et al., 2015), metal exposure (Elkin et al., 2022), UV light (Page et al., 2020), air pollution (Prunicki et al., 2021), smoking facilitates global hypomethylation (van Dongen et al., 2023) etc.

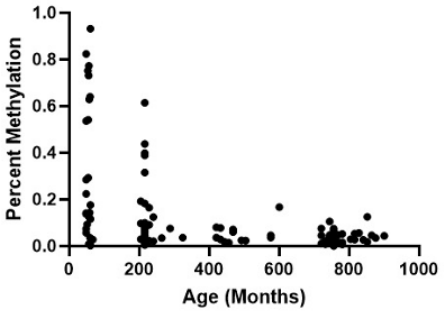


**Figure 1.** Scatter plot (Pearson R = -0.4269, N = 119), y-axis: percent methylation and x-axis: age (months). The figure was visualised by GraphPad Prism V8.0.

**Table 2**. Correlation coefficients of age vs. percent methylation

| R | Age (Months) | Percent Methylation |
|---|---|---|
| Age (Months) | 1.0000 | |
| p-value (2-tailed) | | |
| Percent Methylation | **-0.4269** | 1.0000 |
| p-value (2-tailed) | 1.2880E-6 | |

The correlation in bold is significant at the level (2-tailed). N of valid cases = 119

**The influence of sex on the methylation of the hTERT promoter in ageing.**

The influence of sex on hTERT methylation in ageing can be an important factor in understanding differences in ageing processes and susceptibility to disease between men and women. The relationship between gender, percentage of hTERT methylation, and ageing involves complex interactions influenced by genetic, hormonal, and environmental factors.

In this research, the negative correlation of hTERT and ageing was found in men (r = -0.376) and women (r = -0.43) (Figure 3, Table 3). More studies may be required using case-control designs to determine the correlation between hTERT methylation and chronic diseases in the elderly population. There are gender variations in the ageing process, and women typically live longer than men (Huang et al., 2024). Horvath 2013, showed that the epigenetic clock uses DNA methylation levels to estimate biological age. Horvath's work revealed that the epigenetic ageing rates differ between genders, with women generally showing slower epigenetic ageing rates compared to men (Horvath, 2015). According to Yuan et al. (2015) sex-specific differentially methylated regions (sDMRs) change with age. They discovered that men and women have different sets of sDMRs, indicating gender-specific aging trajectories in DNA methylation (Yuan et al., 2015). Gender differences in DNA methylation patterns show that men and women exhibit distinct DNA methylation patterns across the genome. Due to genetic differences, men only have one X and one Y chromosome, and women have two X chromosomes. Some genes, including DNMT1 (DNA methyltransferase 1), are found on the X chromosome and are involved in the regulation of DNA methylation. Differences in X-linked gene expression can influence DNA methylation patterns, including those at the hTERT promoter. Meanwhile, men have a single X chromosome, which can make them more susceptible to mutations or variations that affect methylation regulation, potentially leading to gender-specific methylation patterns at the hTERT promoter.

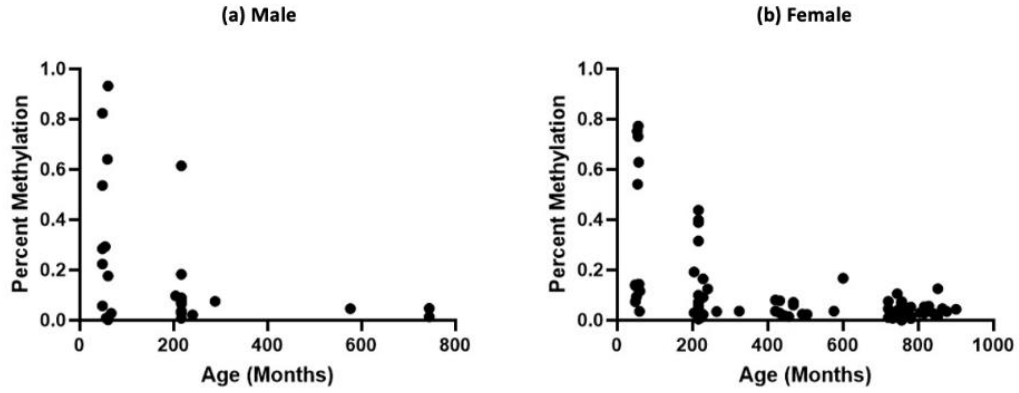


**Figure 3**. Scatter plot sex (a) Male (Pearson R=-0.3768, N28) (b). Female (Pearson R=-0.4309, N 91. Y-axis: percent methylation and x-axis: age (months). The figure was visualized by GraphPad Prism V8.0.

**Table 3**. Correlation coefficients of percent methylation vs age based on gender

| R | Age (Months) | Percent Methylation |
|---|---|---|
| Age (Months) | 1.0000 | |
| p-value (2-tailed) | | |
| Percent Methylation (Male), N28 | **-0.3768** | 1.0000 |
| p-value (2-tailed) | 0.0510 | |
| Percent Methylation (Female), N91 | **-0.4309** | 1.0000 |
| p-value (2-tailed) | 2.1205E-5 | |

The correlation in bold is significant at the level (2-tailed). N of valid cases = 91. In males, not barely significant because of the least sample.

To analyse DNA methylation patterns in men and women of various ages, (Hannum et al., 2013) found that age-related changes in DNA methylation were more pronounced in men compared to women using whole-genome bisulfite sequencing. The methylation patterns in men and women showed that men generally exhibit higher levels of methylation at the hTERT promoter compared to women. Higher methylation is associated with lower telomerase activity and decreased expression of hTERT, which accelerates telomere shortening. As a result of higher expression of hTERT and higher telomerase activity, women generally have lower levels of methylation on the hTERT promoter. This difference can contribute to slower telomere shortening and potentially slower biological ageing. Based on hormonal influences, in women, oestrogen can influence DNA methylation patterns through activation of estrogen receptors, which interact with the DNA methylation machinery (Rawłuszko-Wieczorek et al., 2022). Oestrogen has been shown to protect against global DNA hypomethylation and can modulate the methylation status of specific genes, including hTERT. The protective role of oestrogen can help maintain the length of telomeres and reduce the rate of telomere shortening with age. Meanwhile, in men, testosterone and its derivatives have a less pronounced effect on telomerase activity compared to oestrogen. Additionally, androgens in men can also affect DNA methylation patterns in different ways compared to oestrogen (Harbs et al., 2023). Differences in hormone levels and receptor activity between men and women can lead to gender-specific methylation changes at the hTERT promoter. This could contribute to the observed higher levels of methylation and the reduced telomerase activity in men.

The novelty of this study is the first to confirm a negative correlation between hTERT promoter methylation and ageing in saliva, establishing saliva as a non-invasive biomarker for epigenetic ageing research using buccal swab samples. It also introduces a sex-based perspective on methylation patterns, paving the way for more personalized ageing studies. The contribution of research for the science or society could lead to broader ethical and social applications, making epigenetic research more accessible and impactful, contribute to early detection of age-related diseases, allowing for better prevention and intervention strategies, helps democratize access to scientific advancements in longevity and public health, hTERT methylation is linked to cellular aging and disease risk, this study lays the groundwork for future research on chronic diseases in elderly populations, further studies could explore its role in conditions like cancer, neurodegenerative disorders, and cardiovascular diseases, leading to improved diagnostics and treatment approaches.

## CONCLUSION

This study confirmed a negative correlation between hTERT promoter methylation and aging using saliva, applicable to both men and women. Future studies with case-control designs are suggested to investigate the correlation of hTERT methylation with chronic diseases in the elderly.

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## AUTHOR CONTRIBUTION STATEMENT

SN contributed to the conceptualization, data collection, analysis, and drafting of the manuscript. WS was involved in methodology design, data analysis, and manuscript editing. Y assisted with data collection and review of the research findings. IR participated in investigation and data processing. KP contributed to methodology and drafting of the initial manuscript. ARHU provided supervision,

conceptual guidance, as well as final editing and critical review of the manuscript

## CONFLICT OF INTEREST STATEMENT

The authors declare that there is no conflict of interest regarding the publication of this paper.

## USE OF ARTIFICIAL INTELLIGENCE (AI)-ASSISTED TECHNOLOGY

The authors declare that no artificial intelligence (AI) tools were used in the generation, analysis, or writing of this manuscript. All aspects of the research, including data collection, interpretation, and manuscript preparation, were carried out entirely by the authors without the assistance of AI-based technologies.

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