Telomere length distribution in blood and saliva by RT-PCR in age-varying Thais: A Pilot Study

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Abstract:

The old-age populations are increasing in number in virtually every country. Identifying reliable biological indicators of virtual aging is a key objective in geroscience. Telomeres, the DNA–protein structures located at the ends of chromosomes, have been convincingly proposed a biomarker of aging. Epidemiologic studies show an association between telomere length (TL) in leukocyte and aging. This study investigated TL in blood leukocytes and cells in saliva samples of 108 healthy Thai population at various age ranges. Subjects were divided into four groups (I:21-40 years, II:41-60 years, III:61-80 years and IV:80 years up), The extracted DNA was analysed for TL by qPCR. The mean length of the telomere were found statistically significant different among age groups and decrease with increasing age (p-value < 0.05) in both blood and saliva samples. The means of TL in I, II, III, and IV in blood were 1.03±0.016, 0.92±0.022, 0.82±0.028, and 0.56±0.100, respectively; whereas in saliva were 1.07±0.021, 0.95±0.022, 0.82±0.028, and 0.59±0.040, respectively. In addition, the relationship of TL in blood and saliva was R = 0.418, p-value = 0.00. The preliminary results demonstrated consistent telomere length measurements in saliva as an alternative peripheral source compared to blood. Saliva could be considered as a non-invasive and as a reliable source of DNA for measuring telomere length.

Received: 26 August 2019
Revised: 11 October 2019
Accepted: 22 December 2019
Online publication date: 7 January 2020

Introduction

Aging is associated with multifactorial process that is influenced by genetic, individual lifestyle, and environmental factors. The rate of deterioration or aging depends on various factors such as genetic, environment, behavior, and socioeconomic conditions. Therefore, it is useful to study innovative technique in order to measure biological aging, which could be used as an index to assess the status of the body compared to the actual age.

Telomeres, the specific DNA–protein building blocks and are composed of tandem hexameric nucleotide repeats of the six-nucleotide sequence, 5'-TTAGGG-3', located at both ends of each chromosome. The function of telomeres is to protect the natural ends of chromosomes from being recognized as damaged DNA that may occurred from, for example, nucleolytic degradation, senseless recombination, repair, and fusion of inter-chromosome (Shammas, 2011), hence contributing to chromosomal stability. Telomeres therefore play a crucial role in preserving the genetic information in human genome. During cell division, a small number of telomeric DNA could be lost. Once telomere length becomes critically shorten, the cell lost its function and eventually undergoes apoptosis. Telomere length may then be considered as a biological clock to determine the lifespan of an organism. Certain compounds and factors associated with distinct lifestyles may accelerate telomere shortening by inducing DNA damage in general or more specifically at telomeres and may therefore affect health and lifespan of an individual. Other possibly factors influence on aging include alcohol consumption (Dixit et al., 2019), stress (Mathur et al., 2016), diet (Pérez et al., 2017), exercise (Puterman et al., 2010), disease (Calado & Young, 2012), medication (Newton et al., 2019; Tsoukalas et al., 2019) and genetics (Mirabello et al., 2010).

Telomere measurement for biomarkers of cellular aging received considerable attention, particularly following Nobel Prize in Physiology or Medicine 2009 in which discovering how chromosomes are protected by telomeres and the enzyme telomerase (Chan & Blackburn, 2004). The rate of telomere shortening may either increased or decreased influenced by certain lifestyle factors and environment. Telomere shortening during aging is associated with increasing incidence of diseases, obviously by cancer (Jafri, Ansari, Alqahtani, & Shay, 2016). Richard Cawthon and colleagues discovered the important findings that people with longer telomeres have longer lives than those with short telomeres (Richard M. Cawthon, Smith, O'Brien, Sivatchenko, & Kerber, 2003) and those with longer telomeres live with better health (Terry, Nolan, Andersen, Perls, & Cawthon, 2008).

In addition, telomere shortening, has also been shown to associated with atherosclerosis and cardiovascular aging (Yeh & Wang, 2016). Indeed, accumulated evidences suggested that age-dependent telomere shortening could be used as a biomarker for coronary artery disease and hypertension (Yang et al., 2009), related diseases or syndromes, such as insulin resistance (Gardner et al., 2005), diabetes mellitus (Wang et al., 2016), obesity

and weight loss, (Welendorf et al., 2019), myocardial infarction (Rezvan, 2017), and Alzheimer's (Liu et al., 2016). However, it is not certain known whether short telomeres are just a sign of cellular aging or essentially contribute to the aging process. Similar observation were considered in relation to group of subjects who are exercising on a regular basic have the telomere length longer than those subjects who did not exercise (Puterman et al., 2010). Besides, subjects with high stress have shorter telomere than the group of those having low stress. Therefore, telomere length may be a potential target for a marker of biological aging. Studies in humans strongly suggest that leukocyte telomere length acts as a marker of biological aging. More detailed examinations of mean leukocyte telomere length by age in the earlier studies show that the regional telomere length difference declines at older ages. However, the social and economic determinants of telomere length remain unclear (Needham et al., 2013).

The world's population is aging, elderly group is increasing in number and constitute a growing share of the population in virtually every country. The population of Thailand is entering aging society since 2004 and will enter a super-aged society in the year 2031(WHO, 2012), which has been estimated that the elderly population will increase dramatically as the rank in the future. The elderly is the group that started the decline of the body functions, therefore, health problems increased with advancing age. Common problems of the elderly are cardiovascular diseases, high cholesterol, cancers, diabetes, hypertension, depression, Alzheimer's disease, bone disease and arthritis, etc. The present study aims to fulfil the knowledge gap about health in Thais by studying the telomere length, a marker that is considered an indicator of biological aging. Investigation of the average telomere length in healthy populations may be useful in the surveillance of the elderly population of Thai population. Moreover, the study involved possibility of using minimal invasive technique i.e. saliva for measuring the telomere length.

A precise, reproducible, and simple methods intending to measure telomere length are highly required both in the laboratory and in clinical application. Three main methods are available: Southern blot, which is the gold standard method, but is labor intensive, time consuming, and requires large DNA quantities; flow-FISH, which combines flow cytometry and fluorescence *in situ* hybridization, but is labor intensive and requires intact cells for analysis; and quantitative PCR (qPCR), which requires low quantities of DNA, but the method requires several reagents to stabilize the reaction in a "laboratory-developed" mastermix. The study considered using a simplified and automated qPCR method to measure telomere length using standardized, commercially available chemistry.

Subjects and Methods

1. Study populations

One hundred and eight healthy subjects were included in this study. The subjects were interviewed regarding demographic information, marriage status, education, income, smoking habit, alcohol use, physical activities, medicine history, diet and medical history. Informed consents were obtained from all subjects. The study was approved by the "Ethical Clearance Committee on Human Right Related to Researches Involving Human Subjects", Rangsit University. Blood pressure was measured, after a 5-min rest, on the right arm in the sitting position with a standard mercury sphygmomanometer. Blood was collected from the antecubital vein for 2 ml and saliva samples were collected for approximate1ml.

2. Telomere length assay

An anticoagulant K_3-EDTA (Sigma-Aldrich, Germany) was used as anticoagulant for blood samples, whereas fresh or frozen saliva was used. Genomic DNA was extracted and purified by Macherey-Nagel™NucleoSpin® kits according to the manufacturer's manuals. DNA was quantified by NanoDrop (Thermo Fisher Scientific, Germany) and diluted to a final concentration of 2.5 ng/µl. Telomere detection was performed as previously described (R. M. Cawthon, 2002) with modification. Telomere length ratio (T/S ratio) was measured from DNA, using a real-time quantitative polymerase chain reaction (qPCR)-based assay that compared the ratio of expression level of telomere sequence copy number (T) to single-copy gene copy number (human β-globin gene (T/S) with the same calibrator sample. The primer sequences (5′⟶3′) used for the telomeres and the human β-globin gene were as follows:

tel 1, GGTTTTTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGT;
tel 2, TCCCGACTATCCCTATCCCTATCCCTATCCCTATCCCTA;
36B4u, CAGCAAGTGGGAAGGTGTAATCC;
36B4d, CCCATTCTATCATCAACGGGTACAA.

The thermal cycling profiles for both amplicons began with 35-cycle, 61 min. qPCR was performed on the Rotor-Gene Q real-time instrument with the QIAGEN Rotor-Gene SYBR Green Kit. gDNA from human peripheral cells was diluted at 1:2 in seven serial dilutions in the range of 0.1 ng – 6.25 ng/ PCR in a 20-µl reaction volume. The master mix was prepared with SYBR Green I 5 µl/1xrxn, primer forward 0.1µl/1xrxn, primer reverse 0.1 µl/1xrxn, H_2O 3.8 µl/1xrxn and DNA 1 µl/1xrxn. The PCR condition used in the assay was: 95°C 5s; 98°C 7s, 58°C 10s (35 cycles).

The test results were calculated by the $2^{-\Delta\Delta CT}$ Livak method (Livak & Schmittgen, 2001). The data was analyzed by SPSS version 17.0 software. The statistically data is displayed as mean and standard error (Mean ± SE) comparing the average telomere length

with age in various ranges, considering the differences statistically significant at *p* value ≤ 0.05.

Results

1. Telomere length measurement distribution in blood leukocyte

Analysis of the relationship between age and telomere length in blood as shown in figure 1. The figure shows that the length of the telomere (T/S) is statistically significant decrease with age (year) increasing (R = 0.433, *p* value = 0.001).

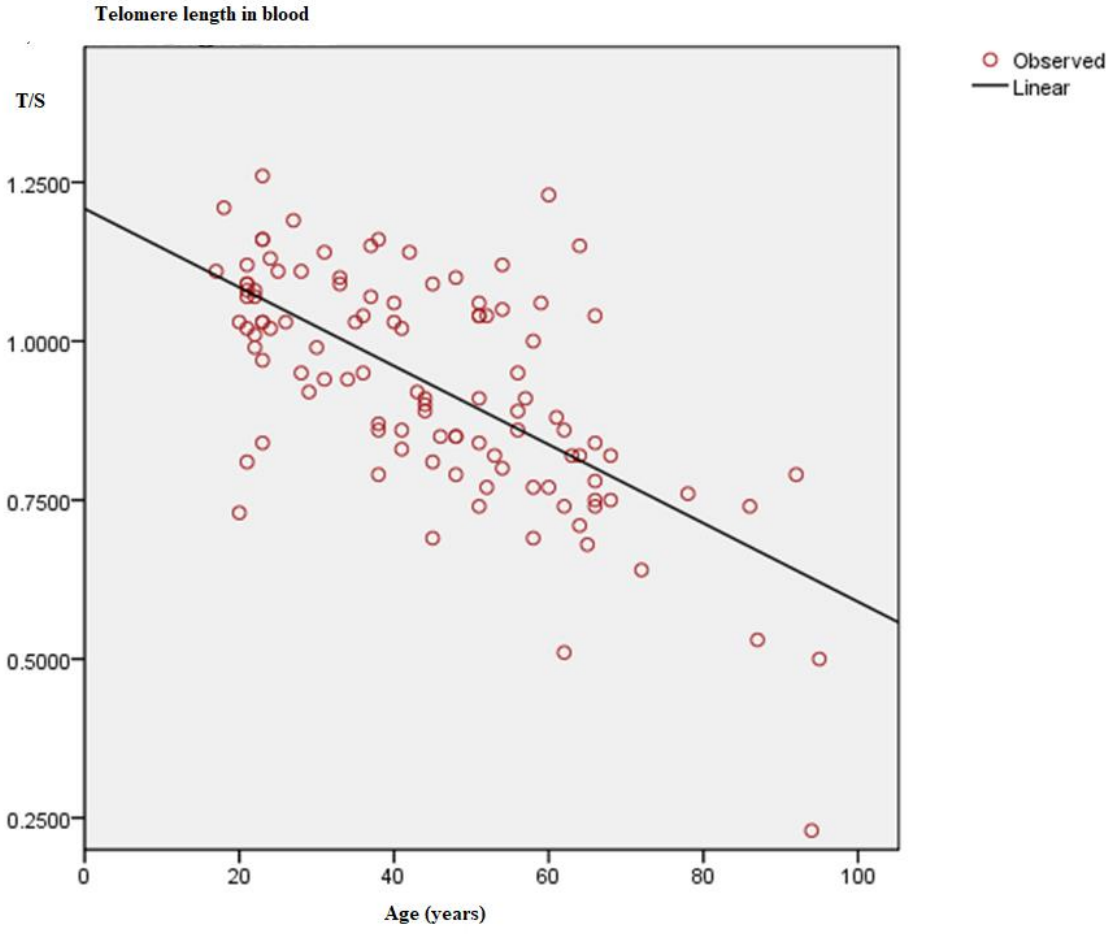

Figure 1 Distribution of telomere length (T/S) in blood of volunteers with age ranging from 21-80 yrs.

2. Telomere length measurement distribution in saliva

Analysis of the relationship between age and telomere length in saliva as shown in figure 2. The figure shows that the length of the telomere (T/S) is statistically significant decrease with age (year) increasing (R = 0.474, *p* value =0.001).

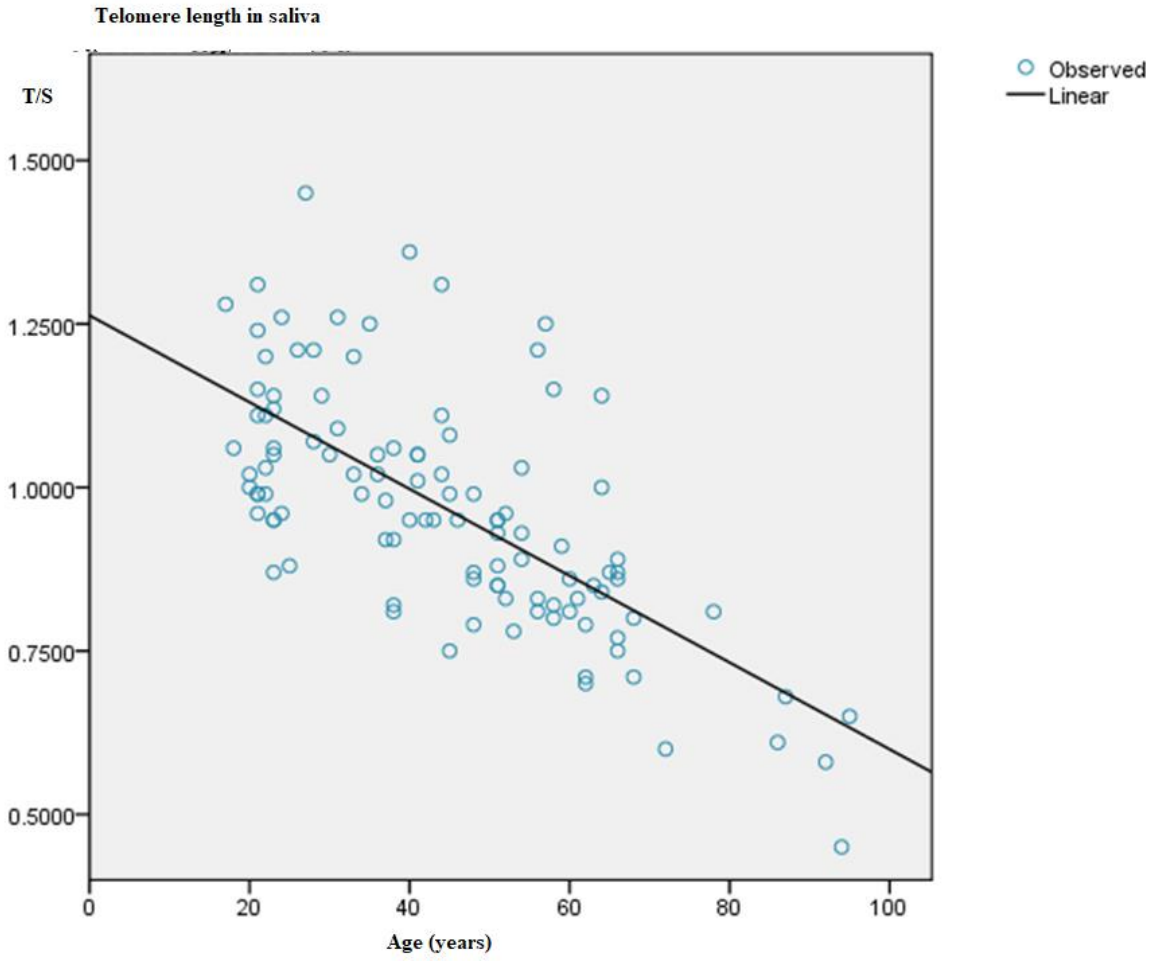

Figure 2 Telomere length in saliva. Distribution of telomere length (T/S) in saliva from volunteers with age ranging from 21-80 yrs.

3. Relationship of telomere length in blood and in saliva

Analysis of the relationship between telomere length in blood and telomere length in saliva is shown in figure 3. The figure shows that the length of the telomere is statistically significant decreased with age increasing (R = 0.418 *p* value =0.00).

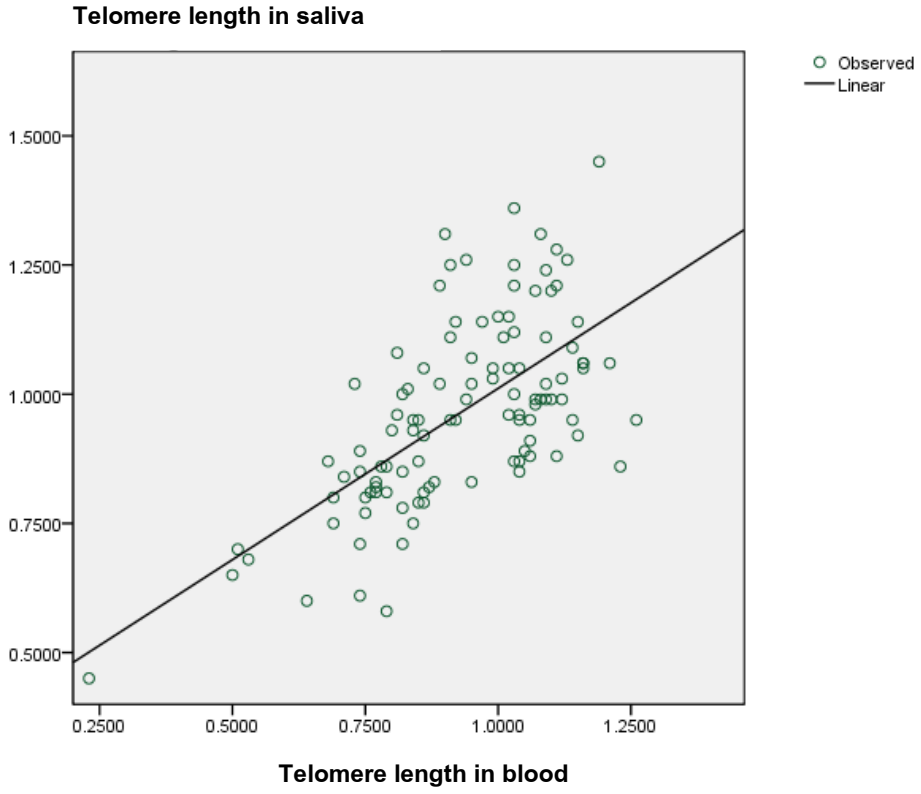

Figure 3 Relationship of telomere length (T/S) in blood and saliva samples from 21 - >80 yrs volunteers.

4. Telomere length in different age group

The table 1. shows the relative between telomere length in saliva and age-varying groups. The subjects were divided into 4 age ranges i.e. I, II, III, and IV. The length of the telomere decreased with increasing age group statistically significant at *p* value = 0.001. Table 3.2 shows the mean TL in blood compared to saliva. The means of TL in I, II, III, and IV in blood were 1.03±0.016, 0.92±0.022, 0.82±0.028, and 0.56±0.100, respectively; whereas in saliva were 1.07±0.021, 0.95±0.022, 0.82±0.028, and 0.59±0.040, respectively.

Table 1: Telomere length in blood and saliva in different age group

Age range	N (person)	TL (blood) * T/S (mean± SE)	TL (saliva) ** TS (mean ± SE)
21 - 40 years old	47	1.03 ± 0.016	1.07 ± 0.021
41 - 60 years old	38	0.92 ± 0.022	0.95 ± 0.022
61 - 80 years old	18	0.82 ± 0.028	0.82 ± 0.028
> 80 years old	5	0.56 ± 0.100	0.59 ± 0.040
Total	**108**		

* average mean ± SE = 0.93 ± 0.017 with minimum-maximum range of 0.23 – 1.26

** average mean ± SE = 0.97 ± 0.017 with minimum-maximum of 0.45-1.45

5. Mean of telomere length in blood and telomere length in saliva in all age groups

Then mean telomere length in blood and saliva was 0.93±0.017, and 0.97±0.017, respectively. It was shown that both the minimum and maximum telomere length in saliva were higher than in blood.

Discussion

The present study investigated telomere length in blood leukocytes as compared to cells in saliva samples to determine whether saliva could be used for an alternative non-invasive source for telomere length measurement. The study was conducted in healthy Thai population at various age ranges. Telomere length ratio (T/S ratio) was measured from DNA, using a simplified and automated qPCR method instead of Southern blot, which is although the gold standard method, but is labor intensive, time consuming, and requires large DNA quantities. The method was demonstrated to be highly reproducible and accurate for human peripheral cells.

The result shows that telomere length is associated with age that is statistically significant decreased with increasing age at *p*-value ≤ 0.05, and in the same manner of blood and saliva samples. The result corresponds to previous study, which reported the telomeres in somatic cells are progressively shortened with aging and illustrating of the relationship between the telomere length and other factors which may affect the frequency of cell divisions (Guan *et al*., 2007; H. Satoh *et al*., 1996). In addition, it was found in the present study that telomere length was not consistent well with age particularly for very old subjects i.e.> 85 years (data not all shown, some data was excluded from the study). This observation was corresponding to a previous study suggesting that TL could not be used as a predictive indicator for age-related morbidity and mortality at ages over 85 years, possibly because of a high degree of telomere length instability in this group.

Cumulative accelerated cell division of leukocytes in inflammatory conditions and continuous oxidative stress with aging are currently regarded as the contributing factors for telomere-shortening with aging (Guan *et al*., 2007; M. Satoh *et al*., 2008). Oxidative stress has been reported to increase in elderly subjects, possibly arising from an uncontrolled production of free radicals by ageing mitochondria and decreased antioxidant defenses (Andriollo-Sanchez *et al*., 2005). It is believed that oxidative stress (OS) plays a vital role not only in the aging process, but also in many degenerative diseases including Alzheimer's disease, cancer, diabetes, and chronic inflammation (Barnham, Masters, & Bush, 2004). Previous studies have reported oxidative DNA damage as a major factor associated with age related diseases. It can interfere with the expression of various genes associated with DNA repair and cellular proliferation (Bohr, 2002). The relative contributions of these different mechanisms to telomere shortening are unknown, although oxidative stress has been suggested as a major factor of telomere shortening. Telomere shortening might impact on the regenerative capacity of human tissue during aging and chronic diseases.

Telomere length fulfills several of the criteria for robust biomarkers of human aging because it does the following: i) decreases progressively with chronological age; ii) varies considerably among individuals; iii) registers the life cycle of proliferative cells; and iv) is strongly linked to inflammation and oxidative stress (Zglinicki & Martin-Ruiz, 2005). These observations support the hypothesis that telomere shortening can be used as a prognostic marker for age-related.

Scientific comparisons of blood and saliva telomere length are limited. However, there is a chance although imperfect correlation between them. In fact, DNA in saliva is derived from blood. Some studies show that up to 74% of the DNA in saliva comes from white blood cells (Thiede, Prange-Krex, Freiberg-Richter, Bornhäuser, & Ehninger, 2000), yielding likely the same amount of DNA per volume and the same DNA quality. Surprisingly, scientists recently found that people who had received bone marrow transplants express

the genes of the donor in both their blood and their saliva, but not in other cells (Thiede et al., 2000). A publication in Nature Communications, it was shown that telomeres shorten at equivalent rates in the somatic tissues of adults (Daniali *et al*., 2013). Therefore, saliva telomere length is probably a reliable indicator of telomere length dynamics. In addition, saliva is one of the most accessible of our body's bio-fluids making saliva sample collection easy and non-invasive. Saliva also harbors a wide spectrum of genetic data that can be used for genetic research and clinical diagnostic applications. There are two complications in using saliva to measure telomeres: even fresh saliva contains a substantial amount of degraded DNA, and saliva contains DNA from bacteria and other microbes. The current bottom line is that saliva is proving to be a very important source for genomic analyses, but substantial research remains to be done to verify previous telomere length discoveries made using blood, and to validate saliva as a source for clinical-grade telomere analyses. Although the TL from the alternative peripheral sources i.e. saliva was slightly higher as compared to blood, the results in this study showed consistent TL measurements. The finding strengthens the belief that saliva could be considered as good and as reliable a source of DNA for measuring telomere length. How, the Cons of using saliva source is the inter individual variability in ratio of cell types in saliva that contains of leukocytes and buccal cells (Lin, Smith, Esteves, & Drury, 2019). Commercial collection kit for saliva samples are also available for long shipping then extract and test purified DNA for reliable testing.

There are many factors that might slow telomere shortening, and initial scientific studies report that there are also ways to reverse this process, to lengthen telomeres. The study that reported to regulate the rate of telomere shortening, including certain dietary factors, sleep, stress, and exercise. Here are a few that have been shown to slow telomere shortening, for examples, i) routine and vigorous exercise (Werner et al., 2009) (Du *et al*., 2012), ii) healthful diet including sufficient omega-3 fats (Farzaneh-Far *et al*., 2010), iv) multivitamins, particularly vitamin C and vitamin E (Qun *et al*., 2009), and v) stress reduction and sufficient sleep (Parks *et al*., 2009). However, individual is different, and it isn't currently known what levels of these different factors are right for each person. Thus, this is the most important reason to measure telomere length.

In conclusion, measurement of telomere length should be a good biological marker and tool for evaluating health status of aging population. The benefits may be for government planning such as preventive measures or may alert people to take care their health. Nonetheless, a single telomere length measurement by itself has limited biological value, but the comparison with the telomere length of a representative sample of the population. In this regard, the knowledge of telomere length of “healthy” individuals of different ages allows the comparison of a given individual telomere length with their age and gender group, providing an idea of their general health status or even an estimation of their

biological age. Moreover, telomere length measured over time (longitudinal studies) may provide a valuable instrument to characterize positive or negative effects of several treatments or changes in lifestyle habits and other factors impact health status. Additional aging markers could be measured in parallel to telomere length to be able to define an "aging signature". Thus, telomere length measurement arises as a new tool for aging and health status characterization with multiple novel applications. This study provides the evidence of strong associations between telomere length in blood and saliva. Therefore, saliva could be used as an alternative and non-invasive specimen source to measure the telomere length. Larger studies with detailed information are warranted to confirm our findings.

Acknowledgments

This research study was supported by grant from Research Institute, Rangsit University

Disclosure

The author reports no conflicts of interest in this work.

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