

## Research Paper

# The Ratio of Biological Health Score to Immune Cell Telomere Length: A Reliable Index Indicating the Association Between the Severity of Injury and Senescence in Mustard-chemical Veterans



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## ABSTRACT

**Background:** Sulfur mustard (SM), a chemical weapon used in the Iraq-Iran war from 1983-1988, has multiple chronic complications. This study aimed to evaluate one of the long-act effects of SM, i.e. the senescence according to the severity of injury in veterans via the estimation of biological health score (BHS) and relative telomere length (TL) of immune cells.

**Materials and Methods:** SM-chemical veterans were categorized into three groups according to the percentage of chemical injury (5-20%, 25-45%, and 50-70%). Healthy volunteers also participated as a control group. Eighteen biomarkers from different physiological systems (inflammatory/immune, endocrine, cardiovascular, and metabolic), and organs (liver and kidney) were used to estimate BHS. The TL of immune cells was also measured for each participant by monochrome multiplex real-time PCR.

**Results:** There were significant correlations between age-adjusted TL (negative), BHS, and BHS/TL ratio (positive) and the percentage of injury. The TL was significantly decreased in all veterans with different injuries compared to healthy participants while it did not change in veterans with the injury percentages of 25-45% and 50-70%. The BHS in three groups of veterans was significantly higher than in healthy individuals. The BHS/TL ratio was significantly changed between all groups and increased with the progress of injury.

**Conclusion:** The BHS and TL may not individually be accurate indices for the determination of senescence and biological aging while the ratio of these two parameters improves this defect and could be a more reliable index indicating biological aging in chemical veterans with different percentages of injury.

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## 1. Introduction

**S**ulfur mustard (SM) gas as a chemical weapon (1-chloro-2-[2-chloroethylsulfanyl] ethane) that had been used in the Iraq-Iran war from 1983 to 1988, possesses multiple chronic complications [1, 2]. Now, the delayed effects of SM are still substantial in many people [3]. SM-exposed individuals suffer from chronic injuries of the dermal, ocular, pulmonary, digestive, hematopoietic, endocrine, immunological, genital, reproductive, and neurological systems [4-6]. These complications are even observed following once-only exposure to SM [7]. In addition, sustained post-traumatic stress disorder is a critical issue in SM-exposed people that disrupts their usual life by inducing psychological complications (e.g. anxiety, depression, and sleep disorders) [8, 9]. Molecular and cellular studies have also determined many intracellular changes after SM exposure, including failed antioxidant systems and then increased reactive oxygen species (ROS), cell membrane/DNA damage, and cellular apoptotic cascade activation [10, 11]. Oxidative stress diminishes the regenerative ability of organs, leading to degenerative diseases [12, 13]. These harmful effects may change the expression of many age-related genes, accelerate telomere attrition, and cause cellular aging and senescence [14, 15].

A telomere is a structure of the repetitive DNA at the end of chromosomes that protects chromosomes from degradation and illegitimate recombination and plays a key role in cell fate [16]. Telomere shortening is known as the principal sign of aging or the reason for age-related damage leading to biological aging. Biological aging, in turn, promotes subsequent aging. Thus, telomere attrition may progress aging via cellular senescence [17, 18]. Immune cells differ from normal somatic cells in that they can regulate telomerase, the telomere-extending enzyme, and limit telomere attrition in activated cells undergoing cell division. Immunosenescence is characterized by antigen exposure and oxidative stress-induced remodeling of the immune system. The immune system deteriorates with age due to a decline in the absolute number of naive T and B cells and T and B lymphocytes [19].

Allostatic load is a failed adaptive response, and like telomere length (TL), it is a biological reflection of progressive stress and harmful health consequences [20]. The allostatic load is influenced by many parameters, like age, gender, livelihood challenges, personal characteristics, social condition, and formative memories [21, 22]. Recently, an improved form of the allostatic load

method has been determined as a biological health score (BHS). Stressful challenges on the physiological wear-and-tear system were represented in this scoring by biomarkers from four systems (endocrine, metabolic, cardiovascular, and inflammatory/immune) and two organs (the liver and kidney) [23].

In this study, the aging indices of relative TL of immune cells and BHS were estimated and compared in SM-exposed people with different rates of SM injury to evaluate one of the long-act effects of SM, i.e. the senescence.

## 2. Materials and Methods

### Study design and sampling

This research was carried out on 373 male chemical veterans from Sardasht City (a city in West Azerbaijan Province, Iran) who were injured by the bombs containing SM by Saddam Hussein's military in 1987. They were categorized into three groups based on the severity of the injury: 5-20%, 25-45%, and 50-70%. The personal information for the percentage of injury was obtained by self-report and confirmed by the [Foundation of Martyrs and Veterans Affairs](#). The control (0% injury of) group included 103 healthy volunteers from Rabat City (a city close to Sardasht with similar culture and environmental conditions). Blood samples were prepared from all participants.

### DNA extraction and monochrome multiplex quantitative polymerase chain reaction (qPCR)

Genomic DNA was isolated from blood samples using the salting-out technique. NanoDrop spectrophotometry was used to determine the amount of extracted DNA. PCR was performed on samples with a 260/280 absorbance ratio of more than 1.8. According to Cawthon (2009) [24] and Nasiri et al. (2021) [25], monochrome multiplex qPCR (MMqPCR) was used to quantify relative leukocyte telomere length (LTL). DNA samples were amplified in triplicate using a kit (HOT FIREPol® EvaGreen® HRM Mix; Solis Biodyne, Korea) and a real-time thermocycler (Rotor-Gene Q 6-plex, Qiagen, Germany). According to Nasiri et al. (2021) [25], primers for telomere (T) and albumin (S) (as a single-copy gene) were generated. Reference DNA (from 12 controls) was diluted to create a standard curve. After estimating T and S from this standard curve, we determined the T/S ratio for each sample to obtain the relative TL.

### Estimation of biological health score through biochemical markers

BHS as an extended allostatic load was calculated for each participant through 18 plasma biomarkers of endocrine, cardiovascular, metabolic, and inflammatory/immune systems, liver, and kidney according to our previous studies [25, 26]. These biomarkers consisted of dehydroepiandrosterone sulfate (DHEA-S), prolactin, luteinizing hormone (LH), and testosterone as candidates for the endocrine function, hemoglobin, high-density lipoprotein cholesterol (HDL), low-density lipoprotein cholesterol (LDL), total cholesterol, and triglycerides as candidates for metabolic function, systolic and diastolic blood pressures and heart rate as candidates for cardiovascular function, C-reactive protein (CRP) and transforming growth factor-beta (TGF-β) as candidates for Inflammatory/immune function, alanine transaminase, aspartate transaminase, and gamma-glutamyl transferase as candidates for liver function, and creatinine as a candidate for kidney function. In order to calculate BHS, each of the 18 biomarkers was assigned a risk quartile (with high risk corresponding to the highest quartile for all but testosterone, DHEA-S, and HDL, which were assigned to the lowest quartile). If a participant's biomarker fell into the high-risk quartile, they received the score one, whereas all other quartiles received the zero score. The total value of all biomarkers was then calculated by adding up each participant's scores in the highest risk quartile. Thus, the BHS might be from 0 to 18 [27].

### Statistical analysis

Table 1 displays the Mean±SD scores of the subjects. The number of volunteers in each group was enough to compare statistically (Table 1). TL data were age-adjusted by

subtracting the subjects' linearly predicted TL from their observed TL, allowing for comparisons across four groups [28]. The Kolmogorov-Smirnov test was run to ensure data normality for both the healthy and chemical veteran groups. The data did not follow a normal distribution, thus, they were analyzed using non-parametric tests, i.e. the Kruskal-Wallis and Mann-Whitney U tests. Statistical significance was assumed for P<0.05. The correlations between BHS, TL, BHS/TL ratio, and the percentage of injury were evaluated using Spearman regression. SPSS software, version 26 (IBM-SPSS, Inc, Chicago, IL, USA) was utilized to do the statistical analyses.

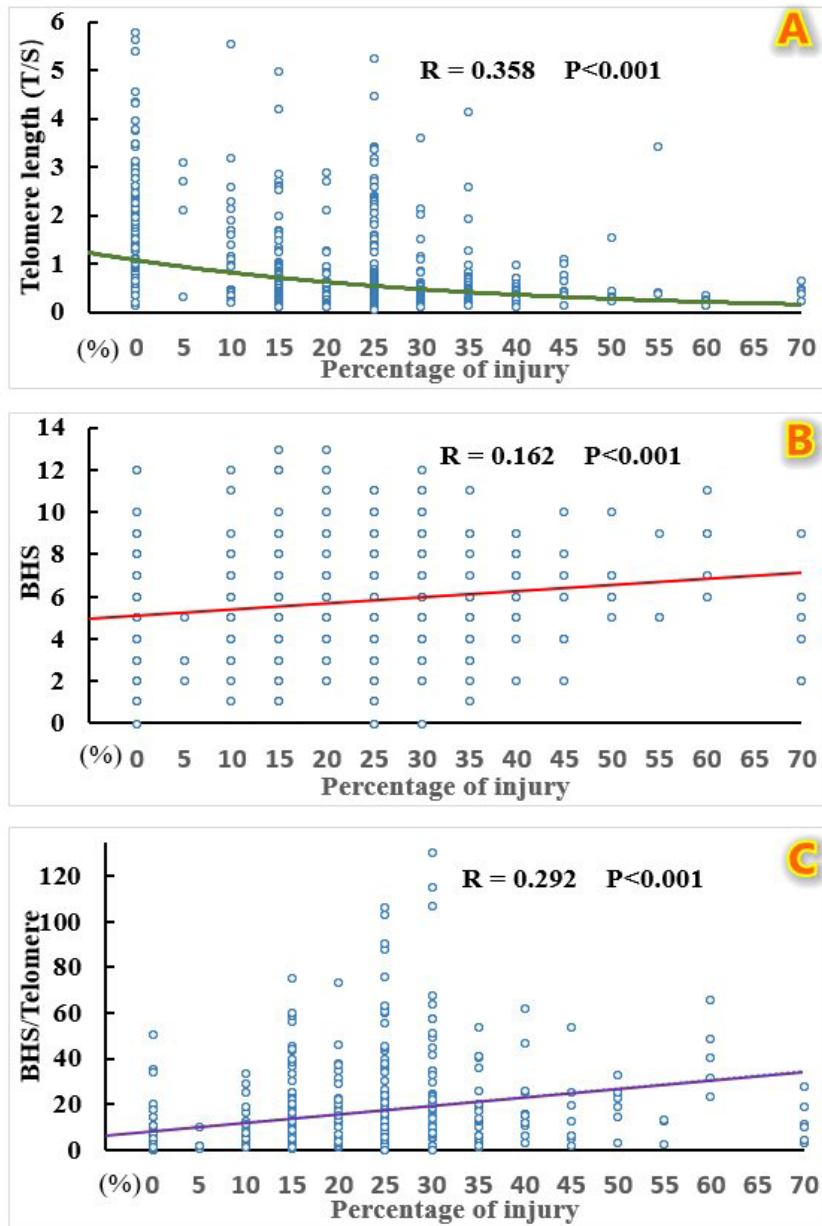
### 3. Results

Figure 1 shows the regression graphs of TL (after age adjustment), BHS, and BHS/TL ratio, and the percentage of injury rate in the chemical veterans. This figure indicated a negative correlation between age-adjusted TL and the percentage of injury while there were positive correlations between BHS and BHS/TL ratio and the percentage of injury (P<0.001).

Figure 2 represents the comparison of TL (after age adjustment), BHS, and BHS/TL ratio between healthy individuals and chemical veterans. TL significantly decreased in all veterans with different injuries compared to healthy participants while it did not differ between the veterans with the injury percentages of 25-45% and 50-70%. BHS in the healthy individuals was lower than one in three groups of the veterans (P<0.05) while this parameter did not significantly change between the veterans with different percentages of injury. The BHS/TL ratio was significantly changed between all groups and increased with increasing the percentage of injury.

Table 1. Descriptive analysis of telomere length and biological health score in chemical veterans with different percentages of injury

Injury Rate (%)	No. (%)	Mean±SD (Min–Max)			
		Age (y)	TL	BHS	BHS/TL
0 (non-exposed)	103(100)	50.6±10.01 (28-74)	1.80±1.18 (0.14–5.79)	4.79±2.29 (0.0–12.0)	5.05±7.10 (0.0-50.6)
5-20	150(40.2)	54.4±12.03 (28–97)	0.88±0.945 (0.09–5.55)	5.87±2.71 (1.0–13.0)	14.92±14.45 (0.4–75.2)
25-45	202(54.2)	58.0±13.76 (28–103)	0.76±0.897 (0.05–5.26)	5.93±2.63 (0.0–12.0)	20.92±23.26 (0.0–130.0)
50-70	21(5.6)	58.6±15.39 (27–84)	0.54±0.710 (0.14–3.42)	6.47±2.36 (2.0–11.0)	21.70±15.78 (2.6–65.9)
Total (5-70)	373(100)	56.6±13.31 (27–103)	0.79±0.911 (0.05–5.56)	5.94±2.65 (0.0–13.0)	18.56±19.98 (0.0–130.0)

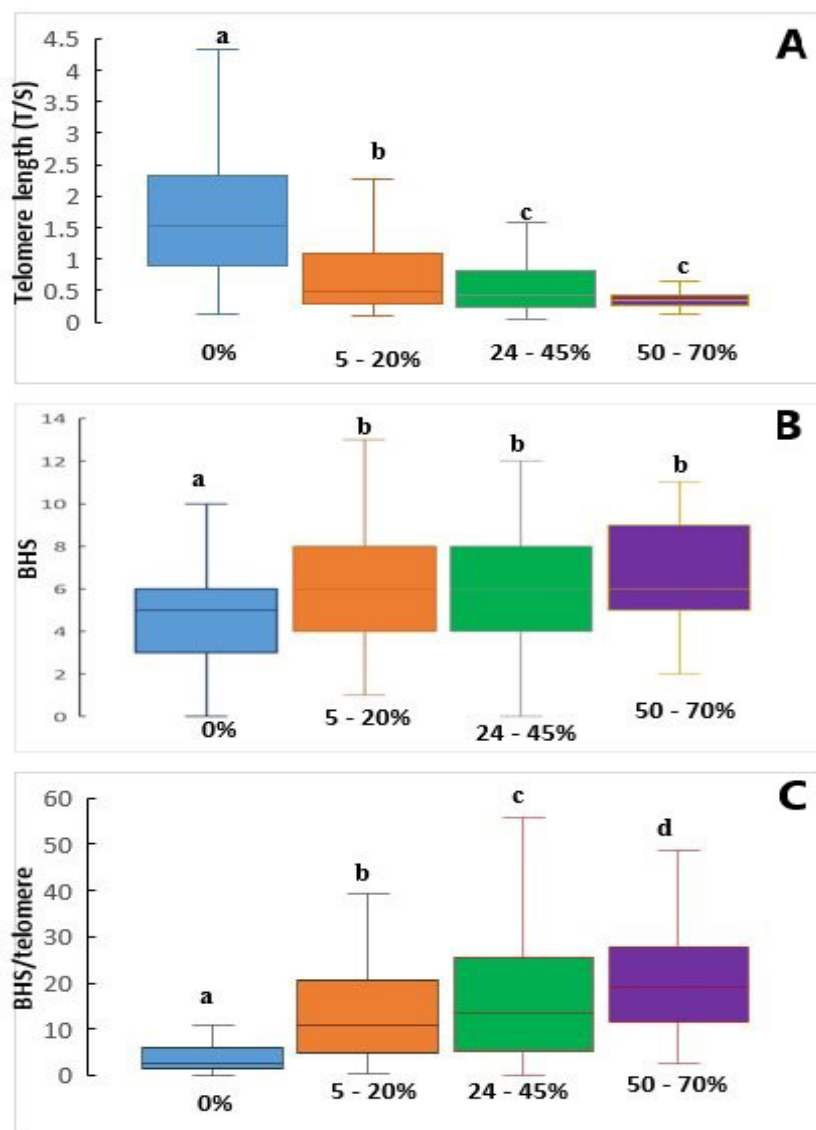


**Figure 1.** Regression graphs indicating the correlation between telomere length (TL, after adjustment for age) A) Biological health score (BHS), B) BHS/TL, C) The percentage of injury in chemical veterans

#### 4. Discussion

SM, a chemical weapon, causes irreversible damage to cell structure and function, accelerating biological aging over time [12]. Relative TL and BHS [26, 29] have been used to quantify biological aging in SM exposure. In this investigation, these biomarkers and their ratio (BHS/TL) were compared to measure biological aging concerning chemical harm.

This method of investigation probably yields more accurate results from the biological aging rate due to SM across all injury severity levels. Although TL has been considered a potential biomarker of aging, it is possible that this biomarker will fluctuate over the person's lifetime. Also, it seems that a single biomarker will not be adequate to reflect aging in all biological systems [30, 31]. Therefore, additional panels of biomarkers can be used in addition to TL to determine biological age [32]. The BHS, which includes 18 biomarkers and is a comprehensive type of allostatic load, has been proposed as



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**Figure 2.** Comparison of telomere length (TL, after adjustment for age)

A) Biological health score (BHS), B) BHS/telomere ratio, C) Between healthy individuals (0%) and three groups of chemical veterans with different percentage of injury (%)

a, b, c, dSignificant difference between groups (P<0.05).

a reliable indication to assess aging in both healthy and chemically-exposed veterans. Previous studies have indicated that people who have been exposed to SM have significantly shorter relative TLs [14, 26, 29]. Also, they are associated with ROS overproduction and increased expression of 8-oxodG and 8-oxoguanine glycosylase (OGG1) transcripts as indicators of oxidative damage. However, it has been found that excess ROS may play a significant role in the diminution of TL and the progression of biological aging [16, 33]. In the present study, TL shortening in SM-exposed chemical veterans was confirmed and this shortening was partially associated with the increasing severity of the chemical injury. However,

this association was not specific for each group of veterans with different injury rates. The observed associations between TL and the risk of many different diseases suggest a critical role for TL in the health at the cellular level of body organs [31, 34]. Despite these strong correlations, the role of TL is not clear as cause or effect. However, many interventions to increase TL for the purpose of preventing disease were not influenced. On the other hand, TL as a biomarker of disease did not have clinical utility. It indicates that the association between TL and diseases is very complicated, and TL alone is not precisely predictive of risk [35].

We also calculated BHS as a measure of biological aging. Previous research was used to determine which biomarkers should be employed to construct this indicator [26]. Higher values of BHS indicate lower individual health; According to BHS as an advanced allostatic load, the biological health in all kinds of societies is influenced by social, physical, and environmental stress [36], and the parameters related to kidney and liver function and different physiological systems that were accessible in the research can reflect the stressful effects [37]. In the previous study, the cardiovascular system had the greatest impact on the determination of BHS of SM-exposed veterans [26]. In this study, the BHS results confirmed that the overall health condition of veterans was substantially worse than the control group but this parameter was not able to differentiate the health condition of chemical veterans with different percentages of injury.

The earlier study established a negative correlation between TL and BHS in healthy and SM-exposed individuals [26]. This correlation was also perceptible in our data. In the present study, BHS and TL were estimated in chemical veterans with different percentages of injury and both have been indices of biological aging in previous studies [38, 39], but it seems that in the chemical veterans, they could not be an accurate index to show this biological aging. For the first time, in our study, the ratio of these two indices (BHS/TL) was expressed in the evaluation of senescence and biological aging in chemical veterans. This ratio synergizes the effect of each parameter and exhibits better the association between biological aging and the severity of injury, as our data confirmed.

## 5. Conclusion

In conclusion, TL and biological health scores may not individually be accurate indices for the determination of senescence and biological aging while the ratio of these two parameters improves this defect and could be a more reliable index indicating biological aging in the chemical veterans with different percentages of injury rate.

## Ethical Considerations

### Compliance with ethical guidelines

The Board of Research Ethics of [Janbazan Medical and Engineering Research Centre \(JMERC\)](#), the Board of Research of the Ministry of Health, and the Research Ethics Committee of [Shahed University](#), all endorsed this study (Code: IR.SHAHED.REC.1399.151). Written informed consent was signed by all participants.

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## Authors' contributions

Study and experimental design: Mohammad-Reza Vaez-Mahdavi, Tooba Ghazanfari and Sussan Kaboudanian Ardestani; Experimental work, data analysis and writing: Leila Nasiri, Hossein Hassanpour and Nayere Askari.

## Conflicts of interest

The authors declared no conflict of interest.

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