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# Exploring telomere length and environmental factors in Alzheimer's disease: insights from molecular genetics and fluorescence in situ hybridization (FISH)

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**Background**
Alzheimer's disease (AD) is a progressive neurodegenerative illness marked by a decline in cognitive abilities, including memory, logic, and thinking. Telomeres, which can reach lengths of up to 15 000 base pairs in humans, are repetitive DNA sequences found at the ends of chromosomes. One indicator of age-related disorders is the progressive reduction of telomere length (TL) in healthy human body cells. The regulation of TL is influenced by a complex interplay of genetic and environmental influences.
**Objective**
A comparison between real-time PCR (RT-PCR) and quantitative-fluorescence in situ hybridization (Q-FISH) methods for determining TL and evaluating environmental factors contributing to the onset of AD. Patients and methods
In all, 10 controls and 30 AD patients participated in this study. Blood samples from all participants were collected, and TL was measured using Q-FISH and RT-PCR techniques.
**Results and conclusion**
Our findings showed a significant variation in TL between patients and controls, showing a *P* value of 0.036. The relationship between the T/S ratio (telomere repeat copy number to single-copy gene copy number), as measured by RT-PCR, was analyzed. TL (kb) was assessed by Q-FISH, and the analysis was performed using linear regression. The correlation coefficient ($r^2$) for the association between telomere repeat copy number to single-copy gene copy number (T/S) ratio and TL (kb) was found to be $r^2$=0.266 with a *P* value of 0.004.
We concluded that a significant association exists between AD and the reduction in TL. Environmental factors, such as diabetes and hypertension, also impact TL in AD. Moreover, RT-PCR is more precise and easier to use than Q-FISH for evaluating TL.

**Keywords:**
Alzheimer', s disease, diabetes, hypertension, quantitative-fluorescence in situ hybridization, real-time PCR, telomere length

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## Introduction

Alzheimer's disease (AD) is a widespread neurodegenerative disorder marked by a gradual decline in cognitive function, memory loss, and a diminished ability to carry out daily tasks independently. AD is the leading cause of dementia, accounting for ~60–80% of all dementia cases. It primarily affects the elderly and is marked by the accumulation of amyloid plaques and tau tangles in the brain, leading to the deterioration of neuronal function and connectivity. This disorder results in the progressive degeneration of brain cells, leading to a gradual decrease in brain size and a loss of nerve cells and their connections. It is increasingly recognized as one of the most costly, deadly, and burdensome conditions of the modern era [1–3].

AD arises from a multifaceted interaction of genetic, lifestyle, environmental, and epigenetic factors [4]. Of the various genetic risk factors identified, apolipoprotein E (APOE) gene is the most prominent and widely recognized, playing a role in over half of all AD cases. The most common form of the disease, which typically emerges after the age of 65 years, is linked to the APOE gene located on chromosome 19q13.2 [5]. Moreover, mutations in genes on chromosomes 1, 14, and 21 are associated with rarer forms of AD, which generally manifest at an earlier age.


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The contribution of the APOE gene to the development of AD remains not fully understood. The APOE gene has three variants: APOE2, APOE3, and APOE4. While carrying the APOE4 variant increases the risk of developing AD, the other variants, APOE2 and APOE3, seem to offer some protection against the disease. It is important to note that inheriting the APOE4 gene does not guarantee the development of AD [6].

AD is a genetically diverse condition. It includes AD2, linked to APOE allele on chromosome 19, and AD3, which results from mutations in the presenilin-1 gene on chromosome 14q; and AD4, associated with mutations in the PSEN2 gene on chromosome 1q31 [7].

Environmental factors likely play a significant role in AD development. These factors include educational attainment, head injuries, use of NSAIDs, obesity, diabetes, physical activity, hypertension, and engaging in cognitive activities. Pollution, especially from heavy metals, is considered a major health threat due to its persistence and bioavailability, contributing to AD pathogenesis [8].

Telomeres are repeated DNA sequences located at the ends of chromosomes. These sequences are composed of double-stranded, noncoding G-rich tandem repeats (5′-TTAGGG-3′), which are vital for proper chromosome replication during cell division and gradually shorten with each cycle [1]. When telomeres become critically short, it can lead to issues such as chromosome fusions, abnormal recombination, and degradation. Thus, maintaining sufficient telomere length (TL) is crucial for chromosome stability and cellular protection [9]. The length of telomeres is linked to age-linked disorders, including AD. The capacity of telomeres to withstand DNA damage serves as an indicator of aging and oxidative stress [10].

TL can be measured using various methods, such as quantitative-fluorescence in situ hybridization (Q-FISH), which allows for the visualization of telomeres during metaphase using peptide nucleic acid probes. These probes bind to the telomeres and are proportional to their lengths, enabling comparisons between different chromosomes [11,12].

Q-FISH, or quantitative-fluorescence in situ hybridization, is a technique that effectively measures TLs in interphase cells and formalin-fixed paraffin-embedded tissue slices by combining fluorescence in situ hybridization with digital picture quantification. Studying the shortest telomeres, which are essential for cell viability and genomic stability, is especially beneficial [9,13].

Real-time PCR (RT-PCR) has emerged as a widely used technique for measuring TL. This approach uses novel primers designed to minimize primer–dimer formation, a common issue with standard assays due to the repetitive nature of telomere sequences [14]. RT-PCR is efficient for analyzing large sample sizes and requires only small amounts of DNA. It can be adapted for various tissues and provides valuable comparative data on TL [15].

The goal of this study is to compare and evaluate the effectiveness of measuring TL using Q-FISH and RT-PCR, as well as to investigate the impact of environmental influences on AD progression.

## Patients and methods

The study included 10 male and 20 female Egyptian patients with AD (10 males and 20 females), along with 10 control volunteers matched for age and sex. Alzheimer's diagnoses were based on comprehensive patient histories, physical and neurological examinations, laboratory tests, neuropsychological assessments, and imaging techniques such as computed tomography scans, MRI, and PET scans. Moreover, questionnaires were administered to the guardians of Alzheimer's patients to identify potential environmental risk factors related to lifestyle and education [16]. Q-FISH and RT-PCR analyses were used to measure TL in peripheral blood samples from the patients. Half of the blood samples were placed in heparin tubes for Q-FISH, and the other half were placed in EDTA tubes for RT-PCR.

This study was conducted in accordance with the principles outlined in the Declaration of Helsinki. The research protocol and procedures received approval from the Ethics Committee of the National Research Centre (NRC) in Cairo, Egypt, under Ethics No. 16/061. Written informed consent was obtained from the guardians of all participants before their inclusion in the study.

### Quantitative-fluorescence in situ hybridization technique

Blood samples, which were collected in heparin tubes, were cultured according to the method proposed by Verma and Babu [17]. Metaphases were photographed using a fluorescence microscope fitted with a CCD

camera and connected to ISIS software as described by Passos *et al.* [12].

Telomere/centromere-FISH (T/C-FISH) was used to measure the fluorescence intensity of both telomeres and the centromere of chromosome 2. This method enabled precise measurement of individual telomeres, as the fluorescence intensity is proportional to both telomeric and centromeric length. The stability of the centromere 2 DNA sequence made it a reliable reference for TL analysis.

Fifteen metaphases were studied from each participant and the median (T/C)-FISH value was calculated. A simple regression analysis was performed to convert the (T/C)-FISH data into telomere restriction fragment (TRF) values. The formula used was $y=c+m\times x$, where $y$ represents base pairs (bp), $c$ the y-intercept, $m$ the slope, and $x$ is the telomere/centromere (T/C) value. The regression equation that was derived is $y=2507+204x$, where $x$ represents the T/C ratio and y represents the TRF value in base pairs. Therefore, TRF values were derived from the T/C data using this equation.

#### Real-time PCR technique

The RT-PCR technique was used to measure TL in DNA samples extracted from peripheral blood leukocytes of patients with AD.

#### DNA extraction

Genomic DNA was isolated from peripheral blood leukocytes collected in EDTA tubes using the salting-out method [18].

#### Determining DNA concentration and purity

DNA concentration and purity were evaluated using a Nanodrop (Thermo Scientific NanoDrop 2000c Spectrophotometer) measuring absorbance at 260/280 nm (Thermo Fisher Scientific 3411 Silverside Road Bancroft Building, Suite 100 Wilmington, DE 19810 U.S.A). A ratio close to 1.8 indicates pure DNA. Deviations from this ratio may suggest contamination by proteins, phenols, or other substances that absorb at 280 nm [19].

#### Real-time PCR

Real-time kinetic quantitative PCR was used to determine the $C_t$ value, which represents the cycle number at which fluorescence exceeds a defined threshold above the baseline. A linear relationship between $C_t$ and the logarithm of the amount of input DNA allows for relative quantification by comparison to a standard curve, which is generated from multiple dilutions of a reference DNA sample.

Telomere tel 1, tel 2 (T) PCR, and single-copy gene 36B4 (S) PCR (T/S) were performed in separate tubes. Two master mixes were prepared: one containing the T primer pair and the other containing the S primer pair. Both mixes contained the same reagents except for the primers [20].

To confirm uniform amplification of the S gene in all samples, the ratio of 36B4 gene copies to β-globin gene copies in the experimental DNA was compared with a reference DNA. The PCR conditions for β-globin were the same as those for 36B4, except for the primer concentration, which was set to 400 nM for β-globin.

**The telomere primer sequence**

| | Primers sequence |
|---|---|
| Tel 1 | 5′-GGTTTTTGAGGGTGAGGGTGAGGGTGAGGGTGAGGGT-3′ |
| Tel 2 | 5′-TCCCGACTATCCCTATCCCTATCCCTATCCCTATCCCTA-3′ |

**Single-copy gene primer sequence**

| | Primers sequence |
|---|---|
| 36B4u | 5′-CAGCAAGTGGGAAGGTGTAATCC-3′ |
| 36B4d | 5′-CCCATTCTATCATCAACGGGTACAA-3′ |

#### Statistical analyses

TL analysis involved capturing 10 metaphases per donor using a fluorescence microscope (Axioscope 2; Zeiss) with a CCD camera and ISIS software (Meta-Systems). Data normalization was performed by calculating the ratio of telomere to centromere intensities (T/C value) [12]. Statistical comparisons were made using $t$ tests for means and SDs. Linear regression was used to correlate TL measurements from RT-PCR and Q-FISH. One-way analysis of variance was used to evaluate the significance of differences in TL measurements between groups.

## Results and discussion

#### Quantitative-fluorescence in situ hybridization technique

The Q-FISH technique showed a significant difference in TL between Alzheimer's patients and controls, with a standardized mean difference of 0.036 (Table 1, Figs 1–3). No significant difference in TL was observed between patients and age ($P$=0.985) nor between sexes ($P$=0.428). However, a significant difference in TL was found between the group with a family history of AD and the healthy control group.

#### Real-time PCR technique

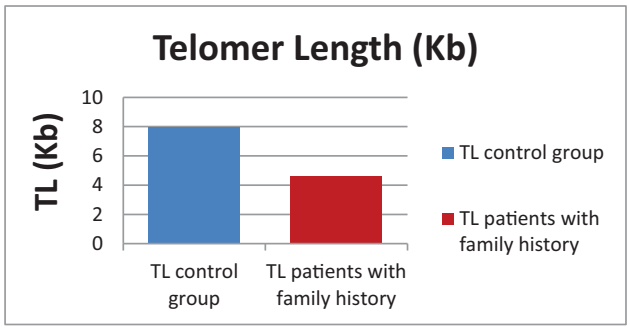
In our study, the fold change (fold change=$2^{-\Delta\Delta Ct}$) was calculated by determining the telomere PCR (T) and single-copy gene PCR (S) ratio (T/S=$2^{-\Delta Ct}$) for each

**Table 1 Mean±SD of telomere length in patients and controls**

| Technique | T/C-FISH and TL (mean±SD), kb AD patients group | T/C-FISH and TL (mean±SD), kb control group | *P* value |
|---|---|---|---|
| Q-FISH | 15.69±6.72<br>5.61±1.39 | 26.98±14.52<br>7.97±2.96 | 0.036 |

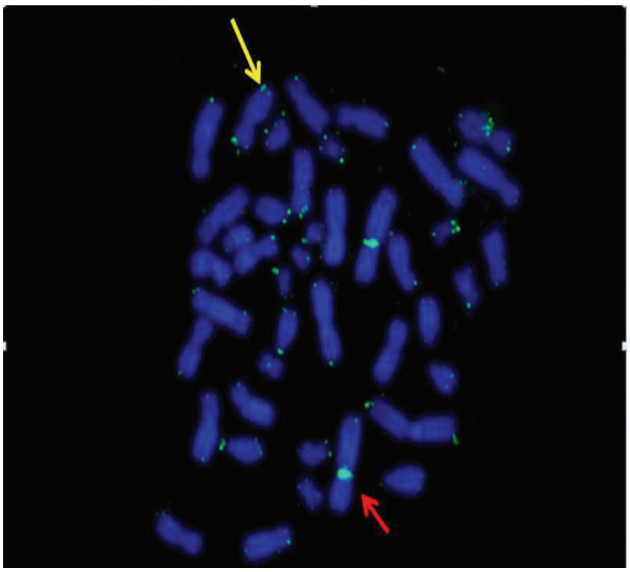
AD, Alzheimer's disease.

**Figure 1**




Telomere length (TL) (kb) of Alzheimer's disease (AD) patients with family history and controls measured by quantitative-fluorescence in situ hybridization (Q-FISH).

**Figure 2**



Metaphase spread hybridized with telomeric PNA probes using quantitative-fluorescence in situ hybridization (Q-FISH). The chromosomes (blue) and the telomere signals (green spot). The centromeric region of chromosome 2 highlighted by red arrows and telomeric signals highlighted by yellow arrows. PNA, peptide nucleic acid.

sample with normalization based on a reference DNA sample.

The results demonstrated a significant difference in the telomere-to-single-copy gene (T/S) ratio and the fold change in TL between Alzheimer's patients, *P*=0.0001 (Fig. 4).

There was evidence of a nonlinear relationship between TL and AD. No significant differences were found between relative TL ratio (T/S ratio) and age (*P*=0.3) or sex (*P*=0.4).

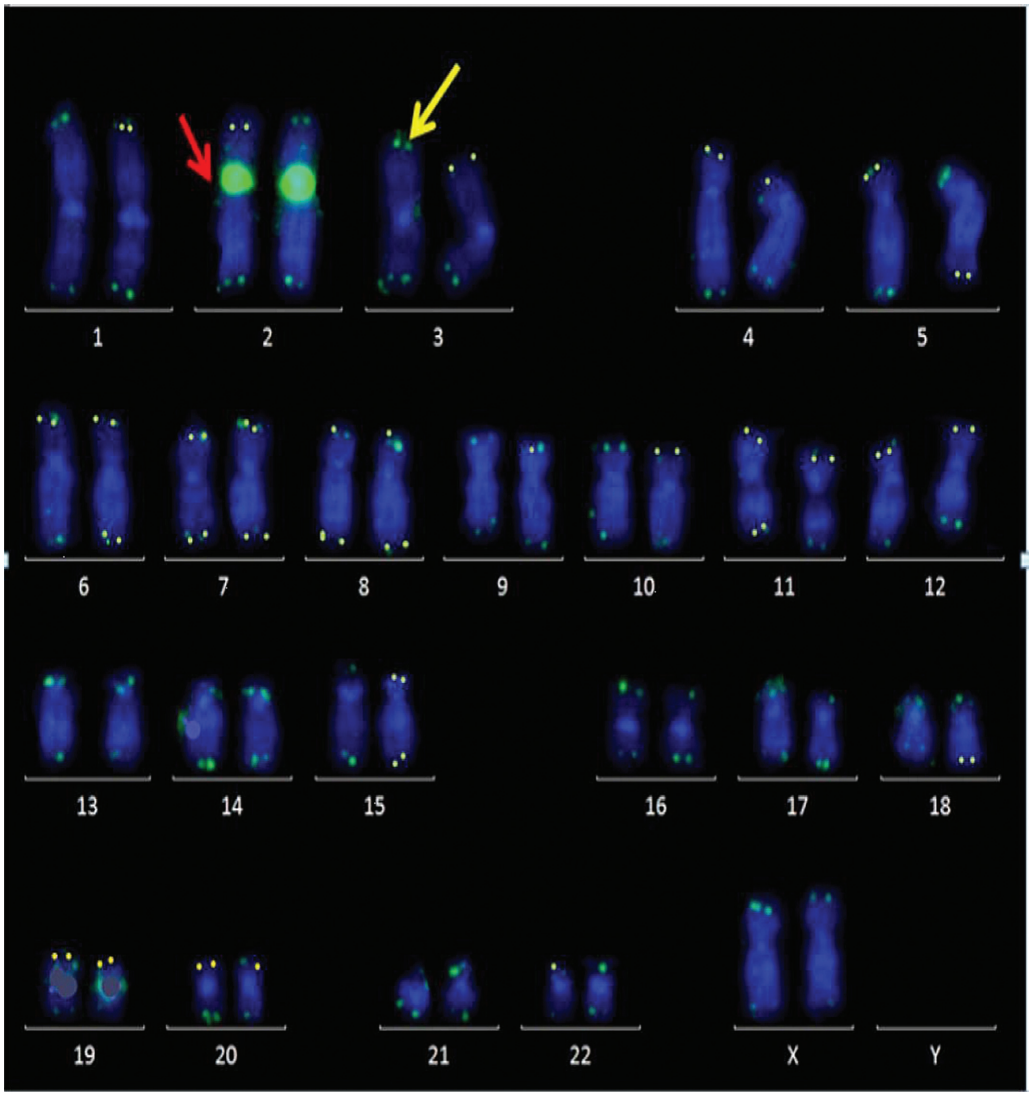
Three AD patients had low fold change from total cases. The first case had a fold change of 0.002 and a relative T/S ratio of 1.738, showing minimal TL difference (0.002) with normal change for a young age (56 years). The second case had a fold change of 0.004 and a relative T/S ratio of 1.669. The third case, aged 85 years, showed relatively normal TL with a minimal fold change (0.005). A significant association was found between low fold change and relative T/S ratio with *P*=0.0002.

After normalizing using the fold change formula for six AD patients with high fold changes, we observed the following: one patient, aged 85 years, had a fold change of 1.42 and a relative T/S ratio of 1.228 with a 0.19 increase in fold change, indicating shorter relative TL. Another patient, aged 82 years, exhibited a moderate fold change of 0.45 and a relative T/S ratio of 1.302 with a 0.85 increase in fold change, also reflecting shorter TL. A significant association was found between high fold change and T/S ratio with *P*=0.018.

Twenty-eight patients were hypertensive which constitutes 93.3%. Among them 24 patients had controlled hypertension, which constitutes 85.7% and four patients had uncontrolled hypertension and they form 14.2% of the total hypertensive patients. The current study revealed a highly significant difference in the T/S ratio and fold change among controlled hypertension patients with a decreased fold change (*P*=0.0001).

Twenty-six (86.6%) patients were diabetes: 22 (84.6%) patients had controlled diabetics and four (15.3%) had uncontrolled diabetics. A significant difference in the T/S ratio and fold change was observed in controlled patients (*P*=0.0004). The relative TL (1.517) indicated a very low fold change (0.020695409) corresponding to only 2% of the patients. The Q-FISH result was 7.95 kb. The original relative length (1.517)

**Figure 3**




Karyograms stained by quantitative-fluorescence in situ hybridization (Q-FISH) to estimate telomere length. Telomeres are stained (green), highlighted by yellow arrows with the centromeric region of chromosome 2 (indicated by red arrows) and telomere-free ends (indicated by green arrows).

decreased to 1.496 after normalization, indicating long or normal TL. A significant difference was observed between the T/S ratio and fold change in uncontrolled diabetic patients ($P$=0.025).

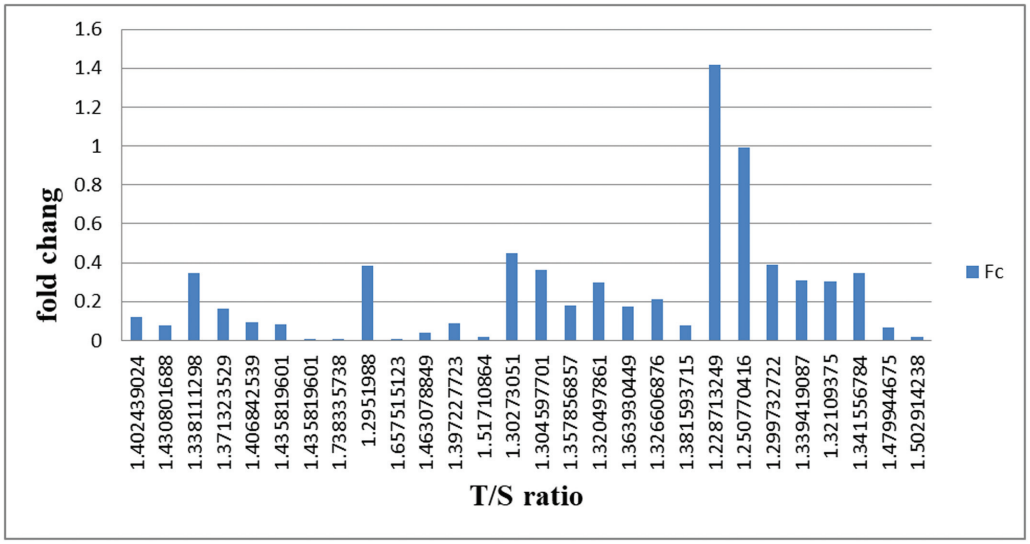
In smokers, a significant difference was observed in T/S ratio and fold change ($P$=0.012). Also, in nonsmokers a significant difference was found between T/S ratio) and fold change ($P$=0.004).

This study found a significant correlation between RT-PCR results and TL (kb) from Q-FISH by linear regression ($r^2$=0.266, $P$=0.004). In addition, a correlation was observed between TL (kb) and fold change by linear regression ($r^2$=0.185, $P$=0.018), indicating an inverse relationship between TL and fold change (Figs 5 and 6).

The study showed a significant variation in TL between patients and controls ($P$=0.036). The mean TL of the patients was 5.61±1.39 kb, which was shorter compared with the controls, who had a mean of 7.97 ±2.96 kb. Forero *et al.* [21] similarly reported reduced TL in Alzheimer's patients relative to healthy participants in a larger cohort of 860 patients and 2022 individuals.

In this study, Alzheimer's patients aged 56–90 years (mean 5.61±1.39 kb) had TLs significantly shorter than those of their grandparents (42–72 years; mean 9.6±0.8 kb) and great grandparents (62–82 years; mean 8.0±1.1 kb). Saretzki observed a TL reduction of ~2.4 kb in Alzheimer's patients compared with age-matched controls. They also reported a decline in TL from a mean±SD of 16.4±1.2 kb in the newborns to

**Figure 4**




This figure represents the telomere and single-copy gene (T/S) ratio for all Alzheimer's patients versus the value of fold change.

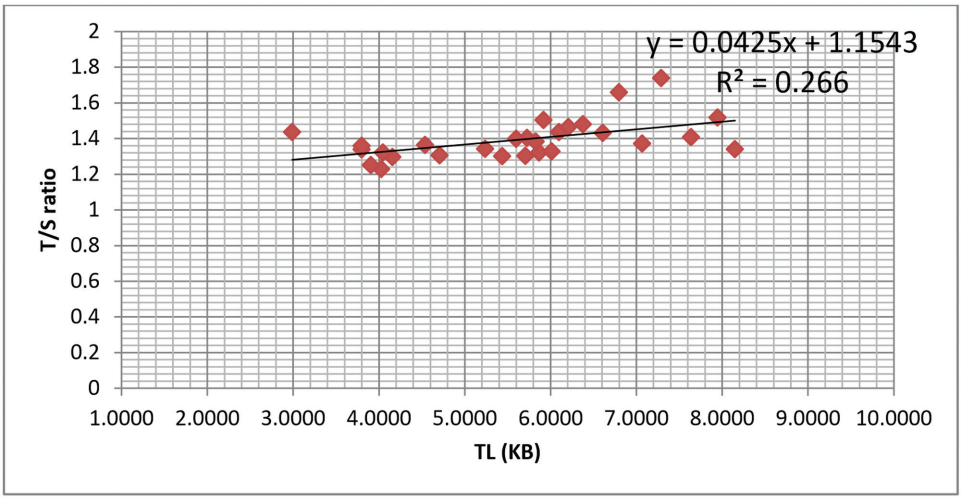
11.6±1.2 kb in their parents, and further reductions in grandparents (42–72 years) and great grandparents (62–82 years) [22].

The current findings suggest that individuals with a family history of AD may be more susceptible to the disease. Thorin noted that familial AD, though rare, represents less than 2% of cases and follows an autosomal dominant inheritance pattern, with symptoms potentially appearing as early as age 24 years in the most aggressive familial mutations identified [23].

Among the patients in our study, a 72-year-old female with the shortest TL (2.99 kb) was a smoker with a family history of diabetes and hypertension, showing a significant reduction of about 4 kb from the normal range. In contrast, a 75-year-old female patient with the longest TL (7.98 kb) was a nonsmoker without diabetes or hypertension. Kiecolt-Glaser *et al.* [24] highlighted that TL is influenced by both genetic and environmental factors. Notably, a 71-year-old patient with a TL of 7.52 kb, which is close to the control group's average, suggests that in some cases, age may not significantly affect TL, and that other environmental factors may play a role [25].

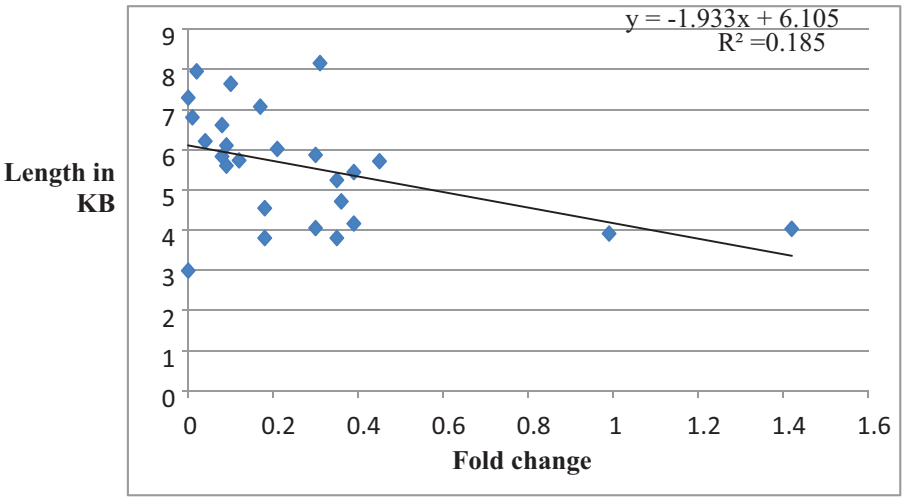
Telomere shortening may be accelerated by oxidative stress, inflammation, lifestyle factors, and other contributors to AD [26]. Our study revealed that 75% of controlled diabetic patients had reduced

**Figure 5**




Correlation of relative telomere length (TL) determined by quantitative-fluorescence in situ hybridization (Q-FISH) (kb) and telomere and single-copy gene (T/S) ratios by real-time PCR (RT-PCR).

**Figure 6**




Correlation of relative fold change of telomeres by real-time PCR (RT-PCR) and telomere length (TL) (kb) determined by quantitative-fluorescence in situ hybridization (Q-FISH).

relative TL, while 25% of uncontrolled diabetic patients showed increased fold change. Significant differences were observed between T/S ratios and fold changes in controlled diabetes ($P$=0.0004) and uncontrolled diabetes ($P$=0.025). This supports previous studies linking shorter TL with type 2 diabetes mellitus [27,28]. In contrast, other studies have found a negative correlation between TL and type 2 diabetes mellitus [29]. In addition, several studies have highlighted the variability of TL across different population groups [30].

Similarly, significant differences were noted in controlled hypertension patients between T/S ratios and fold changes ($P$=0.0001) and in uncontrolled hypertension patients ($P$=0.0003). Webb *et al.* [31] found that systolic blood pressure variability is associated with dementia and may predict faster AD progression.

In our study, smoking was associated with telomere shortening with 43.3% identified as smokers (13 cases). Significant differences in T/S ratios and fold changes were found between smokers ($P$=0.012) and nonsmokers ($P$=0.004). Some studies have shown that TL, a marker of aging and survival, is heritable and varies among individuals, influenced by factors such as oxidative stress and smoking [32,33]. While smoking may contribute to cerebrovascular diseases, it may not directly cause AD but could indirectly increase the risk of dementia [34].

Our study found that TL measured by RT-PCR correlated with results from Q-FISH with a correlation coefficient ($r^2$) of 0.266 ($P$=0.004). To our knowledge, this is the first comparative study conducted in Egypt. In addition, there was an inverse relationship between TL (kb) and fold change ($r^2$=0.185, $P$=0.018), indicating that as TL increases, the fold change decreases, reflecting a reduction in TL.

Gutierrez-Rodrigues *et al.* [35] reported that flow-FISH is more precise and reproducible than RT-PCR for measuring TL. In addition, other studies have highlighted that techniques such as TRF, RT-PCR, FISH, and telomere length combing assay are effective for measuring average TL, while methods like Q-FISH and single telomere length analysis are better suited for assessing the shortest telomeres [9].

Based on these results, both RT-PCR and Q-FISH methods have their advantages and limitations, and their results are complementary. RT-PCR is straightforward, requiring only a small amount of starting DNA, but it provides only an average TL as a relative ratio. However, Q-FISH can detect telomeres on all chromosomes but struggles with very short telomeres that do not hybridize with probes, appearing as telomere-free ends. In addition, Q-FISH requires highly skilled cytogeneticists for chromosome-specific analyses and expensive probes.

Although Q-FISH on metaphases is unique in its ability to measure all individual telomeres per metaphase, it is time consuming, often taking a week to process a small number of samples, making it impractical for large-scale studies. Currently, no

single method perfectly combines accuracy, efficiency, and speed for measuring TL. Nevertheless, RT-PCR remains a key high-throughput option for TL assessment due to its ease of use and accessibility.

## Conclusion

We concluded that there is a significant correlation between AD and the reduction in TL. Environmental factors, such as diabetes and hypertension, also impact TL in AD. Moreover, RT-PCR is more precise and easier to use than Q-FISH for evaluating TL.

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### Conflicts of interest

There are no conflicts of interest.

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