DNA Methylation as a Coming Clue for Age Prediction

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ABSTRACT

Background: DNA methylation (DNAm) is a biochemical modification which occurs over the lifespan of an individual and it is a substantial constituent in the aging process. The degree of methylation was significantly related to age. So far, the ELOVL2 locus has been the most thoroughly studied age marker. This locus has been demonstrated to be reliable in ancient and fresh human bloodstains, which constitute a major source of DNA in forensic laboratories. **Aim of work:** The current study aimed to assess the use of DNA methylation on the ELOVL2 gene from blood samples as biomarkers for chronological age estimation using pyrosequencing in Egypt. **Material and methods:** Eighty whole blood samples from individuals aged 18-69 years divided into 4 groups were analysed using a DNA methylation quantification assay based on bisulphite conversion and DNA pyrosequencing of 7 CpG sites in the ELOVL2. **Results:** Our results display significantly strong correlation between DNAm and chronological age; the model supporting DNAm as a strong age predictor. The age prediction accuracy was most accurate in age group III (>40- 50y) and was least accurate in age group IV of the elderly individuals (>50-69 y) on choosing1, 2.5-, and 5-years as difference threshold.

Keywords: DNA methylation, aging, whole blood, ELVOL2.

1. INTRODUCTION

In human existence, ageing is a natural and continual process. The chronological age is different from the biological age which refers to how old a person seems influenced by genetic and environmental factors independent of the passage of time alone ***(Freire-Aradas et al., 2017; Jung et al., 2017).*** Age discrimination of anonym human bodies is an important issue in the field of forensic medicine. It can provide valuable information to the medicolegal interrogator in crime inquiry as well as utility in mass catastrophe situations where age may be difficult to estimate ***(Elmadawy et al., 2021).*** Estimation of chronological age from biological materials such as bloodstain is a pivotal important point in forensic investigations ***(Meissner and Ritz-Timme, 2010).*** Procedure based on forensic genetic analysis expected to provide some advantageous information than conventional methods of age estimation as there is no adequate morphologic or biochemical information ***(Ou et al., 2012).*** Since then, new DNA tests have been investigated to deduce individual age from a biological trace ***(Freire-Aradas et al., 2017).***

The application of DNA methylation (DNAm) to obtain additional information in forensic investigations has shown to be a promising and growing topic of study (Naue et al., 2017). For a long time, DNAm was a "black hole" for forensic experts, but it can now provide forensically relevant information that matches the DNA profile ***(Vidaki et al., 2017).*** DNAm is the addition of a methyl group to the

5' cytosine of a CG dinucleotide, which has an impact on transcription factor binding sites, insulator components, and chromosome architecture, as well as gene regulation and cell differentiation ***(Ziller et al., 2013).*** These functions explain why DNAm at key places in the genome displays a cell-type-specific pattern and how it can be utilised to differentiate between tissues and body fluids ***(Lee et al., 2012).*** Multiple DNA methylation regions that can be useful for age prediction have been identified in recent advances epigenomics. Some of these indicators have been employed in the development of age prediction models that could be useful in forensics. DNA methylation markers have been demonstrated to outperform other forms of possible age predictors, such as telomere lengthening, age-dependent changes in T cell DNA, and age-altering mRNA levels ***(Spólnicka et al., 2017).*** The promoter of the ELOVL2 gene is regarded the most promising locus for age prediction in the forensics profession ***(Park et al., 2016).***

Forensic genetics have been recently burgeoned in Egypt. Until recently, the data about age prediction in Egyptians by using molecular genetics methods still finite. Therefore, Egyptian forensic DNA data extension is essentially recommended. So, the current study aimed to use the DNA methylation from blood samples for chronological age prediction. The novelty of our study is in the application of epigenetic studies to an Egyptian population that has never been studied previously. Also, this study aimed to establish methodology and evaluated results on the Egyptian population.

2. MATERIALS AND METHODS

2.1. Sample collection, DNA extraction and Quantification:

-A total of 80 healthy unrelated volunteers of both sexes were included in this study. The volunteer's age ranged from 18–69-year-old which divided into 4 groups as following:

Group (1): 18 – 30 years.
Group (2): >30 – 40 years.
Group (3): >40 – 50 years.
Group (4): >50 – 69 years.

-Inclusion criteria: All are healthy unrelated Egyptian donors aged from 18 – 69 years.
-Exclusion criteria: Individuals with age-associated disease (such as cardiovascular, Parkinson's disease, Alzheimer's disease, immunological disease and cognitive impairment), human genetic syndromes (such as Down syndrome and Werner's syndrome), alcohol drinking, smoking. Obesity, metabolic syndrome and cancer.

-Whole blood samples were collected from all individuals into EDTA tubes to be stored at -80^{o}C for further molecular analysis. The DNA isolation kit (G-spin^{TM}, Korea) was used to extract genomic DNA from the collected blood samples according to the manufacturer instructions. DNA was then measured and evaluated using nano drop spectrophotometer and gel electrophoresis to determine the quality of each individual's amplicon pool.
Written informed consents were attained from all participants for their legally authorized representative. This research was approved by the Benha University Research Ethics Committee, approval number 00084

2.2. Bisulfite conversion and quantification

The extracted DNA was exposed to bisulfite conversion using Thermo Scientific™ EpiJET™ Bisulfite Conversion Kit.

2.3.PCR amplification and methylation analysis

PCR amplification of DNA was done using COSMO PCR RED M. Mix and primer set. The Primer sequence was Biotin-AGGGGAGTAGGGTAAGTGAGG (sequence forward), AACAAAACCATTTCCCCCTAATAT (sequence reverse) and ACAACCAATAAATATTCCTAAAACT (sequencing).
PCR product purification and Agarose Gell Electrophoresis were done to check the quality of the product. DNA Pyrosequencing: The final DNA pool will be sequenced:
We act on ELVOL2 (ELVOL fatty acid elongase 2 chromosome location):

- CpG1: Chr6:11,044,661.

- CpG2: Chr6:11,044,655.
- CpG3: Chr6:11,044,647.
- CpG4: Chr6:11,044,644.
- CpG5: Chr6:11,044,642.
- CpG6: Chr6:11,044,640.
- CpG7: Chr6:11,044,634.

Lastly this formula was used to obtain intact result:

Zbiec-Piekarska 1-42.8393176902677 + 0.63266203860581 × ELVOL2 (CPG5) + 0.877474742612866 × ELVOL2 (CPG7) (Zbieć-Piekarska et al., 2015).

2.4. Statistical Analysis

After data collection, data was revised, coded, and served to statistical software IBM SPSS version 21. All values at $P \leq 0.05$ were considered to be significant. Data are presented as Minimum, Maximum, mean ± SE. Comparison of the correlation coefficient of the two population means of independent samples was done using the Student's T-test. Correlations among variables were studied by using the Pearson`s coefficient. The mean absolute deviation (MAD) and the standard error of estimate (SEE) were used to check the accuracy of predictions made with the regression line.

3. RESULTS

In all age categories, there were no statistically significant differences between chronological age and estimated age **(Table 1).** The mean of absolute value of predicted age minus chronological age in the four age prediction models ranged from 1.12 to 1.63 years, while the median value ranged from 0.11 to 1.96 years **(Fig. 1).**

Considering intergroup comparisons assessment was performed using the values of the predicted age minus chronological age of each group. There were no statistically significant differences between groups regarding mean values of age differences **(Table 2).**

Correlation analysis indicated a strong positive statistically highly significant correlation present overall ($0.790 \leq r \leq 0.892$, mean absolute r = 0.826) between predicted and chronological age for the four groups, which explained 62.4% to 79.6% of the age variation according to R square **(Fig. 2).**

The performance and accuracy of the age prediction model were calculated for all individuals, with a difference of 1, 2.5, and 5 years between the predicted and chronological ages, as well as for the four groups based on their chronological age. The model presented with the least performance observed in group IV (MAD of 3.932 and SEE of 4.816). When a threshold of 1 year difference was chosen, the age prediction accuracy was better in age group III (70% of correct predictions) than in age group I (65% of correct prediction) and in age groups II, IV (60% for both). As regards 2.5 years difference as threshold, the age prediction accuracy was better in age group III (80% of correct predictions) than in age group I (75% of correct predictions) and in age groups II and in IV (70% of correct predictions for both). Considering 5 years difference as threshold, the age prediction accuracy was better in age groups I and III (85% of correct predictions for both) than in age group IV (80% of correct predictions) and in age group II (75% of correct predictions) **(Table 3).**

The average and SE of DNA methylation of ELOVL2 gene at CpGs sites in blood samples at different age levels increase as age increased at different CpGs sites **(Table 4).** Correlation analysis indicated an excellent positive statistically highly significant correlation between DNAm status at CpG(s) sites of ELOVL2 gene and blood estimated age **(Table 5).**

Table (1): Descriptive statistics of chronological age and estimated age in the four age-groups prediction model.

Groups	All Groups (18-69 y) (n=80)	Group I (18-30 y) (n=20)	Group II (>30-40 y) (n=20)	Group III (>40-50 y) (n=20)	Group IV (>50-69 y) (n=20)
Chronological age					
Mean ± SE	40.35±1.52	23.70±0.85	34.50±0.66	44.50±0.66	58.70±1.40
Median	39.50	24.00	34.50	44.50	58.00
Min-Max	18.0-69.0	18.0-29.0	30.0-39.0	40.0-49.0	50.0-69.0
Estimated age					
Mean ± SE	40.09±1.22	24.93±0.78	35.60±0.84	46.10±0.84	57.62±1.52
Median	39.04	26.05	33.21	45.61	57.09
Min-Max	12.52-69.57	12.52-34.75	29.53-42.68	37.71-49.90	48.18-69.57
Test of Significance (P-value)	0.949	0.223	0.230	0.172	1.974
	0.345	0.829	0.821	0.865	0.063

SE: Standard error, Min-Max: Minimum-Maximum, Significant (P<0.05).

Table (2): Intergroup comparisons assessment using the values of the predicted age minus chronological age of each group.

Groups	Group I vs II	Group I vs III	Group I vs IV	Group II vs III	Group II vs IV	Group III vs IV
T test	0.918	0.856	0.283	0.118	0.874	1.258
P-value	0.308	0.624	0.778	0.907	0.388	0.184

Significant (P<0.05)

Table (3): Evaluation of the accuracy of the age prediction models.

Individuals	All Groups (n=80)	Group I (n=20)	Group II (n=20)	Group III (n=20)	Group IV (n=20)
MAD	**3.343**	**2.136**	**1.917**	**3.188**	**3.932**
SEE	**4.422**	**3.756**	**3.680**	**4.003**	**4.816**
PCP					
≤ 1 years	**63.8%**	**65%**	**60%**	**70%**	**60%**
≤ 2.5 years	**76.3%**	**75%**	**70%**	**80%**	**70%**
≤ 5 years	**82.5%**	**85%**	**75%**	**85%**	**80%**

MAD: Mean absolute deviation, SEE: Standard error of estimate, PCP: Percentage of correct predictions

Table 4: Average and SE of DNA methylation of ELOVL2 gene at CpG sites in blood samples at different age levels

ELOVL2 site	Group I (18-30 years) (n=20)	Group II (>30-40 years) (n=20)	Group III (>40-50 years) (n=20)	Group IV (>50-69 years) (n=20)
CpG_1	67.70±0.44	74.74±0.50	82.02±0.53	89.72±0.53
CpG_2	47.56±0.50	54.50±0.43	62.0±0.58	69.53±0.51
CpG_3	46.80±0.46	54.18±0.45	60.68±0.51	69.28±0.57
CpG_4	58.06±0.50	65.15±0.47	71.87±0.47	79.99±0.49
CpG_5	33.54±0.58	40.10±0.48	46.44±0.69	55.05±0.97
CpG_6	22.26±0.47	29.22±0.47	35.53±0.48	44.30±0.84
CpG_7	51.49±1.06	59.34±0.65	66.16±0.62	74.79±1.17

*Values are expressed as Mean ± Standard Error.

Table 5: Correlation analysis between the DNA methylation status at different CpG(s) sites of ELOVL2 gene and estimated age.

CPG(s) sites	Estimated age				
	All Groups (n=80)	**Group I (n=20)**	**Group II (n=20)**	**Group III (n=20)**	**Group IV (n=20)**
CpG_1	0.986 0.000*	0.902 0.000*	0.865 0.000*	0.788 0.000*	0.923 0.000*
CpG_2	0.984 0.000*	0.902 0.000*	0.862 0.000*	0.752 0.000*	0.929 0.000*
CpG_3	0.986 0.000*	0.902 0.000*	0.865 0.000*	0.788 0.000*	0.924 0.000*
CpG_4	0.986 0.000*	0.902 0.000*	0.865 0.000*	0.788 0.000*	0.923 0.000*
CpG_5	0.979 0.000*	0.922 0.000*	0.816 0.000*	0.832 0.000*	0.894 0.000*
CpG_6	0.983 0.000*	0.884 0.000*	0.826 0.000*	0.688 0.000*	0.925 0.000*
CpG_7	0.991 0.000*	0.983 0.000*	0.931 0.000*	0.934 0.000*	0.944 0.000*

*Significantly different at ($P<0.05$).

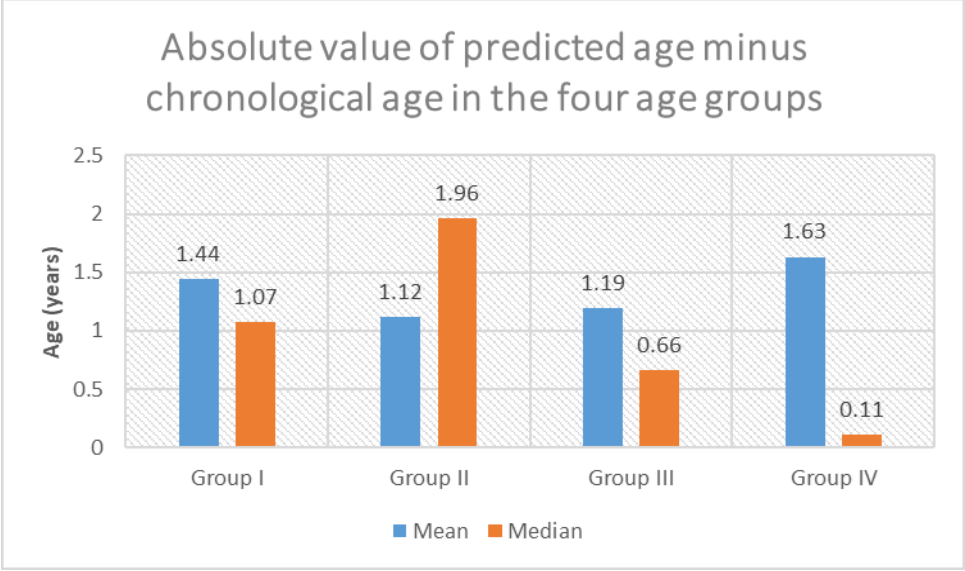

Figure (1): Absolute value of predicted age minus chronological age in the four-age group prediction model.

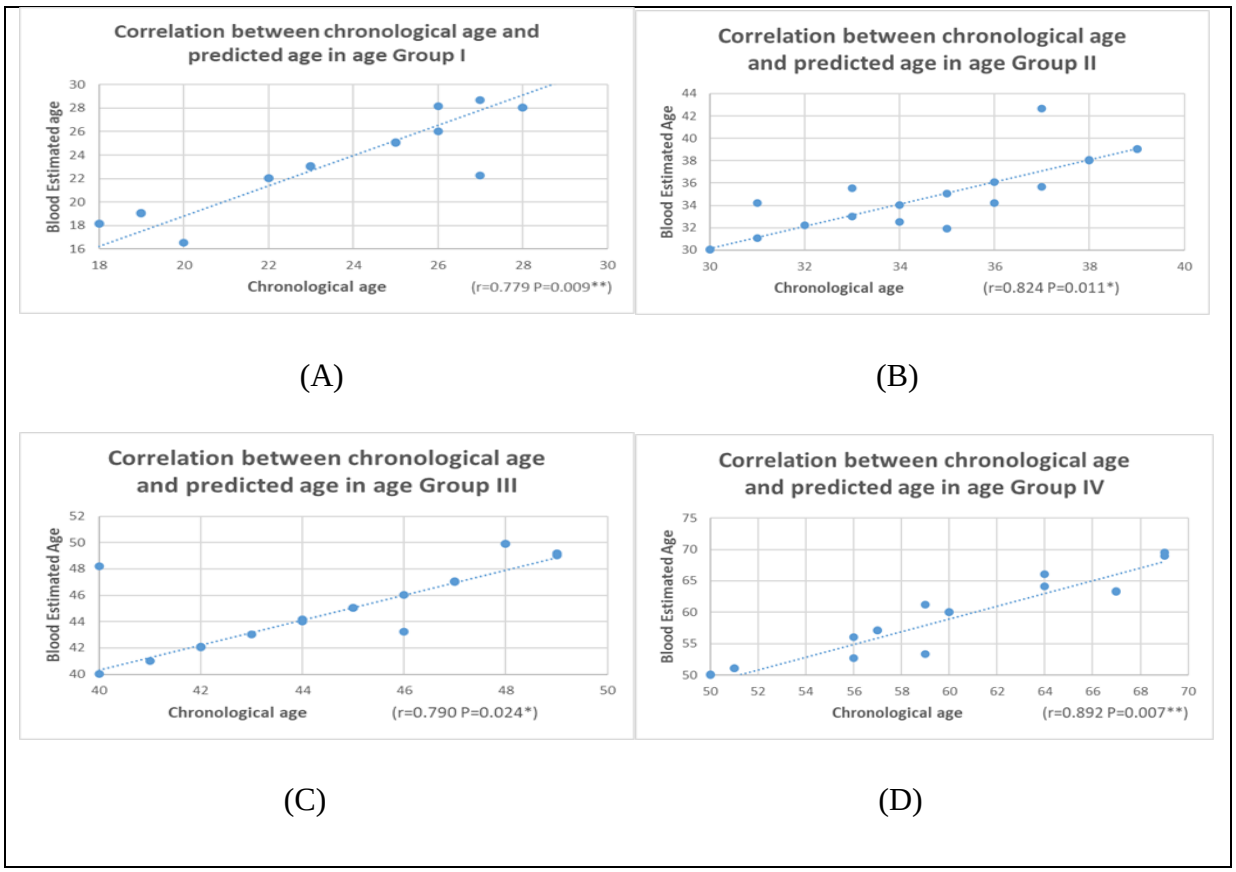

Figure (2): Correlation between chronological age and predicted age (A; age group I, B; age group II, C; age group III, D; age group IV).

4. DISCUSSION

For a long time, forensic science has been hunting for a relevant form of marker that may help speed up the age prediction process using biological clues found at a crime scene. Recently, Because the regulatory areas of multiple genes get progressively methylated with increasing age, DNAm is considered the most promising information source about human age in forensic science. This suggests a functional relationship between age, DNA methylation, and gene expression ***(Sukawutthiya et al., 2021).***

The present study exposed a high correspondence between predicted age and chronological age supporting DNAm as a strong age predictor. Remarkably, across a population, this DNAm age correlates strongly with chronological age ***(Field et al., 2018).*** Because the human genome has a large number of DNAm loci that are linearly associated with age and may match each other, a very precise final estimate of chronological age can be obtained ***(Zbieć-Piekarska et al., 2015).*** These results are matched with the results of previous studies (Huang et al., 2015; Xu et al., 2015; Mcewen et al., 2017; Jung et al., 2019). Also these findings are in accordance with those reported by, ***Daunay et al. (2019)*** who found strong correlation between DNAm of all CpGs and the chronological age of all individuals. In contrast, ***Jung et al. (2017)*** who discovered that methylation measurements at several to hundreds of CpG sites are expected to measure biological age, which is not always coincident with chronological age but can help predict life expectancy, implying that DNA methylation age could be useful in the search for age accelerators or decelerators for a longer human lifespan.

In our data, the MAD between predicted and chronological age was largest in the groups of older people. This was in agreement with ***Bekaert et al. (2015)*** who found that, the MAD between expected and chronological age was the largest for people ≥ 60 years old. Additionally, ***Zbieć-Piekarska et al. (2015)*** reported that age prediction accuracy is lowest in the age group comprising individuals aged 60–75 years. Furthermore, this finding is consistent with earlier research indicating that DNAm patterns predict age more accurately in younger people than in older people, as older people have a higher variance and their age is under-predicted ***(Naue et al., 2017; Correia Dias et al., 2020).*** The association between ELOVL2 DNA methylation and age was not a straight line, but rather a curve that climbed steeply throughout childhood before leveling? out later in life ***(Bekaert et al., 2015).*** This finding could be explained by environmental factors having a greater impact on DNA methylation status in older people with different medical histories or lifestyles, which increases age estimation error ***(Zbieć-Piekarska et al., 2015).*** In contrast, Huang et al. (2015) reported that, there was no significant difference between the young and elderly groups because they used a limited sample size to screen the potential markers, which comprised 10 blood samples from younger donors (aged 10 to 25 years) and 10 blood samples from older donors (aged 55 to 65 years).

In the present study, the age prediction accuracy was more accurate in age group III **(**>40-50 y**)** and less accurate in age group IV (>50-69 y) on choosing 1, 2.5-, and 5-years difference as threshold. This result in accordance with ***Freire-Aradas et al. (2016)*** who found that Category III (40–59) was successfully predicted (76.47% of the population), when the predicted age coordinated with the actual age 5 years. Also, in the same vein of Al-Ghanmy et al. (2021) who reported that predicted age correlated well with chronological age in the 40–59 year age groups, but less accurate in the ≥60 year age group. Also, in agreement with Bekaert et al. (2015) and Zbieć-Piekarska et al. (2015), who found that 55.2% success rate for samples of 60–75 years and 54.9% for study samples of 60–91 years, respectively. Changes in DNA do not occur at the same rate throughout a lifetime, although they do accumulate quickly until adulthood ***(Freire-Aradas et al., 2016)***. Certain age-related DNA mutations appear to be pre-programmed, whereas others are the result of environmental and stochastic factors. Increased methylome age has been linked to lower mental

and physical fitness in the elderly, as well as greater mortality in those aged 69-79 years ***(Marttila, 2016).***

The present study illustrated that ELOVL2 appears to be a good candidate marker for age estimate. ***Freire-Aradas et al. (2016)*** confirmed that ELOVL2 has been widely reported as a main age predictor and consequently is integrated in all forensic expectation models to date, as the most useful age marker. The underlying reason could be that DNA methylation levels in the ELOVL2 specific locus are very consistent across samples ***(Sukawutthiya et al., 2021).***

In the present study, there is an outstanding positive statistically highly significant correlation between DNAm status at studied CPG(s) sites of ELOVL2 gene and predicted age. These result in accordance with Garagnani et al. (2012) who stated that the methylation level of CPG sites in the ELOVL2 promoter is strongly related to age, and the change in methylation level with aging is large, ranging from 7% to 91%. In contrast with ***Zbieć-Piekarska et al. (2015)*** who stated that CPG sites 5 & 7 were found to be most considerably correlated with age and Johansson et al. (2013) who reported that the strongest affirmative correlation of methylation with age is seen in a CPG_1 in the promoter of ELOVL2.

5. CONCLUSION

Based on our satisfactory and promising results regarding the correlation of methylation patterns and chronological age, this study suggests that methylation of ELOVL2 can be used as an indicator of age prediction. These advances in technology and molecular biology can be used as very important tools in forensic sciences.

6. RECOMMENDATIONS

- The process of creating age prediction models using DNA methylation necessitates procedures and equipment that forensic laboratories don't typically count or use on a regular basis, implying additional costs and proper staff training.
- Methylation is an epigenetic mechanism that regulates gene expression. Age prediction analysis cannot be completely based on previous studies' findings, because different population groups are exposed to different environmental factors that may alter gene regulation and thus methylation patterns. Each population group must be sequenced and analyzed to standardize not only the technique, but also the reference parameters used for the results interpretation.
- More research is needed to increase the forecast accuracy for older age categories.

CONFLICTS OF INTEREST

There are no conflicts of interest declared by the authors.

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الملخص العربى

مثيلية الحمض النووى الديوكسي ريبوزى كوسيلة للتنبؤ بالعمر

مثيلية الحمض النووي (DNAm) هو تعديل كيميائي حيوي يحدث على مدى عمر الفرد وهو مكون مهم في عملية الشيخوخة. درجة مثيلة الحمض النووي مرتبطة بشكل كبير بالعمر. الجين ELOVL2 هو أكثر علامات العمر تقييمًا بدقة حتى الآن. وقد ثبت أن هذا الجين يمكن تحليله بشكل موثوق في بقع الدم البشرية القديمة والحديثة ، والتي تعد مصدرًا رئيسيًا للحمض النووي في مختبرات الطب الشرعي. الهدف من البحث: هدفت الدراسة الحالية إلى تقييم استخدام مثيلة الحمض النووي على جين ELOVL2 المأخوذ من عينات الدم للمصريين كمؤشرات حيوية لتقدير العمر الزمني باستخدام التسلسل الحراري. طريقة البحث: تم تحليل 80 عينة دم كاملة من الأفراد الذين تتراوح أعمارهم بين 18-69 سنة مقسمة إلى 4 مجموعات باستخدام مقايسة مثيلة الحمض النووي على أساس تحويل ثنائي الكبريتيت والتسلسل الحراري للحمض النووي في 7 مواقع CpGفي ELOVL2. نتائج البحث: تُظهر نتائجنا ارتباطًا قويًا بين الحمض النووي والعمر الزمني ، وهو النموذج الذي يدعم الحمض النووي كمتنبئ قوي بالعمر. كانت دقة التنبؤ بالعمر أفضل في الفئة العمرية الثالثة (أكثر من 40-50 سنة) وكانت أقل دقة في الفئة العمرية الرابعة للأفراد المسنين (أكثر من 50-69 سنة) في اختيار الفرق 1 و 2.5 و 5 سنوات.