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# DNA methylation of *ELOVL2* gene as an epigenetic marker of age among Egyptian population

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# Abstract

**Background** Cellular and molecular changes occur during aging, decreasing organ function. The aging process was measured by several biomarkers, including DNA methylation (DNAm), an epigenetic change regulating gene expression, which is highly accurate at predicting biological age. DNAm is heritable and therefore varies between different populations.

**Aim** To assess blood DNA methylation changes as epigenetic clocks in the male and female Egyptian population. Pyrosequencing was used to measure the methylation of nine CpG sites in blood samples from 100 healthy Egyptians (18–69 years) using a cross-sectional study. Two age predicted models based on the *ELOVL2 gene* were compared in three age categories and correlated in all age groups despite decreasing accuracy with increasing age.

**Results** The mean absolute deviation (MAD) using the 1st and 2nd age predicted models for 18–40 years was 1.06 and 2.7, respectively; for 41–60 years, it was 4.4 and 3.8, respectively; and for > 60 years, it was 7.7 and 7.0, respectively. No significant differences in DNA methylation were found between the sexes.

**Conclusion** DNA methylation of the *ELOVL2* gene can be used as an accurate biomarker for age estimation. Additionally, this method has the potential to be more accurate than traditional methods of age estimation.

Keywords Aging, DNA methylation, ELOVL2, Egyptian individuals

## Introduction

Aging has attracted curiosity and provoked imagination throughout history. Because of the subconscious fear of aging accompanying morbidity and mortality, a novel aging measure is sought to differentiate a person's risk for a variety of health outcomes. Although age is measured chronologically, it inaccurately correlates with biological age, which reflects an individual's health status; therefore,

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DNAm levels have been used as a biomarker for biological age. DNAm-based age is also known as "epigenetic age" or "epigenetic clock". Epigenetics refers to heritable gene function changes that cannot be explained by DNA sequence changes [3]. The epigenome is defined as the complete description of all chemical modifications to DNA that regulate gene expression within the genome. A number of epigenetic factors control transcription, such as DNA methylation and histone modifications, which monitor protein synthesis. The epigenetic pattern is preserved throughout generations in the same way that the DNA sequence is inherited. However, during an individual's lifetime, they can be altered or modified over time. However, these changes are reversible and occur without



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alterations to the primary DNA sequence. Environmental exposures can modify epigenetics, such as nutrition, life-style, and smoking [4, 5].

DNAm relationship with the aging process, which varies throughout life, is vital for understanding the molecular mechanisms of normal and premature aging. This is also vital for their application to predicting an individual's age. Aging is the slow, complex, and time-dependent decline of multiple biological and physiological functions. Aging has profound implications for death risk. Aging is also associated with increased risk for several chronic diseases; therefore, measuring the aging rate is critical for clinical, basic, and observational research. Persons of the same chronological age may vary in their aging rate, suggesting that chronological age is a poor representation of biological aging [6].

Biological aging can be predicted through DNA methylation levels. This primarily affects cytosines and is followed by guanines in a 5'-3' direction in the DNA double helix. This process results in the addition of a methyl group (-CH<sub>3</sub>) to their 5' carbon (C5), which varies over time. These specific 5'-3' cytosine-guanine methylation sites in DNA are called "CpG" dinucleotides [7, 8].

Methylation of specific CpG sites of many genes, such as *ELOVL2*, FHL2, and PENK, correlates with age. Among them, *ELOVL2* is a very promising biomarker for age prediction. It shows a strong correlation with the age-progressive increase in methylation levels throughout maturation and aging. The *ELOVL2* gene is found on the p arm of chromosome 6. It encodes *ELOVL2*, which is a transmembrane protein involved in the synthesis of polyunsaturated fatty acids (PUFA), which are involved in many crucial biological functions, including energy production, modulation of the inflammation response, and maintenance of cell membrane integrity. *ELOVL2* is hypermethylated in the elderly as it shows a progressive increase in methylation levels throughout maturation and aging [9–11].

Prior studies have evaluated the applicability of estimating biological age using age-associated CpG sites in humans [12, 13]. But none of these studies were conducted on the Egyptian population, considering our different lifestyles compared with the European population and their corresponding studies. In this study, we used genome-wide methylation data on Egyptians to identify epigenomic changes with age. It is imperative to study methylation changes with age, to understand the biological processes associated with aging and the role of epigenetics in susceptibility to age-related diseases.

The aim of our study was to assess blood DNA methylation changes as epigenetic clocks. Furthermore, blood DNA methylation can help to identify the suspect of unknown blood samples in a medico-legal case, decreasing the number of people in the search range.

## Methods

All study subjects signed a written, informed consent. The study protocol was approved by Al-Azhar University's Local Ethical Committee. Healthy volunteers were recruited et al.-Azhar University and the Theodor Bilharz Research Institute for this study.

## **Study subjects**

This study was conducted on 100 healthy Egyptian participants, including 57 males and 43 females, with ages ranging from 18 to 69. The participants were divided into three groups as follows: Group 1 with individuals aged between 18 ando 40 years (35 participants, 20 males, and 15 females), Group 2 with individuals aged between 41 to 60 years (34 participants, 21 males, and 13 females), and Group 3 with individuals aged over 60 years (31 participants, 15 males, and 16 females).

Healthy participants were included after investigations for complete blood count (CBC), chemistry, and hepatitis markers. Patients with chronic diseases, individuals having an infection within the two weeks prior to blood sample collection, and individuals who received any vaccinations within the two weeks prior to blood sample collection were excluded.

A volume of seven milliliters of peripheral venous blood samples was collected in vacutainer tubes from each participant under complete aseptic conditions. Blood samples were divided into two tubes: a plain tube (2 mL) for routine chemistry investigations and an EDTA tube (5 mL). The EDTA samples were centrifuged at 1000g for 10 min. The buffy coat was separated and frozen in aliquots at -80 °C for further molecular assays.

## **DNA** extraction

DNA extraction was performed to isolate DNA using the En-Mag Core<sup>®</sup>-nucleic acid extraction kit according to the manufacturer protocol.

#### **Bisulfite conversion**

Four hundred nanograms of genomic DNA were used to convert unmethylated cytosines to uracils leaving 5-methylcytosine residues unaffected using Thermo Scientific<sup>TM</sup> Epi JET<sup>TM</sup> Bisulfite Conversion Kits (Qiagen) according to the manufacturer's instructions. In every assay, internal control is included to ensure that the input target sequence has been bisulfite modified successfully.

### PCR amplification of DNA

PCR was conducted in a SimpliAmp thermal cycler with bisulfite-treated DNA as a template. The DNA

template (1  $\mu$ l) and *ELOVL2* primer (0.2  $\mu$ M) were mixed with bisulfite-treated DNA (20 ng), and the volume was adjusted to 30  $\mu$ l. Table 1 lists the *ELOVL2* PCR primer.

PCR was performed using thermal cycling conditions including an initial denaturation step performed for 10 min. at 95 °C, followed by 50 cycles of 30 s denaturation at 95 °C, 30 s annealing at 60 °C (primer time minus 5 °C), and 30 s elongation at 72 °C. The final step included 5 min. elongation at 72 °C and resulted in more than 10 million copies of template DNA being generated after 25–35 cycles.

#### **DNA pyrosequencing**

To prepare for pyrosequencing, 10 µL of each PCR product sample was purified and processed using the primer listed in Table 1. The technical steps were done according to the previous published method [13]. Briefly, streptavidin sepharose High Performance columns (manufactured by GE Healthcare, USA) were utilized to bind PCR products. The biotinylated PCR products were then purified using the Sepharose beads, washed, and denatured with a 0.2 M NaOH solution. After denaturation, the samples were rewashed using the Pyrosequencing Vacuum Prep Tool (manufactured by Qiagen) according to the instructions provided by the manufacturer. Pyrosequencing primers (Table 1) were annealed to the PCR products, and the PSQ96 HS System (manufactured by QiagenF Inc., USA) was used to sequence the samples as per the manufacturer's instructions. The methylation status of each locus was analyzed as a T/C single-nucleotide polymorphism (SNP) using Q-CpG software (manufactured by Qiagen). The methylation scores were calculated as the percentage of methylated alleles at each CpG site, with each locus being analyzed individually divided by the total number of methylated and unmethylated alleles. The rates of methylation were determined by computing the percentage of methylation at the CpG sites of every gene. The Pyromark Gold Q96 SQA Reagents (Qiagen) were used for DNA methylation analysis in

**Table 1** *ELOVL2* primer sequences are denoted in the 5'-3' direction. Biotin labels (-bio) are indicated at the 5'-end of the respective forward or reverse primers

Primer	Sequence (5 <sup>′</sup> –3 <sup>′</sup> )
Forward	GGAGGGGAGTAGGGTAAGTGA-bio
Reverse	ACCATTTCCCCCTAATATATACTTCA
Sequence	ΑCAACCAATAAATATTCCTAAAACT

accordance with the manufacturer's instructions on the Pyomark Q96 instrument.

## ELOVL 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: Chr6:11,044,634. CpG8: Chr6:11,044,638. CpG9: Chr6:11,044,625.

## Data analysis

Two formulas were used to obtain the intact result:

1. Zbiec-Piekarsa 1 model: -42.8393176902677+0.6 3266203860581×*ELOVL2* (**CpG5**)+0.87747474261286 6×*ELOVL2* (**CpG7**) [14]

2. Sukawutthiya model: - 25.9 + 0.7 (**CpG6**) + 0.6 (**CpG9**) [9]

## Statistical analysis

All statistical analysis and graphical representations were performed using MS Excel (Microsoft) for calculation of MAD and RMSE and Minitab 18 software for ANOVA. All continuous data were evaluated for normal distribution or homogeneous variances. Data are presented as a mean and standard error of mean (±SEM) for quantitative parametric data with a 95% confidence interval (CI).

For each of the three age groups, the accuracy of age prediction was evaluated by the mean absolute deviation (MAD) and the root mean square error (RMSE) and the correlation analyses were assessed using the Pearson R correlation coefficient (r) and coefficient of determination ( $R^2$ ). p values were also calculated for each formula in each group with P < 0.05 considered statistically significant and P < 0.001 highly significant.

$$MAD = \frac{\Sigma |Predicted age-Chronological age|}{n}$$
$$RMSE = \sqrt{\frac{\Sigma (Predicted age-Chronological age)2}{n}}$$

Mean absolute deviation (MAD) differences between different genders (males and females) and p value was calculated for each group.

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## Results

The study subjects were examined for full laboratory investigations to ensure that they are healthy within normal values. The methylation data were derived from the amplification of CpG islands within the *ELOVL2* gene using bisulfite conversion-pyrosequencing. The methylated CpG sites investigated were nine CpG sites (Table 2). The linear correlation between age and the DNA methylation level of the CpG sites on *ELOVL2* gene was measured using the two formulas (Zbiec-Piekarska 1 and Sukawutthiya age prediction models) as follows:

Zbiec-Piekarska 1 model

Sukawutthiya model

 $-25.9 + (0.7 \,\text{CpG6}) + (0.6 \,\text{CpG9}) \tag{2}$ 

DNA methylation of the *ELOVL2* gene was proved to be significantly correlated with age (P < 0.001). The *ELOVL2* gene showed nine CpG sites with the strongest positive correlations and significant hypermethylation linearity with chronological age. The individuals were divided into three age groups. Then, the age was predicted of each individual in each group by using age prediction model formulas using nine CpG sites in the *ELOVL2* gene.

The correlation in the group 1 (18–40 years) was discovered of R=0.96,  $R^2=0.93$ , mean absolute deviation (MAD)=1.05898917, and root mean square error (RMSE)=1.70815873 (P<0.001) using the Zbiec-Piekarska 1 formula in this age group (Fig. 1A). These data confirmed the positive correlation between the predicted age and the chronological age from 18 to 40 years (Table 3 and 4). However, when using the Sukawutthiya formula in this age group, a correlation was found of R=0.86,

 $R^2 = 0.74$ , MAD = 2.70142857, and RMSE = 3.29759609 (P < 0.001) (Fig. 1B).

When using the Zbiec-Piekarska 1 formula in group 2 (41–60 years), a correlation was observed of R=0.72,  $R^2$ =0.52, MAD=4.43809221, and RMSE=5.32826265 (P<0.001) (Fig. 1C). Also, when using the Sukawutthiya formula in this age group, a correlation was observed of R=0.65,  $R^2$ =0.42, MAD=3.825, and RMSE=5.42830409 (P<0.001) (Fig. 1D).

By applying the Zbiec-Piekarska 1 formula in group 3 (>60 years), a correlation was observed of R=0.60,  $R^2$ =0.40, MAD=7.68415232, and RMSE=8.3539714 (P<0.001) (Fig. 1E). Moreover, by applying the Sukawut-thiya formula to this age group, a correlation was observed of R=0.62,  $R^2$ =0.38, MAD=6.97774194, and RMSE=7.58755241 (P<0.001) (Fig. 1F).

All age groups were inferred from data, calculated by both models, showed strong positive correlations between DNA hypermethylation and age. When comparing predicted age with chronological age categories to assess age estimations with correlation coefficient (R) = 0.83 and mean absolute deviation (MAD) = 4.45, we found different accuracies.

The predicted age and the chronological age categories differences in males and females were compared in all age groups. The MAD using Zbiec-Piekarsa 1 model (MAD 1) and the Sukawutthiya model (MAD 2) was 5.2 and 4.9 for males, and 3.6 and 4.1 for females, respectively.

In group 1, there were 20 male and 15 female participants with MAD1 of 1.4 and MAD2 of 2.9 and MAD1 of 0.6 and MAD2 of 2.3, respectively. In group 2, there were 21 male and 13 female participants with MAD1 of 4.6 and MAD2 of 3.6 and MAD1 of 4.16 and MAD2 of 4.2, respectively. In group 3, there were 15 male and 16 female participants with MAD1 of 9.6 and MAD2 of 8.2 and MAD1 of 5.9 and MAD2 of 5.8, respectively.

**Table 2** Correlation analysis of CpG sites in the *ELOVL2* gene and chromosomal coordinates of nine CpGs at *ELOVL2* (Elongation of Very Long Chain Fatty Acids-Like 2) gene DNA methylation loci

(1)

Gene	Site	Assay location	GRCh38	R	R <sup>2</sup> (adj)	SE	P value
ELOVL2	CpG1	Intron 1	Chr6:11044628	0.785	0.61	9.7	4.08×10 <sup>-22</sup>
	CpG2	Intron 1	Chr6:11044631	0.687	0.47	11.4	$2.84 \times 10^{-15}$
	CpG3	Intron 1	Chr6:11044634	0.682	0.46	11.5	$5.93 \times 10^{-15}$
	CpG4	Intron 1	Chr6:11044640	0.897	0.80	6.9	$2.0 \times 10^{-36}$
	CpG5	Intron 1	Chr6:11044642	0.926	0.85	5.9	2.91×10 <sup>-43</sup>
	CpG6	Intron 1	Chr6:11044644	0.921	0.84	6.1	6.73×10 <sup>-42</sup>
	CpG7	Intron 1	Chr6:11044647	0.918	0.84	6.2	4.80×10 <sup>-41</sup>
	CpG8	Intron 1	Chr6:11044655	0.806	0.65	9.3	4.54×10 <sup>-24</sup>
	CpG9	Intron 1	Chr6:11044661	0.881	0.77	7.4	1.37×10 <sup>-33</sup>

Map coordinates refer to the genomic positions in human reference genome 38 (GRCh38)

R-correlation coefficient, R<sup>2</sup>(adj)-adjusted R<sup>2</sup> (coefficient of determination), SE-standard error, CpG-5'-cytosine-phosphate-guanine-5', Chr-chromosome



Fig. 1 Chronological age versus predicted age for group 1, 2, and 3 using the Zbiec–Piekarsa 1 formula (A, C, E) and the Sukawutthiya formula (B, D, F)

Table 3	Comparison	between	1st	and	2nd	age	prediction
models (	using MAD						

Age	1st Age predicted model	2nd age predicted model	p
18–40 years	1.06	2.7	0.0001
40–60 years	4.4	3.8	0.47
>60 years	7.7	7.0	0.39
Gender (sexes)	Male (4.8)	Male (4.6)	0.79
	Female (3.6)	Female (4.2)	0.38

## Discussion

DNA methylation is highly divergent between populations. This divergence may be mainly due to differences in allele frequencies and complex epistasis or gene and environment interactions. Although DNA methylation is a very stable epigenetic mark, numerous environmental influences have been associated with variation in DNA methylation and other epigenetic marks. These include nutritional factors, exposure to environmental pollutants, and the social environment [15, 16].

Age (years)	Zbiec–Piekarska 1 model				Sukawutthiya model			
	R	R <sup>2</sup>	MAD	RMSE	R	R <sup>2</sup>	MAD	RMSE
18–40	0.96	0.93	1.1	1.7	0.86	0.74	2.7	3.3
41-60	0.72	0.52	4.4	5.3	0.65	0.42	3.8	5.4
>60	0.6	0.4	7.7	8.4	0.62	0.38	7	7.6
Male	0.77	0.59	5.2	6.15	0.54	0.29	4.9	5.95
Female	0.61	0.37	3.6	5.04	0.52	0.27	4.1	5.18

**Table 4** Correlation results in the three studied groups using different models

R—correlation coefficient (R=0.70-1 represents very strong relationship, R=0.40-0.69 represents strong relationship, R=0.30-0.39 represents moderate relationship, R=0.20-0.29 represents weak relationship, R=0.01-0.19 represents no or negligible relationship, R=0 represents no relationship),  $R^2$  (adj)—adjusted  $R^2$  (coefficient of determination), MAD—mean absolute deviation, RMSE—root mean square error

In the current study, results showed high correlation between DNA methylation changes in all nine CpG sites of ELOVL2 gene with aging. Paprazzo et al. [17] confirmed that ELOVL2 was highly correlated with age and showed hypermethylation of nine CpG sites with aging from blood samples. Similar results were observed by Correia Dias et al. [12] who reported that the *ELOVL2* locus exhibited highly significant values for all selected CpG sites reflecting that DNA methylation changes are strongly correlated with aging across all nine CpGs in blood samples. Also, these findings are in accordance with those published by Daunay et al. [18] who found a strong correlation between DNAm of seven CpGs and the chronological age of all individuals in the blood sample. Bacalini et al. [19] previously reported DNA methylation of ELOVL2 CpG islands from whole-blood DNA samples was strongly correlated with age and demonstrated hypermethylation with age but using different CpG sites (CpG 11,12,13,14). Al-Ghanmy et al. [13] confirmed that ELOVL2 was highly correlated with age and showed hypermethylation of nine CpG sites with aging from blood samples.

This study revealed that DNA methylation is positively correlated with chronological age in all age groups. This is in agreement with other similar studies regarding the positive correlation of DNA methylation and age [20, 21]. Likewise, a study by Gensous et al. [20] found a high correlation between predicted ages and chronological ages of 278 healthy individuals of ages between 20 and 80 years old.

In a study conducted by Bernabeu et al. [22] on donors from Scotland aged between 18 and 99 years, correlation analysis indicated a strong link between predicted and chronological age. Other studies have also reported a significant positive correlation between the DNA methylation of *ELOVL2* and age [23]. Similarly, nonparametric correlation analysis showed a high correlation between predicted and chronological age, confirming the positive correlation of DNA methylation of *ELOVL2* and age [24]. Additionally, correlation analysis using Zbiec-Piekarska 1 age prediction models from blood samples of 100 French donors between 19 and 65 years of age found a strong correlation between predicted and chronological age [18]. These results demonstrate that DNA methylation of *ELOVL2* gene is a reliable biomarker for predicting chronological age. These findings are consistent with previous studies that have shown a robust correlation between DNA methylation and age.

According to the study data, the level of age-related methylation was highest in the age group of 18–40 years, then decreasing in the 40–60 age group, and lowest in the group over 60 years old. This finding is consistent with a recent study that found age-related methylation to be highest in the age group of 1–20 years, then decreasing in the 21–40 age group, with a further decrease in the 41–60 age group and the lowest in the 61–80 age group. The last group, aged 81–100 years, had a lower level of age-related methylation [25]. Similar observations that the difference between predicted and chronological age was the largest for people more than 60 years old, and accuracy between DNAm and aging decreased with increasing age [26].

No significant difference in DNA methylation between males and females was revealed in this study. This was also observed in other studies that gender had no effect on age prediction accuracy from blood sample [18]. Also, there were no significant differences in methylation levels between men and women for any CpG marker [13]. Gender effects did not impact the accuracy of model prediction with insignificant differences between males and females with a tendency to slightly overestimate male age. Recent study stated that although men predicted higher ages than women, sex did not influence age prediction [27]. This study findings indicated that gender effects did not have a significant influence on the accuracy of age prediction. Thus, the model was found to be unbiased toward gender. In the current study, each group was separately examined for the impact of gender revealing no significant differences in methylation levels between men and women in groups 1 compared to group 2. Group 3 revealed significant difference in methylation levels between men and women, with men showing more methylation denoting increased biological age. These results may suggest one of the reasons why men live for fewer years than women [28]. No similar reports could be reached concerning these results.

The paper has two main limitations. Firstly, a larger sample size should have been considered, including individuals of extreme age to conduct a more comprehensive survey of the Egyptian population. Secondly, a longitudinal study would have been more suitable than a crosssectional study in revealing the factors that influence biological aging, but it was limited due to subject compliance [29]. The study's raw data of all groups was provided in the Excel sheet of the Additional file 1.

## Conclusion

ELOVL2 gene can be used for prediction of age as it is significantly highly correlated with chronological age. There is a high correlation between DNA methylation changes in all nine CpG sites with aging. There is more probability to get accurate age prediction in younger ages. Zbiec-Piekarska 1 formula is more strongly correlated with chronological age in ages 18-60 years. Zbiec-Piekarska 1 formula is more accurate with chronological age in ages 18–40 years. Sukawutthiya formula is more strongly correlated with chronological age in ages above 60 years. Sukawutthiya formula is more accurate with chronological age in ages above 40 years. Younger people tend to have fewer health issues and lead healthier lifestyles, which means that the Zbiec-Piekarska 1 formula is more accurate for them. As people age, their lifestyles and physical health can change, which means that the Sukawutthiya formula is better able to take these factors into account and provide a more accurate prediction.

In conclusion, these findings indicate that DNA methylation of *ELOVL2* could be used as an accurate biomarker for age estimation. Additionally, this method may have the potential to be more accurate than traditional methods of age estimation.

#### Abbreviations

ANOVA	Aspartate aminotransferase
С	Cytosine nucleotide
CBC	Complete blood count
CH3	Methyl
Chr	Chromosome
CI	Confidence interval
CpG	Cytosine phosphate guanine
DNA	Deoxyribonucleic acid

DNAM	DNA methylation
EDTA	Ethylene diamine tetraacetic acid
ELOVL2	Elongation of Very Long Chain Fatty Acids-Like 2
FHL2	Four and a half LIM domains protein 2
G	Guanine nucleotide
MAD	Mean absolute deviation
PCR	Polymerase chain reaction
PENK	Proenkephalin
PUFA	Polyunsaturated fatty acids
R	Pearson R correlation coefficient
$R^2$	Coefficient of determination
RMSE	Root mean square error

## **Supplementary Information**

The online version contains supplementary material available at https://doi. org/10.1186/s43042-024-00477-7.

Additional file 1. Raw data of the study's group were listed in the Supplementary file.

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#### Author contributions

FM and HE designed the study and reviewed the manuscript. NS completed the practical part, collected the data, analyzed it, and wrote the original manuscript. All authors read and approved the final manuscript.

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#### Availability of data and materials

The data are available with corresponding author.

#### Declarations

#### Ethics approval and consent to participate

Has been approved by Al-Azhar University Ethical Committee 2022.

#### **Consent for publication**

This article has been approved for publication by all authors.

#### **Competing interests**

The authors declare no conflicts of interest related to this work.

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