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DNA Methylation Markers for Age Estimation

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Degree of Science in Biology / Zoology

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بِسْمِ اللَّهِ الرَّحْمَنِ الرَّحِيمِ
يَوْمَ تَشْهَدُ عَلَيْهِمُ أَلْسِنُهُمْ وَأَيْدِيهِمْ وَأَرْجُلُهُمْ بِمَا كَانُوا
يَعْمَلُونَ (24) يَوْمَئِذٍ يُوفِّيهِمُ اللَّهُ دِينَهُمُ الْحَقَّ وَيَعْلَمُونَ
أَنَّ اللَّهَ هُوَ الْحَقُّ الْمُبِينُ (25).....

صدق الله العلي العظيم

سورة النور..... من الآية (24-25)

DEDICATION

This thesis is dedicated.....

To my honorary and supporting in all time

My father ...

To who continually learns, grows and develops and who has been a source of encouragement and inspiration throughout my life,

My mum

To my life candlelight and operatives in the happiness moments

My sisters & brothers

To who taught me the meaning of friendship, fidelities and benevolentness

My friends

To everyone who prayed for my success I present my modest effort ...

Hiba

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Abstract

Age prediction is useful to narrow down the number of potential suspects because it can provide some general characteristics for predicting appearance. Epigenetic marks consider a promising method of the age prediction model. DNA methylation has an important role in the personal information of chronological age prediction. Recent studies reported DNA methylation levels of many CpG sites in the human genome have a relationship with aging. Therefore, DNA methylation levels of specific genes provide the key information of forensic investigation. In this study, 120 blood samples were collected from healthy persons, Arabic Iraqi population age range 18-years. The genomic blood samples were subjected to bisulfate conversion and 95 amplification followed by pyrosequencing. The results included 92 samples out of 120 that detected 27 methylated CpG sites of ELOVL2, TRIM59, FHL2, KLF14, and MIR29B2CHG genes. The statistical analysis used to multivariate regression analysis to finding a correlation between the methylated CpG sites and age. The 15 CpG markers of three genes (ELOVL2, TRIM59, and FHL2) showed linear correlations with chronological age, it could age estimation with a mean error of ± 6.92 years and correction coefficient was ($R^2 = 96.8\%$). For the most statistical satisfaction, only nine of age-associated methylation markers of ELOVL2 gene that including strong correlation and good fitting ($R^2 = 98\%$) to a prediction model based on a multiregression model to assess the prediction accuracy. The nine CpG sites of ELOVL2 gene have appeared strong linear relationship with age (all P-values < 0.05 , 0.01). The Mean Absolute Deviation (MAD) between predicted and real chronological age was ± 2.893 years. Notably, the DNA methylation marks of ELOVL2 gene strongly suggest the prediction model of age in this work. Identification of novel single nucleotide polymorphism in the Iraq population. rs7808283 G>A associated with the loss of one CpG site of human KLF14 located at Chromosome Chr7:130734413-130734373 of Promoter gene.

Title	page
Dedication	
Acknowledgments	
Abstract	I
List of contents	II- III- IV
List of Table	V-VI
List of Figures	VII- VIII
List of abbreviations	IX

Chapter One : Introduction

1.1	Introduction	1
	The Aim of the study and objectives	3

Chapter Tow: Literature Review

2.1	Ageing Theory	4
22.	Epigenetics and DNA methylation	7
32.	Age and its association with Epigenetic	9
.132.	CpG islands	10
.232.	Mechanism of gene silencing	12
.332.	Gene-body methylation in mammals	15
42.	Changes in the DNA Methylation in Cancer Cells	16
.142.	Hypomethylation	16
.242.	Hypermethylation	16
52.	DNA demethylation	17
62.	Methods used for Age Estimation	19
.162.	Telomeres	19
.262.	Dental age	20
.362.	Immunological age	21
72.	biomarkers of this study	22
.172.	ELOVL2 Elongation of very long-chain fatty acids–like 2 gene	22
.272.	The tripartite motif (TRIM)	23

.372.	FHL2 gene	23
.472.	KLF14 gene	24
.572.	MIR29B2C gene	24
.82	Age estimation and Forensic Applications	25
2.9	DNA in Forensic Science	26
Chapter Three: Material and Method		
3.1	Material	28
3.1.2	Kits and Reagents	29
3.1.3	Primers	29
3.1.4	Chemicals	32
3.1.5	Experimental design	33
3.2	Method	34
3.2.1	Preparation of Buffers and Reagents	34
3.2.1.1	Preparation of 10X TBE buffer	34
3.2.1.2	Preparation of 1% agarose gel	34
3.2.1.3	Prepare 1X (ME) Wash Buffer	34
3.2.1.4	Sampling	34
3.2.1.5	Sterilization	35
3.2.1.6	Preparation PCR primers.	35
3.2.2	Extraction of DNA from Whole blood	35
3.2.3	Agarose Gel Electrophoresis	36
3.2.4	Concentration and purification of genomic DNA	37
3.2.5	Bisulphite Sodium Conversion	38
3.2.6	Sterilization of Working Station	39
3.2.7	PCR	40
3.2.8	Pyrosequencing	40
3.2.9	Statistical Analysis	41
Chapter Four: Results and Discussion		
4	Results and discussion	42

4.1	Bisulfite Modification	42
4.2	PCR	42
4.3.1	Assays Validation	42
4.3.2	PCR Bias Testing Using Pyrosequencing	43
4.4	Relationship between CpGs sites of all genes and Age	45
4.4.1	Relationship between CpGs sites of ELOVL2 and Age	45
4.4.2	Relationship between CpGs sites of FHL2 gene and Age	48
4.4.3	Relationship between CpGs sites of TRIM59 gene and Age	50
4.4.4	Relationship between CpGs sites of MIR29B2CHG gene and Age	52
4.4.5	Relationship between CpGs sites of KLF14 and Age	55
4.4.6	The correlation and regression model for Five gene	58
4.5	Multivariate regression model	58
4.6	Relationship between methylation and gender.	63
	Chapter five : Conclusion and Recommendation	
5.1	Conclusion	75
5.2	Recommendation	75
	References	76

List of Tables

Table No.	Title	Page
3.1	Apparatus used in this study.	28
23.	The commercial kits and reagents	29
3.3	Description of primers for bisulfate DNA amplification	29
3.4	CpG Methylation Assays of Five Genes.	30
3.5	Target CpG Sites Coordinates	31
3.6	Primer Sequences in the Assays	31
3.7	Chemical and disposable materials used in this study	32
3.8	DNA Conversion: Treatment of DNA with sodium bisulfite.	38
3.9	The components of PCR reaction	40
3.10	PCR cycling conditions	40
14.	Results of correlation between the age and CpGs sites of ELOVL2	45
24.	Fitting simple linear regression for the CpGs sites of ELOVL2 gene.	45
34.	Results of correlation between the age and CpGs sites of FHL2 gene.	49
44.	Results of regression analysis of CpGs sites of FHL2 gene.	49
54.	Results of correlation between the age and CpGs sites of TRIM59 gene	51
64.	Results of regression analysis of CpGs sites of TRIM59 gene.	51
74.	Results of correlation between the age and CpGs sites of MIR29B2CHG gene	53
84.	Results of fitting simple linear regression for the CpGs sites of MIR29B2CHG gene.	53
94.	Results of correlation between the age and CpGs sites of KLF14 gene	55
04.1	Results of regression analysis of CpGs sites of KLF14 gene	56

14.1	Regression statistics of the age prediction model with the 15 best age predictors:	59
24.1	Results of variance analysis of multi regression model	59
34.1	Regression statistics of the age prediction model with the 9 best age predictors	62
44.1	Results of variance analysis of multi regression model.	62
54.1	Descriptive statistics of the CpGs of TRIM59 gene classified according to the gender	63
64.1	Results of variance analysis for difference between the CpGs of TRIM59 gene and gender	64
74.1	Descriptive statistics of the CpGs of FHL2 gene classified according to the gender.	65
184.	Results of variance analysis for difference between the CpGs of FHL2 gene and gender	65
194.	Descriptive statistics of the CpGs of MIR29B2CHG gene classified according to the gender	66
04.2	Results of variance analysis for difference between the CpGs of MIR29B2CHG gene and gender	67
14.2	Descriptive statistics of the CpGs of ELOVL2 gene classified according to the gender	69
24.2	Results of variance analysis for difference between the CpGs of ELOVL2 gene and gender (ANOVA).	70
34.2	Descriptive statistics of the CpGs of KLF14 gene classified according to the gender.	72
44.2	Results of variance analysis for difference between the CpGs of KLF14 gene and gender	73

List of Figures

NO	Figures	Page
2.1	Schematic Summary of DNA methylation changes during aging	6
2.2	Schematic representation of DNA methylation, which converts cytosine to 5'methyl-cytosine by the catalyzed of DNA methyltransferase (DNMT)	8
2.3	Schematic representation DNA m of promoter.	12
2.4	Mechanism of transcriptional repression by DNA methylation	14
2.5	DNA demethylation processes.	18
3.1.5	Experimental design	33
3.2	Genomic DNA extraction of blood samples showed that electrophonic on agarose gel 1% voltage 120 v. for 15 minutes.	37
4.1	PCR Bias Testing Using Pyrosequencing for ELOVL2 gene	43
4.2	PCR Bias Testing Using Pyrosequencing for FHL2 gene	43
4.3	PCR Bias Testing Using Pyrosequencing for TRIM59 gene	44
4.4	PCR Bias Testing Using Pyrosequencing for KLF14 gene	44
4.5	PCR Bias Testing Using Pyrosequencing for MIR29B2CHG gene.	44
4.6	Regression line drawn on scatter diagram regarding each CpG site of the ELOVL2 gene that hypermethylated with age	47
4.7	Regression line drawn on scatter diagram regarding each CpG site of the FHL2 gene.	49
4.8	Regression line drawn on scatter diagram regarding each CpG site of the TRIM59 gene that hypermethylated with age.	51
4.9	Regression line drawn on scatter diagram regarding each CpG site of the MIR29B2CHG gene that hypomethylated with age.	54
4.10	Regression line drawn on scatter diagram regarding each CpG site of the KLF14 gene.	57
4.11	Chronological age versus predicted age for CpGs sites selected with dentin samples with an MAD of 6.9262 years	60

4.12	Chronological age versus predicted age for the CpGs sites of ELOVL2 gene with an MAD of 2.8939 years	62
4.13	The CpG sites of TRIM59 gene and gender show that there is no significant difference between them	64
4.14	The CpG sites of FHL2gene and gender show that there is no significant difference between them	66
4.15	The CpG sites of MIR29B2CHG gene and gender show that there is no significant difference between them	68
4.16	The CpG sites of ELOVL2 gene and gender appeared that there is no significant difference between them.	71
4.17	The CpG sites of KLF14 gene and gender appeared that there is no significant difference between them.	73

List of Abbreviation

Abbreviations	Whole phrase
5mC	5-methyl –cytosine
AIDS	Acquired immune deficiency syndrome
AR	Androgen receptor
CGI	Cytosine- guanine island
CPGs	Cytosine –guanine di- nucleotides
DNA	Deoxyribonucleic acid
DNMTs	DNA methyltransferases
ELovL2	Elongation of very long –chain fatty acids-like 2gene
FHL2	Four and half LIM domains protein2
KIF14	Kruppel –like factors
LTR	Long terminal repeat
M	Methylation
MDBP	Methylated DNA binding protein
MeCP2	Methyl CPG binding protein 2
MIR29B2C	Micro RNA
mRNA	Messenger ribonucleic acid
PCR	Polymerase chain reaction
RING	Really interesting new gene
ROS	Reactive oxygen species
SAH	S- adenosylhomocysteine
SAM	S- adenosyl – methionine
SNP	single nucleotide polymorphism
sjTRECs	Signal joint TCR excision circles
TCR	T-cell receptors
TRIM	Tripartite motif
TSS	Transcriptional start site

Chapter One

Introduction

1.1 Introduction

Aging is a complex process characterized by a global decline in physiological functions and related to an increased risk for several diseases [1]. The continuous changes happen during the process of aging can be noticed not only in the individual's anatomy and physiology but also at the cellular and molecular levels. Many studies

revealed that an extensive remodeling of gene-expression profiles driving physiological and/or pathological changes in several tissues take place with aging [2]. Age estimation has an important role in the forensic field for detection of chronological age and besides various methods with different accuracy is available. The physical methods included dental morphology and bone epiphyseal ossification, chemical methods based on aspartic acid racemization, lead accumulation, collagen cross-links, the chemical composition of teeth, and glycosylation of proteins [3]. On the molecular biology front, telomere shortening, mitochondrial mutations, and single joint T cell receptor excision circle rearrangements were firstly corresponded with aging but the low accuracy of these assays has quickly decreased hope for these methods to become routinely implemented in forensic investigations. The aging process leads to changes in tissues and organs on a molecular level but only recently, it has been related to changes in DNA methylation patterns [4]. The methylation states of the 28 million CpG dinucleotides in the human genome were observed to alter with aging [5]. Particularly, local CpG sites can become either hypo or hypermethylated with aging [6]. DNA methylation is a process by add methyl groups to DNA molecule. DNA methylation usually acts to repress gene transcription when located in a gene promoter and DNA methylation necessary to normal development. DNA methylation based biomarkers usually referred to as “epigenetic age” or “epigenetic clock” are robust estimators of chronological age of an individual [7].

During the last years, DNA methylation was a “black spot” for forensic scientists. Then, DNA methylation analysis can give more forensic relevant information in parallel to the DNA profile [8]. DNA methylation mostly happens in a CpG sequence context represents an epigenetic mark and plays an important role in cell regulation to establish and save cell identity [9]. These functions explain why DNA methylation at particular sites in the genome shows a cell-type specific pattern and how it can be used for tissue and body fluid discrimination [10]. The epigenetic landscape supply new perspectives for biomarkers. In specific, DNA methylation is well known to modify with aging multiple CpG sites, which are either hypermethylated or hypomethylated [11]. Undoubtedly, representing that epigenetic regulation is indispensable for several aspects of genome functions, the changing of epigenetic landscapes could potentially account for the complex nature of aging by describing the perturbation of the multitude of gene families and cellular pathways that are known to contribute to this process

[12]. Some of the systemic changes arise in response to aging related environmental effectors, while others are interpreted as part of an “aging program” which is part of a development [13]. Independently of the causes of these systemic modifies recent knowledge proposes that global and locus-specific DNA methylation levels are promising markers of human aging that can explain the inter individual variability in functional decline and vulnerability to diseases in the elderly. After arrival to maturity, a methylome and coded chromatin histone are presumably established in each tissue type and multiple DNA methylation biomarkers displaying a good association with age have been described [14].

The advantage of DNA pyrosequencing technology enabled be automated in a massively parallel manner and hundreds of thousands of sequence reads can be obtained in a single run, generating sequence information data that are orders of magnitude larger. Moreover, the effective cost for pyrosequencing when compared with another methods. The regression model chooses the most informative CpG sites for the age estimation model. Thus, the supervised machine learning analysis yields both a group of CpG sites and a corresponding mathematical algorithm that incorporates the DNA methylation levels into an age estimate. Methylation level of CpG sites in the ELOVL2 promoter strongly associates with age and the modify in methylation level with aging is significant spanning from 7% to 91%. Interestingly, they hypothesized that regulation of expression of ELOVL2 through methylation may be included in aging. Epigenomic study on whole blood from persons of different age were concordant in demonstrating that ELOVL2 and FHL2 CpG islands get hypermethylated with aging [15], and a correlation between methylation levels of the CpG sites in ELOVL2, TRIM59, KLF14, C1orf132, and FHL2 and age of the individuals is conformity with previous studies, all CpG sites displayed strong correlation with age. Most CpG sites of ELOVL2 appeared the strongest correlation with age [16, 17].

Aim of the Study

To estimate the age by using DNA methylation markers.

Objectives

1-Detection of CpG sites that characterized by a correlation with chronological age.

2- Designing of age prediction model by measure DNA methylation level of five genes (ELOVL2, TRIM59, FHL2, KLF and MIR29B2CHG) that providing important information in forensic field on human appearance and to less number of potential suspects.

Chapter Two

Literature Review

2.1 Ageing Theory

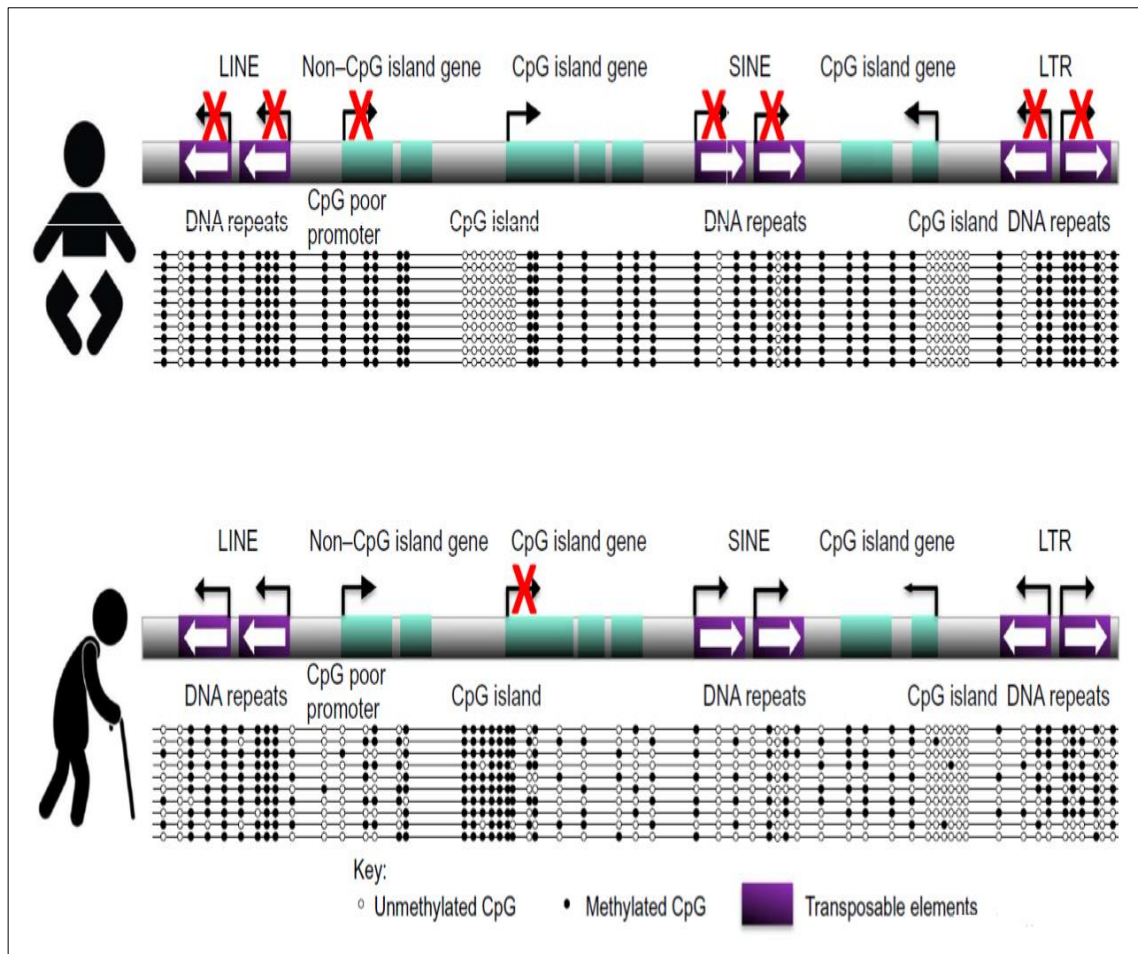
The aging process is a continuous decrease in the physiological capacity reduced ability to respond inconstant to environmental stimuli and raised probability to infection of diseases. Aging is a complex process affected by several factors such as genetics and environmental conditions [18]. There are continuous changes during the process of aging that not only in the physiology and in the individual's anatomy but including molecular and cellular levels. Several studies report that an extensive remodeling of gene-expression profiles driving pathological and physiological []. The molecular basis of the 2changes in different tissues happening with aging [physiological changes with aging has appeared by a branch of molecular genetics regulation. Its "aging epigenetics" that refers to changes in genomic known as changes and do not definition, this term comprehends only heritable phenotypic [19]. gene sequence change in the underlying

Epigenetic patterns show at the gestational and preconceptional level. The exposure to environmental factors during gametogenesis or gestation of both maternal and 0]. 2[paternal has been demonstrated to be responsible for the offspring's epigenome On the other side, during the early stages of life, the inherited epigenetic status undergoes several changes to get a suitable process of cell differentiation and development. Non-random mechanisms, for example, stochastic errors or environmental stimuli can stimulate changes in epigenetic profiles at both early and late life stages and in most cases, they manage many processes occurring that during the lifespan including pathological conditions, differentiation, development and stress 1]. Another important fact that organ function also affected with aging that 2response [depends on how well its component cells function. When aging cells die without being replaced that cell number will decrease and organ function affected for example bones become weaker and less dense thus more prone to break, their calcium content reduced as the amount of absorbed calcium from nutrients decreases [22]. Muscle strength and muscle mass also reduce, it is partly due to muscles being physically used less and as a result, reducing in size but because of decreased levels of testosterone and growth hormone also decreased the production of collagen in the skin which responsible for its strength and of elastin which responsible for its flexibility. The aging of skin becomes drier, less elastic, thinner and finely wrinkled because of decreased production of collagen. The fatty layer beneath with aging also becomes thinner, reducing promoting wrinkling and cold tolerance [22]. Sensory defeats and cognitive

change (sight and hearing) ability are reduced. The various theories, each with supporting proof have trying to explain age-dependent DNA changes [23]. The most commonly suggested mechanisms are: Telomere shortening, DNA damage, and reactive oxygen species (ROS) mediated declination to macromolecules [24]. The amount of DNA damage also increases with increasing age, such DNA age-dependent changes could have an important application in forensic science and allowing age determination from samples collected from crime scenes. In an attempt to describe aging at the genetic level, there are theories like error theory, somatic mutation theory, disposable soma model and the dedifferentiation hypothesis [24]. The gradual drifting of cells from their original differentiation state results in aging with time [25].

The control of differentiation leads to the production of the organism and is maintained for as long as it is important to ensure the organism's evolutionary success. The slow random loss of this differentiation starts happening. Multicellular organisms include during the life span stages of development, reproduction, and senescence. There is evidence explaining early development regulated by successive activation and suppression of specific genes. The theory of gene regulation suggests that aging (senescence) results of changes in the gene expression because of reproduction and other adult activity. The gene expression needed for both reproductive activity and growth depends on multiple factors such as optimum levels of nutrition and can affected by various types of stresses such as pollution, temperature, radiation, and starvation [26].

DNA methylation is one of the more extensively studied and excellent characterized epigenetic modifications during aging (Figure 2.1). In young cells, the majority of CpG sites within the genome have cytosine methylation. Within promoters, CpG methylation leads to transcriptional repression by the formation of compact chromatin structures, such as heterochromatin but promoters of genes that are highly expressed are devoid of DNA methylation. DNA methylation is important during development when it is used to silence genes in tissues in which their expression is never going to be needed or at developmental stages in which they no longer need to be expressed [27].



Schematic Summary of DNA methylation changes during aging. Young :**Figure (2.1)** mammalian cells are characterized by DNA hypermethylation over the genome with the exception of CpG islands within the promoters of expressed genes. In particular DNA repeats, such as SINE, LINE and long terminal repeat (LTR) transposable elements are heavily DNA-methylated helping to maintain them in a constitutive heterochromatin state. During aging, there is general DNA hypomethylation over the genome, which mostly occurs in a stochastic manner within the cell population. Loss of DNA methylation leads to activation of normally silenced DNA sequences like the transposable elements. However, DNA methylation also increases in a nonstochastic manner over the CpG islands of certain genes correlating with their heterochromatinization and silencing [27].

2.2 Epigenetics and DNA methylation

Epigenetics are modifications of DNA molecule that produce changes of DNA function with no involvement alterations of the genomic sequence [28]. Many of these known biochemical processes are classify to DNA and protein epigenetics. One involves, modifications of histone proteins for example methylation of lysine 4 on histone 3 (H3K4me and H3K4me3), which appeared in a relation with regulatory regions and chromatin structure of the genome [29]. DNA methylation is another example of epigenetic phenomenon that means a process of addition of a methyl group to a cytosine base of a DNA molecule.

In mammalian cells, DNA methylation especially occurs at cytosine-guanine dinucleotides (CpG) sites while in prokaryotes, DNA can be methylated on both adenine and cytosine and these sites has an important role in various processes, including defense against foreign DNA and DNA repair. In eukaryotic methylation of cytosine can cause epigenetic gene silencing. Recent studies of DNA methylation across mammalian genomes appeared widespread methylation in intergenic regions and repeats but limited DNA methylation at regulatory regions, this is appropriate with the idea that hypermethylation serves in maintaining genome safety. DNA methylation patterns at regulatory regions are generally stable but a minor group of regulatory regions represents variable DNA methylation between cell types, suggesting that promoter de novo methylation might be contributory in the maintenance and to the initiation of silencing genes during development [30].

Normally, a percentage 70% - 80% of the CpG sites in the human genome are methylated. DNA methylation can estimate in various genomic contexts: transcriptional start sites with CpG islands or without CpG islands at regulatory elements and at repeat sequences in gene structure. The function of DNA methylation seems to a variety with context and the relationship between DNA methylation and transcription is clear more than past time, the recent studies have explained that the position of the methylation in the transcriptional unit effect of its relationship to gene control. Methylation in the immediate proximity of the TSS blocks initiation, but DNA methylation does not block that might even stimulate transcription elongation in the gene body and new proof suggests that gene body methylation may have an influence on splicing and in repeat regions such as centromeres methylation is important for chromosomal stability and also possibly suppress the expression of transposable elements and thus have a role in genome stability [31]. DNA methylation of cytosine at the 5' position to form 5-methylcytosine (5mC) is catalyzed by DNA

methyltransferases (DNMTs) and methyl intake from diet and utilization of the methyl group that supply methyl group during DNA methylation. The major dietary methyl group `donor's choline, betaine, and methionine (Figure 2.2). In mammals, S-adenosylmethionine (SAM) donated a methyl group during the cytosine methylation process [32].

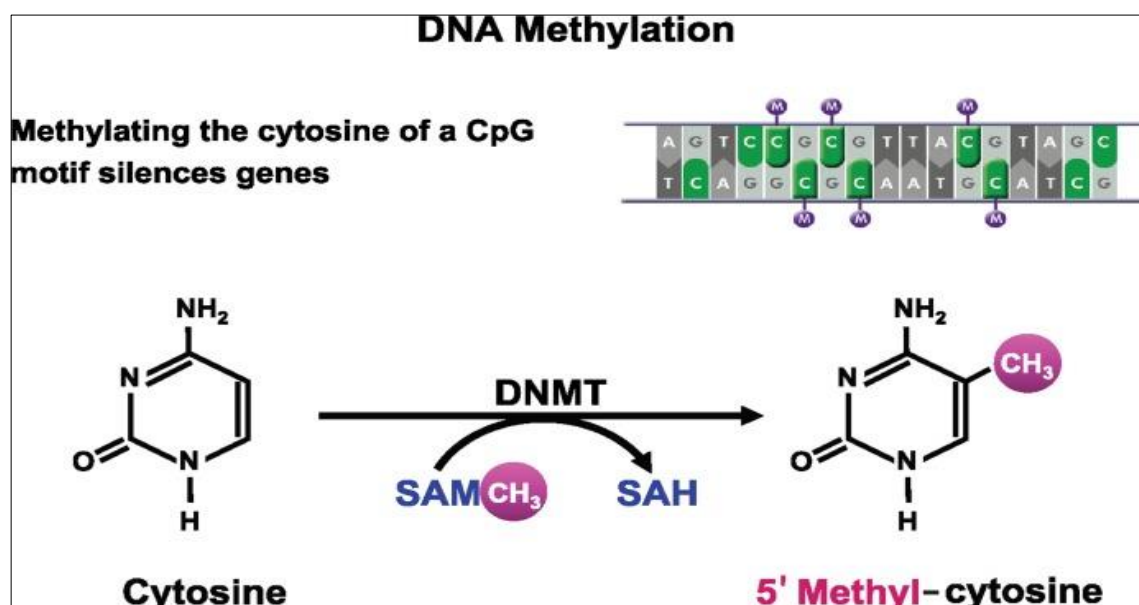


Figure (2.2): Schematic representation of DNA methylation, which converts cytosine to 5'-methyl-cytosine by the catalyzed of DNA methyltransferase (DNMT). DNA methylation occurs at cytosine that are followed by a guanine (CpG motifs). SAM = S-adenosylmethionine; SAH = S-adenosylhomocysteine [35]. About 60% of the CpG islands spread of gene promoters. The CpG islands of promoters in many developmentally regulated or housekeeping genes are generally hypomethylated and this characteristic raise active transcription. On the contrary, some suppressed genes have methylated promoter CpG islands whose shutdown is preserved in the long term [33]. DNA methylation is generally noticed as a signal of gene silencing, during early embryonic development either hypermethylated or hypomethylated DNA patterns are established and are maintained throughout life in the somatic cells [34].

2.3 Ageing and Epigenetic

The process of aging causes several of the changes at the molecular and cellular levels, which involve telomere shortening, senescence and changes in gene expression.

Epigenetic patterns also change over the lifespan, suggesting that epigenetic changes may constitute an essential component of the aging process [36]. DNA methylation is the epigenetic mark that has been most highly studied that means the presence of methyl groups at CpG dinucleotides. The CpG dinucleotides are often placed near gene promoters and may be associated with gene expression. Global DNA methylation levels increase over the first few years of life and later decrease beginning in late adulthood [37]. Researches have reported age-associated DNA methylation alterations across various tissue and cell types led to attempt to predict the chronological age of an individual and found a relationship between the state of the epigenome and age. As well, the group of modifications to DNA do not change in the primary nucleotide sequence. In specifically, DNA methylation associated with chronological age over a long time and maybe changes linked to complex diseases such as metabolic disease and cancer. The idea of epigenome as a fixed imprint is giving as a dynamic landscape that reflects a variety of chronological changes. The new challenge is to determine possibility these changes can be help of described and modeled to detect various rates of human aging and to bind these rates to related clinical or environmental variables [38].

DNA methylation is modification happens primarily in CpG dinucleotide-containing regions, sometime in regulatory sequences that suppress gene expression. The CpG methylation is essential for transcriptional repression of transposons and repeats elements for X-chromosome inactivation and imprinting [39]. Methylation of cytosine can occur not within CpG sequences and is important for regulation of gene expression in embryonic stem cells. DNA methylation controlling of cellular differentiation that patterns of methylation vary in different cell types and various studies have previously documented differences in DNA methylation between cell types and disease state [40]. Epigenetic patterns changes are found to occur either during development or childhood/adulthood. The main difference during the development of embryonic and germ cell changes to the epigenetic landscape are biologically reprogrammed and essential for lineage determination and cellular identity [41]. Important conclusions emerge from the studies about our life span is large epigenetically determined diet and other environmental influences can influence our life span by inhibitors of epigenetic enzymes and changing the epigenetic information that can influence life span of organisms that providing a better understanding of the mechanisms involved in aging. Epigenetic information gives the

reversible nature, the highlight exciting avenues for a therapeutic involvement in aging and age-associated diseases, including cancer [42].

The modifications called “epigenetic clock” or “epigenetic drift” have come from the continuous interactions between genetics and environment that occur during the individual’s lifespan and DNA methylation was not minutely maintained over cell divisions, resulting in a gradual loss and increase in variability over the lifespan [43]. For example, observations some identical twins seem to age more rapidly than their siblings. Speculation is that epigenetic shifts will show to be the most significant class of altered gene expression that can help the biogerontologists work with hundreds of genetically identical individuals in their experiments and they do a very good job at controlling environmental variations [44].

2.3.1 CpG Islands

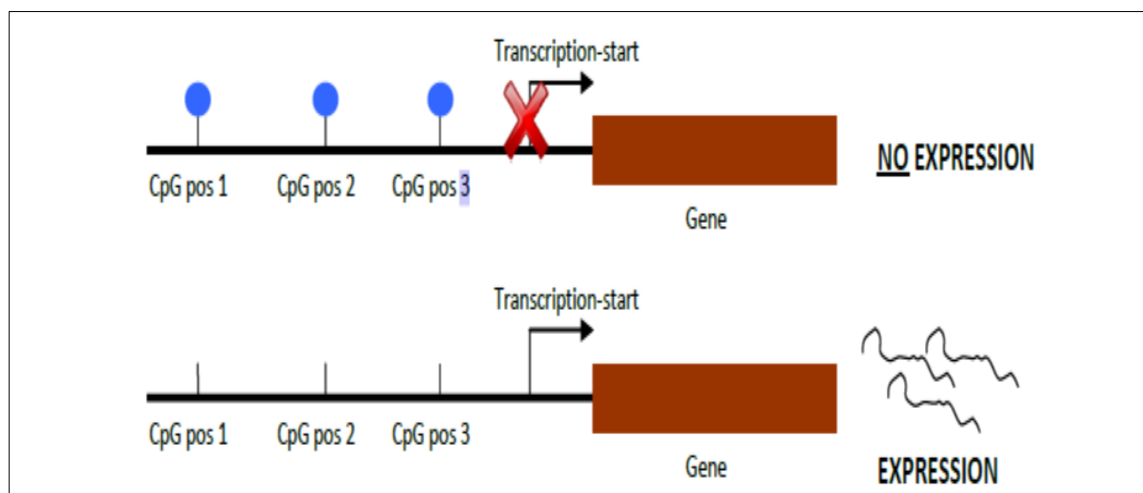
The CpG islands are small-interspersed DNA sequences that deviate significantly from the average genomic pattern by being GC-rich and mostly nonmethylated. Most CpG islands are sites of transcription initiation including thousands distant from annotated promoters. DNA sequence shared features adapt CpG islands for promoter function by destabilizing nucleosomes and attracting proteins that create a transcriptionally permissive chromatin state. Silencing of CpG island promoters is completed through dense CpG methylation or polycomb recruitment, again using their CpG islands are effective to distinctive DNA sequence composition and generically influence local chromatin structure and simplify regulation of gene activity [45]. Most CpG sites in the human genome are methylated, occurring mainly in gene bodies, intergenic regions, and repetitive elements. The unmethylated form occurs preferentially in the context of CpG islands, which settle down with gene promoters [45]. CpG islands are DNA regions of more than 500 base pairs identified by at least 50% of GC content 60% of CpG islands are related to annotated gene promoters and are unmethylated permitting the transcription of the correlated gene [46]. Oppositely, the methylation state of CpG dinucleotides localized in gene body does not affect gene transcription but probably impedes the activation of alternative promoters [47].

The remaining 40% of CpG islands is localized in intergenic and intragenic regions and are defined as “orphan CpG islands” because they are not associated with annotated promoters although presenting promoter-like features. Up to 2 Kb distant from promoter CGIs, particular regions characterized by a lower density of CpG sites

(around 1/10 of CGI) and defined as CGI shores are also involved in regulation of gene expression. CGI shores often overlap with tissue specific-differentially methylated regions (DMRs) in normal tissues and closely associated with somatically heritable [48]. DNA methylation is a central effector involved in this process established by the covalent addition of a methyl group to cytosine residues in the context of cytosine-guanine di-nucleotides so-called “CpG sites” by DNA methyltransferase (DNMT) enzymes [49]. CpG sites are often not in isolation actually, they are embedded in the genome as separated clusters of CpG sites (termed CpG islands) that frequently located in the 5’ nearby regions of transcriptional start sites [50]. In the genome, more than half of the genes contain CpG islands within their promoter and these islands are usually unmethylated in normal cells [51]. Methylation of CpG islands, however, can be detected at small subgroups of autosomal genes (<10%) in a tissue-specific manner

that has a functional role of CpG island [52].

or in distal Shores and shelves are CpG sites located in regions just outside CGIs exhibit the highest variability in DNA regulatory elements especially enhancers [53]. DNA methylation can control gene expression for example with methylation promoter DNA methylation typically associated with gene silencing. On the contrary, unmethylated gene promoters can associate with either active or inactive (poised) as of (figure 2.3) expression states and depending on the levels of nearby histone marks [54].



methylation of promoter [54]. **Figure (2.3):** Schematic representation DNA

2.3.2 Mechanism of Gene Silencing

DNA methylation is an essential mechanism for the suppression of gene activity [55]. Gene silencing refers to general processes of interruption or suppression of transcription or translation of the mRNA of the target gene by mechanisms of epigenetics. Gene silencing maintains, differentiation and maintenance of stem cells. The genomic structure provides a promising treatment for diseases like cancer modification and AIDS [56].

DNA methylation is frequently identified as a ‘silencing’ epigenetic mark and in fact the function of 5-methylcytosine was originally suggested in the 1970s and by improved genome-scale mapping of methylation, DNA methylation can estimate in different genomic contexts: transcriptional start sites with or without CpG islands, in gene bodies, at regulatory elements and at repeat sequences [40]. Gene expression with promoter DNA methylation generally associated with gene silencing. DNA methylation changes appeared to act by stabilizing of preexisting ‘baseline’ expression levels. Thus, age-associated hypermethylation in blood is preferentially noted in promoters of genes that are normally not expressed in blood and hypomethylation is founding in promoters of genes that are normally expressed [57].

The 5-methylcytosine (5mC) have a strong effect in mammalian promoter regions appeared that DNA methylation inhibits transcription by interfering with transcription initiation in addition to DNA methylation has been appeared to reduce the binding affinity of sequence-specific transcription factors and methylation-dependent sequence-specific DNA-binding proteins, such as MDBP may work as transcriptional repressors [58]. However, the range of transcription factors that able to be influenced directly by DNA methylation is usually be limited to the specific areas containing the CpG sequence in their binding site also DNA methylation seems to effect general of promoter sequences. Transcriptional repression by DNA methylation can be affected by a sequence-independent process involving changes in histone acetylation levels and chromatin structure (Figure 2.4) [55].

The methylated DNA binds with transcriptional repressor MeCP2 in a sequence-independent fashion and by MeCP2 the transcriptional repression is non-linearly depending on the 5mC density and it is the recruitment of a complex containing an essential transcriptional co-repressor and a histone deacetylase. Deacetylation of

histones is associated with decreasing levels of transcription may be by allowing compact nucleosomal packing [59]. In normal cells unmethylated promoter's repetitive elements are usually hypermethylated add to that loss of repetitive DNA methylation has been linked to cancer and chromosomal instability [59].

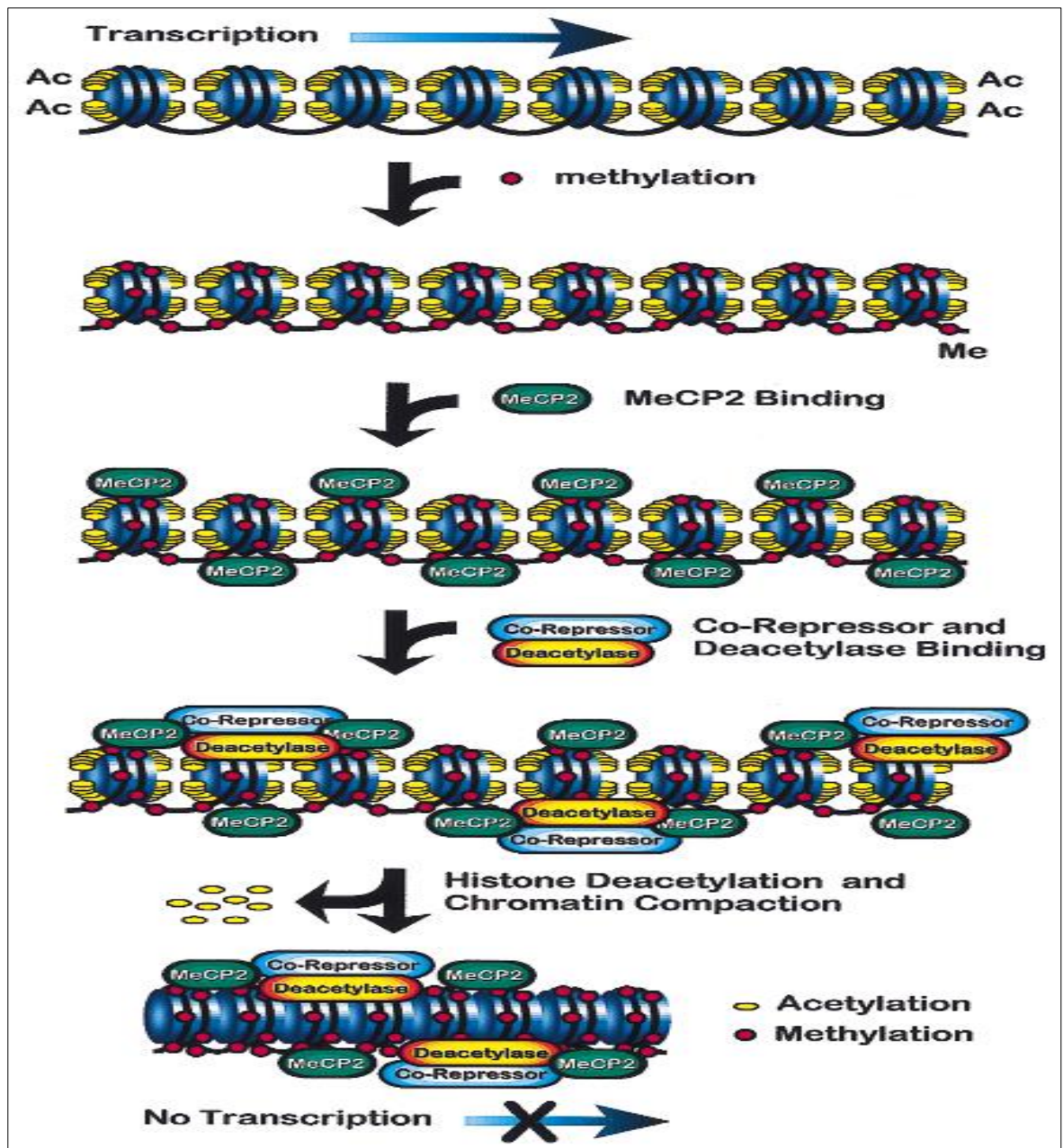


Figure (2.4): Mechanism of transcriptional repression by DNA methylation. The structure of transcriptionally active chromatin is shown schematically at the top. Nucleosomes are shown as blue cylinders representing the core histone complexes with DNA indicated by the black line wrapped around the cylinders. Yellow ovals

(Ac) denote acetylation of histones. DNA methylation (Me) is indicated by red circles [55].

3 2.3 Gene Methylation in Mammals

The human epigenome project aimed to identify catalogue and explain genome-wide DNA methylation phenomena that occurring naturally on cytosine bases at cytosine-guanine dinucleotides (CpG). DNA methylation is usually involved in various biological processes and the aetiology of many diseases. Differentially, methylated cytosines give increase to distinct profiles thought to be specific for tissue type, gene activity and disease state. The identification of such methylation in various positions will significantly develop an understanding of genome biology and develop the ability for diagnosis diseases Mammalian genomes [58]. Such as those of all vertebrates tested yet, are globally methylated in the sense that all concatenation of DNA sequence (genes, intergenic DNA and transposons) are targets for CpG methylation unlike methylated genomes that are methylated and unmethylated domains coexist in almost equal proportions mammalian genomes are dominated by methylated DNA [60].

However, whole-genome studies have shown that there might be alternative functions for DNA methylation in gene bodies that has shown the exons are more highly methylated than introns and transitions in the degree of methylation occur at exon–intron boundaries possibly suggesting a role for methylation in regulating splicing [61]. This process represents an important mechanism of stable tissue-specific regulation of gene expression because DNA methylation of the gene promoter region leads to the repression of the gene activity [61]. Active genes usually show hypomethylation around the transcriptional start site (TSS) and high levels of methylation in the gene body, which increase with gene expression. The methylation of the gene body is believed to block aberrant transcription initiation inside the gene and therefore to help in avoiding the production of truncated mRNAs and proteins. In addition, DNA methylation levels slightly change at splicing sites with exons showing higher DNA methylation than introns which suggests a role of DNA methylation in splicing as well [60].

. 2.4 Changes in the DNA Methylation

Aging and age-related diseases include defined changes in 5-methylcytosine content and are generally characterized by genome-wide hypomethylation and promoter-specific hypermethylation. These changes in the epigenetic landscape represent potential disease biomarkers and are thought to contribute to age-related pathologies, such as cancer, osteoarthritis, and neurodegeneration. Some diseases, such as a hereditary form of sensory neuropathy accompanied by dementia are directly caused by methylation changes. Epigenetic modifications, however, are reversible and are therefore a prime target for therapeutic intervention [62].

2.4.1 Hypomethylation

A lot of new data proves that DNA methylation patterns are altered during aging in a wide range of tissues and species that during the aging process mammalian genome undergoes an erosion of DNA methylation patterns along with directional, *locus* specific hypermethylation and hypomethylation. Age associated hypomethylation occurs in heterochromatic regions of the genome, such as repetitive elements and transposons, which contain the majority of methylated CG dinucleotides in human DNA. In addition to repetitive elements, loss of methylation with aging was also demonstrated in CG-poor regions located next to genes [63].

.2.4.2 Hypermethylation

Irregular DNA methylation is a feature of human cancers that characterized by generalized regional hypermethylation and hypomethylation [55]. The regional exons. CpG islands located in the promoter and upstream hypermethylation includes Hypermethylation of CpG islands recruits methyl subsequently histone deacetylases DNA and DNA binding proteins. Deacetylation of the histone backbones makes the to transcription structure inaccessible structure of the promoter into a closed chromatin [62]. Aberrant hypermethylation of CpG islands factors that lead to gene inactivation as an alternative to genetic changes for placed in the upstream exons or promoter acts tumor suppressor genes. CpG islands are normally protected from the inactivation of methylated in relation to cancer or aging but they are aberrantly DNA methylation [64]. There have been also many studies of regional increases in DNA methylation levels like Baylin and coworkers have explained regional hypermethylation on

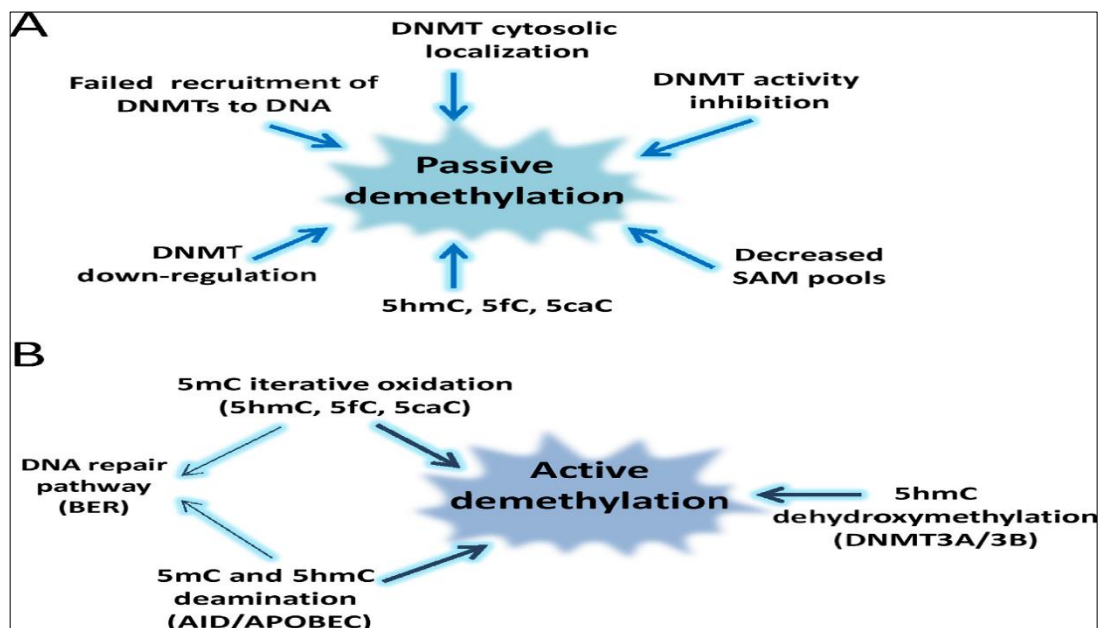
chromosomes 3p, 11p and 17p in a multiple of human tumors. The CpG island include regions that are normally never methylated in vivo but on the contrary they are found to be methylated in tumor tissues. There are guides for inactivation of tumor-suppressor gene function by hypermethylation of the Rb gene in sporadic retinoblastoma. Therefore, that hypermethylation of tumor-suppressor genes leading to gene inactivation leads to a selective growth advantage of the transformed cells [65].

. 2.5 DNA demethylation

The methyl group when added to cytosine does not interfere with the Watson–Crick base pairing of the nucleotide due to the methyl group is situated in the major groove of DNA, where it can be worthily recognized by DNA-interacting proteins. The initial methylation pattern (Figure 2.5) is set by so-called de novo MTases (Dnmt3 enzymes in mammals). This method is perpetuated for the rest of the life of the animal with small tissue-specific changes by a mechanism first suggested by Riggs and Holliday in 1975 [66]. After each round of DNA replication the hemimethylated DNA is formed with the parental strand carrying the methylation marks and newly synthesised daughter strand being freed from methylation. The daughter strand is remethylated by a maintenance DNA methyltransferase, which is specific for hemimethylated DNA (Dnmt1 in mammals). This enzyme preference of hemimethylated sites prevents methylation of previously unmethylated sites and there by keeps the methylation pattern through cell divisions. When the maintenance MTase activity is suppressed passive demethylation of the DNA takes place lead to the generation of unmethylated DNA after another round of replication equivalent to the loss of 50% of the methylation per replication cycle failure of DNA methyltransferase action during DNA replication stimulates a loss of 5mC mark known as passive DNA demethylation process [67]. Multiple mechanisms can be involved in passive DNA demethylation including down-regulation of DNMT enzymes [68]. DNMT cytosolic localization impairment of DNMT recruitment on DNA decrease of the DNMT substrate SAM inhibition of DNMT enzymatic activity (Figure 2.5A). Recently, several pieces of evidence have sustained the existence of molecular mechanisms involved in the active removal of 5mC through the formation of 5-hydroxymethylcytosine(5hmC). 5hmC is catalyzed by three Fe (II) and -ketoglutarate-dependent DNA hydroxylases, the ten-eleven translocation (TET) family enzymes and it is a pivotal intermediate of active

DNA demethylation [69]. In contrast active demethylation occurs independently of DNA replication and needs enzymatic activity to remove the methyl group from the methylated base directly or to exchange the whole base by an unmethylated one. Moreover, a previously unmethylated region (such as a CpG island) might become methylated by the action of a DNA methyltransferase targeted to that locus [70]. DNMT3A and DNMT3B are possibly able to convert 5hmC directly into cytosine although much more evidence suggests the need of further modifications of 5mC and 5hmC in order to trigger DNA damage response for the reintroduction of unmethylated. An additional mechanism that involves the deamination of 5mC and cytosine [71]. 5hmC by the deaminase enzymes AID/ APOBEC (Figure 2.5 B) [72]. Specially, 5hmC is not recognized by DNMT1 enzyme leading also to a passive loss of 5mC. Add to that 5fC and 5caC can undergo replication-dependent dilution (Figure 2.5 A) [73].

Figure (2.5): DNA demethylation processes. A. Mechanisms affecting DNMT action during DNA replication and thus inducing passive DNA demethylation. B.



Mechanisms involved in the active removal of 5mC via enzymatic modifications [69].

2.6 Methods of Age Estimation

Age estimation could help investigators in the absence of known DNA samples for comparison. To date, the age-dependent accumulation of a 4977-bp deletion in mitochondrial DNA, accumulation of D-aspartic acid and telomere shortening have all been analyzed as possible age markers [74]. Moreover, the use of these techniques practically has been limited for several reasons, including the disease effects and role

of environmental effects the low methodological accuracy tissue and gender specific variations [75]. Such as striking heterogeneity of mitochondrial deletions in various tissues and different telomere dynamics in each portion of the body has been found. Therefore, standardized, more stable and accurate methods for age estimation are needed [76]. In forensic application, DNA typing is mainly used for personal identification and paternity testing. In recent years, multiple studies have been made to explain other useful information from DNA samples including ethnicity age and individual phenotypic traits [77].

.1 Telomeres62.

Telomeres are short tandem repeated sequences of DNA found at the ends of linear eukaryotic chromosomes that along with related nucleoproteins function in stabilizing chromosomal end integrity. Telomeres in somatic tissue tend to shorten with age due to the inability of DNA polymerase is cannot to replicate completely the 3' end of linear DNA [78]. The long lifespan of humans and their short telomeres, attrition in telomere length may be a major determinant of human aging not only at the cellular level but also at the organ and maybe the systemic levels. It is crucial to notice that telomere length indicates the number of times a cell has divided; whether a cell ages each time it divides is another matter altogether. The ticking of the epigenetic clock seems to be independent of the number of times the cells in a tissue have divided (brain and blood cells from the same individual appear similar ages) suggests that it is distinguished from aging that is associated with telomere length, cells continue to undergo epigenetic aging, in spite of the fact that their telomeres are maintained by telomerase [79].

The distinctiveness between telomere length and epigenetic aging is further confirmed by Marioni et al who found that telomere length and epigenetic clock estimates are independent predictors of chronological age and mortality risk [80]. However, a universal sign of old age was also appeared to be related with epigenetic age and not with telomere length [81]. Epigenetic age possesses considerable predictive power in regards to time of death telomere length does not [82]. This is given that the lack of connection between telomere length and aging is made apparent in mice (and many other small animals), which age and die in spite of possessing extremely long telomeres that do not become critically short within their lifetime. Obviously, senescent cells that are present in mice are not the result of replicative senescence but to stress from the environment and normal metabolism of the animal.

It must be stressed that while there is no association between epigenetic aging and telomere length, this does not necessarily remove a possible link between epigenetic aging [82].

.2.6.2 Dental Age

Age assessment using teeth was first published by Edwin Saunders in 1837, who assumed that teeth provided the most reliable guide to age comparing with age estimation from height which was a standard method used during that time [83]. Age assessment by teeth is regarded as one of the interesting applications of forensic odontology. Chronologic age estimation by tooth development has been used over a long time. The teeth develop in a regular and serial manner until the age of 15 years and permitting an age estimation within 1 year. The dentition is better precision than any other anthropologic measurement during this period of development. The deciduous dentition starts to develop during the 6th week of intra-uterine life.

Mineralization of these teeth starts at 14 ± 2 weeks and continues after birth [84].

Dental age is regarded as one of the few measures of physiologic development that is regularly applicable from infancy to late adolescence, after about 14 years, age estimation becomes difficult since all the permanent teeth but except the third molar would have completed their development rendering them to be the only evidence used for age estimation. Other alternative methods during the interval between mid-teens& early 20's are available and the third molar offers a unique advantage over other teeth due to its development tends to continue over a longer period and until a later age. [85] On the other hand, humanity needs the dignity of identification of its dead and proper interment in accordance with religion and family wishes. More compelling is the anguish shared by the living relatives and friends of missing persons that remain unknown after being found dead. This make legal problems exist for these families. If a person is missing, no death certificate can be issued for a period of up to 7 years or longer [86]. Visual recognition by acquaintances and reliance on personal effects are regarded as common practical means of identifying the dead. However, these methods have margin of error. Many methods are utilized to determine the age from dentition and may be described in four categories namely, clinical, histological, radiographic, physical and chemical analysis [87].

.3 Immunological age62.

The age of human can be estimated accurately and reliably from blood using T-cell DNA rearrangements T lymphocytes employ specific receptors called T-cell receptors

(TCRs) to recognize foreign antigens and to create a broad repertoire of TCR molecules each immature T lymphocyte undergoes unique somatic rearrangements in its TCR loci during development of intra-thymic. During this rearrangement process, the intervening DNA sequences in the TCR loci are deleted and circularized into episomal DNA molecules also called signal joint TCR excision circles (sjTRECs) [88]. One particular sjTREC is the δ Rec- ψ J α sjTREC that arises through an intermediate rearrangement in the TCRD/TCRA locus in developing TCR $\alpha\beta$ ⁺ T lymphocyte. This sjTREC is seen in approximately 70% of all newly-formed mature TCR $\alpha\beta$ ⁺ T lymphocytes [89]. As shown previously, there is a log linear decline in sjTREC number with increasing human age, reflecting a life-long process of thymus involution that begins shortly after birth by replacement with adipose tissue and resultant loss of thymic function [90].

The application of this method is restricted to blood samples and body parts having blood and is impossible for other body parts or fluids like semen or saliva, that do not contain T cells in quantities required for sjTREC detection. First studies detected a slight gender difference in age-dependent sjTREC numbers [91]. Also a small but statistically significant gender effect on sjTREC quantification have been observed. However, including gender as an additional factor into regression and categorical prediction modeling would not alter age estimates to any significant level. The effect of the immune system health status on the sjTREC copy numbers can be considered a potential drawback of the proposed age estimation approach [90]. Future studies will address the possibility of severe pathological conditions involving the immune system such as leukemia or HIV/AIDS substantially influence the accuracy of this method. Great attention must be paid to the potential influence of a person's biogeographic ancestry on approach for age estimation. Further improvement of accuracy in individual age estimation from biological materials might be offered by future research into other aspects of age-dependent biological changes [91].

2.7 Biomarkers of This Study

A biomarker of aging is described as “a biological parameter of an organism that either alone or in some multivariate composite will in the absence of disease better predict functional capability at some late age than will chronological age” [92]. Its typical features have been drawn up extensively. A biomarker of aging should have a major requisite that it should have a good correlation with the chronological age of the individuals in a population add to that reflect the inter-individual heterogeneity in

the aging ranges of the individuals, that is biological age. Biological age is really a difficult concept to define, as an individual could display a youthful phenotype for some health parameters but not for others. It could be agreed that the ultimate readout of successful biological aging is a long life span (longevity) or a long (disease-free) health span. This means that suitable human study designs like middle aged and elderly prospective populations and family studies are essential to demonstrate the effectiveness of a biomarker of aging [93].

.1 ELOVL2 Elongation of very long-chain fatty acids–like 72. 2gene

The methylation status of ELOVL2 may appear a large proportion of variation in age and is a very good age predictor and that may be considered as a basis for a future age estimation models from blood. The optimized pyrosequencing method provides for quick and reliable analysis of the methylation level in 7 CpG sites take place in the ELOVL2 gene promoter [94]. Studies of ELOVL2 seem to be a very promising candidate marker for age estimation in forensics not only because of strong correlation but also because a wide range of change in methylation, which is observed with aging [95]. ELOVL2 encodes for a transmembrane protein involved in the synthesis of long (C22 and C24) x3 and x6 polyunsaturated fatty acids (PUFA) [96]. It is basically expressed in the liver date, ELOVL2 has not been associated with aging also in light of our results and considering that PUFAs are involved in definite biological functions including energy production, maintenance of cell membrane integrity and modulation of inflammation it is possible that ELOVL2 methylation plays an important role in the aging process through the regulation of multiple biological pathways [97].

.2 The Tripartite Motif (TRIM)72.

Family proteins Tripartite Motif, comprising over 60 members are evolutionarily preserved proteins that share a RBCC motif, which consisting of a common N-terminal Really Interesting New Gene (RING) finger domain and one or two B-box motifs and a coiled-coil region [98]. These proteins are involved in a plethora of cellular processes such as apoptosis, cell proliferation, migration, cell cycle regulation, differentiation and development [99]. Recently, several studies reported that TRIM proteins including TRIM11, TRIM28, TRIM29, TRIM44 and others seemed to work as oncogenes in lung cancer [100], indicating significant roles of the TRIM family in lung tumorigenesis. TRIM59 a novel TRIM family member is characterized by the presence of a RING finger domain, a B-box 2domain, two coiled-coil domains and a

transmembrane domain in its structure [101]. It is implicated in a wide range of biological processes in lung cancer and other multiple tumors. It may be used as a novel multiple tumor biomarker in immunohistochemical detection for early tumorigenesis [102]. Upregulation of the TRIM59 gene promotes gastric carcinogenesis by facilitating the p53 ubiquitination and degradation [103].

.3 FHL2 Gene 72.

FHL2 gene located on chromosome 2q12–q14 and has 7 exons [104]. It is transcribed into a transcript of 1.5 kb in which the first three exons are non-coding and finally translated into a protein of 279 amino acids. Its cDNA was first cloned by two independent groups almost simultaneously [105]. FHL2 is expressed in a cell/tissue specific manner but mostly in heart, a little lower expression level in ovary, a marginal expression level in liver, brain and lung [106]. While it is not expressed in other tissues including thymus, spleen, blood leukocytes, skin and smooth muscle or skeletal muscle [108]. FHL2 belongs to the four-and-a-half-LIM protein (FHL) family [107]. It was also identified as downregulated in rhabdomyosarcoma LIM (DRAL) domain protein which consists of four LIM domains and one N-terminal half LIM domain with specific distribution of cell [109]. In HepG2 cells, the endogenous FHL2 has been localized in both the nucleus and cytoplasm [110], which is consistent with the fact that it passively shuttles between the cytoplasm and the nucleus possibly due to its small molecular mass (32 kDa) [104]. The LIM domains of FHL2 are double zinc finger motifs which supply the protein–protein binding interfaces and allow FHL2 to interact with more than 50 different proteins including receptors, signal transducers, structural proteins and metabolic enzymes [104]. FHL2 plays multiple roles corresponding to its universal distribution for example, it serves as functional modifiers, adaptors and transcriptional co-activators for a series of transcription factors including androgen receptor (AR) [111].

.4 KLF14 Gene 72.

The Krüppel-like factors (KLFs) of mammalian are a family of zinc-finger containing transcription factors with many patterns of expression and a numerous array of cellular functions and their roles in mammalian physiology are well known, there is a growing assessment for their roles in modulating the fundamental progression of aging [112]. KLF14 acts as a transcriptional activator for the generation of lipid signaling molecules [113]. The Krüppel-like factors are a family of transcriptional regulators with a C-terminal DNA-binding domain containing three C2H2-type zinc

fingers recognizing a 5'-C(A/T)CCC-3' sequence also other GC-rich sequences that domain is well-conserved across the family. The N-terminal regions are much less so, allowing multiple protein-protein interactions and transactivation or repression. In mammalian There are at least 18 KLFs with roles in nearly every major organ system regulating an array of cellular functions [114]. KLF14 also has roles in protecting the genome and its deletion in a mouse leads to centrosome aneuploidy and amplification while promoting spontaneous tumorigenesis through its regulation of polo-like kinase 4 [115].

.5 MIR29B2C Gene72.

C1orf132 gene useful as predictors of chronological age in forensic investigations. In addition, studies demonstrate other potential confounders of DNA methylation will be particularly interesting in the case of C1orf132 that is a very powerful age predictor. This locus encodes for a long noncoding RNA and may play an important role in epigenetic regulation. Like mentioned earlier, previous study shown its potential involvement in graft function after HSCT [116]. In particular, the effect appeared in that study was a result of hypermethylation of a single C1orf132 locus but the remaining loci included in the model did not appear this pattern. It means that in some cases as HSCT, C1orf132, which encodes for long noncoding RNA MIR29B2C may not be a perfect predictor of chronological age [117]. Recently the C1orf132 has been shown to encode a long no-coding RNA (lncRNA) which at its 3' end includes microRNA MIR29B2C [118]. The C1orf132 function remains unclear, that in mice the miR29 family (miR29ab1 and miR29b2c) has been contribute to in the self-renewing ability of HSCs and determination of organ and body size [119]. The C1orf132/MIR29B2C locus could also be essential for graft function after HSCT and thus be explained as a potential graft function/ prognostic marker [117].

. 2.8 Age Estimation and Forensic Applications

In forensic science, age estimation is a key factor analysis that can supply very useful information applicable to legal, criminal and anthropological investigations. Forensic age conclusion was initially based on radiography or morphological inspection and only later started to take on molecular approaches. The essential point occurred during an individual's lifespan when the epigenetic signature of DNA methylation was observed to gradually change with age. Recently, numerous of publications reporting DNA methylation age-correlated changes has gradually augmentation and age methylation tests now has a range of applicable to forensic

casework of the forensic community based on blood DNA sample. Most forensic age predictor models have been developed but additional tissues are now also being recognized [121].

The past few decades of research have discovered a powerful relation between aging and DNA methylation. A good example simple and elegant study explained this relation very nicely, by taken monozygotic twins and analyzing locus and global specific differences in DNA methylation the authors found that younger twins had indiscernible methylomes, while older twins had remarkably different methylomes [122]. Another study quantifying the methylation status of 27,578 CpG loci in both non twin and monozygotic twin samples showed 88 sites in or near 80 genes whose 5mC content changed significantly with age. In some genes Methylation of CpG was mostly correlated with age and by using two cytosine from these loci building a regression model, that were able to evaluate the age of persons with an average error of 5.2 years [123]. A complicated interact between multiple biological effects shapes of the aging process. The emersion of genome wide quantitative approaches in the epigenetic field that was highlighted the effective impact of epigenetic deregulation particularly of DNA methylation on aging. DNA methylation alterations with age are commonly grouped in the phenomenon known as “epigenetic drift” which is identified by gradual extensive of hypermethylation of a number of promoter associated CpG islands and demethylation of genome. Specific DNA regions appeared epigenetic changes in aged persons suggesting the importance of these events for the aging process but the epigenetic information obtained recently in aging needs reconsideration because of the discovery of 5- hydroxymethylcytosine a new epigenetic mark present on genome [124].

2.8 DNAinForensic Science

Forensic science can be defined as specialty that aims to help investigator solve legal cases. DNA analysis has developed to become an essential and routine part of modern forensic casework and using extremely sensitive PCR-based techniques to analyze biological material and using DNA evidence from DNA databases can be rapidly interrogated for matches to DNA profiles found at the scene of a crime or even partial matches to close relatives of a perpetrator. Suspects can be linked to crime scenes or one crime scene to another [125]. Forensic biology is the application of biological

fluids in law enforcement [126]. The molecular biology use in forensic science is now well confirmed and it is an accepted method for the identification of individuals. This is usually on the basis of DNA recovered from body fluids and blood or other tissues such as bone marrow [127]. Body fluids and Blood composed of a variety of different cell types. Blood cells are found in a watery fluid called serum. Traditionally, haematology is referred to the study of blood, whilst serology referred to the study of serum (and in particular the immune factors within it) and ‘forensic serology’ is sometimes taken to including not only the study of serum but also blood cells, semen and saliva for forensic serology analysis. In the forensic laboratory refers to the screening of guides for body fluids, while DNA analysis care about to the efforts to individualize body fluids to determine a specific personal [128].

The DNA analysis developed DNA profiles from questioned samples and that help to known standards for comparison purposes. DNA profiles are specific for each person (except for identical siblings) profiles saved from one case will benefit others and leads for law enforcement [129]. In police investigations to link between a crime scene and a specific individual who is a suspect in the case using ‘DNA fingerprint’. DNA guides have become the ‘gold standard’ of forensic testing [130]. The high trustiness of DNA evidence in court stems from the indeed that it uses a statistical approach based on population empirical testing and genetics. It is considered more reliable than eyewitness evidence, which is known to suffer from a relatively high rate of errors [131]. Age estimation is an important point of forensic sciences [132]. Methylation is to a large range influenced by the ageing process and genome-wide methylation analysis has discovered numerous differentially methylated regions (tDMRs) which different in levels of methylation in different tissues and cell types. TDMRs have been useful in various applications specifically in medicine and forensic sciences. Forensic scientists are constantly seeking novel methods to help in the reconstruction of crime scenes and the analysis of tDMRs represents a reliable and new technique to identify tissues and biological fluids found at the scene of a violent act. Not only research has been able to unmistakable identify various fluids and tissues but methods to determine the age, sex and phenotype of individuals has been developed. The research of new tDMRs in genes are being to serve as novel markers in forensic DNA analysis [132]

Chapter Three

Materials and Methods

3. Material and Method

3.1. material

3.1.1 Apparatuses and Disposable materials

Apparatuses used in this study, model, companies and origin listed in table 3-1.

Table (3.1): Apparatus used in this study.

No	Name of Item	Model	Company	Origin
1	Analytical Balance	ABS/ABJ	KERN	Germany
2	Autoclave	DE-23	Systec	Germany
3	Biological safety cabinet	BC-11B	Jelo-Tech	Korea
4	Centrifuge	CL008	Cypress Diagns.	Belgium
5	Filter- Cellulose Acetate Membrane	Sterile syringe filter 0.2 microns	Chmlab	Spain
6	Gel Documentation system	Mthna-Gel Doc System	Al Muthanna University	Iraq
7	Gel electrophoresis	Mupid-one	Mupid	Japan
8	Incubator	CL011	Cypress Diagns.	Belgium
9	Lab. Electrical Oven	LEO-080F	DAIHANLabTec	Korea
10	Gradient Thermal Cycler	TC9600-G	Labnet	USA
11	Microcentrifuge	Mikro 120	Hettich	Germany
12	Micropipette	0.1-2.5 µl, 0.5-10 µl, 50-200 µl	Nanalytik	Germany
13	Microwave Oven	LMH2	LG	Korea
14	pH / Temperature meter	Mi150	Martini	Italy
15	Thermocycler	TC-3000	Labnet	Japan
16	Deep freezer -20°C	Serie 4	Bosch	UK
17	UV sterilization cabinet	GLE-UVSC	SCI-PLAS	UK
18	Vortex mixer	LVM-202	DAIHAN LabTec	Korea

19	Water Bath	LWB-111D	DAIHAN LabTec	Korea
20	Nano drop	1000 UV-VIS	Thermo scientific	USA

3.1.2 Kits and Reagents

The commercial kits and reagents are uses in this study as illustrated in table (3.2).

Table (3.2): The commercial kits and reagents

No	Item	Code	Company	Origin
1	Quick Blood Genomic DNA Extraction kit	1131	DSBIO	China
2	MethylEdge Bisulfite Conversion System	1301	Promega	USA
3	Hot star Taq polymerase Qiagen	203205	Qiagen	USA
4	Streptavidin kit	PSQ96HS	EpigenDX	USA
5	Methylation control	80-8060H	EpigenDX	USA

3.1.3 Primers

Methylation specific PCR primers were designed for the analysis of DNA methylation for five genes (ELOVL2, TRIM59, FHL2, KLF14 and MIR29B2CHG). The primers were designed and synthesized by EpigenDX (USA). Each primer was diluted to final concentration of 100 pmole/μl and kept at -20 C° for storage.

Table (3.3): Description of primers for bisulfate DNA amplification.

Assay ID	Gene Name	Type of assay	PCR Size(bp)	CpGs
ADS9043-RS1	Human ELOVL2 antisense RNA 1 Gene ID: ENSG00000230314	Bisulfite sequencing	193	9
ADS9042-RS1	Human four and a half LIM domains2 Gene ID: ENSG00000115641	Bisulfite sequencing	168	2
ADS5739-FS1	Human MIR29B2CHG Gene ID: ENSG00000203709	Bisulfite sequencing	133	4
ADS570-FS2	Human MIR29B2CHG Gene ID: ENSG00000203709	Bisulfite sequencing	156	4

ADS5738-FS2	Human tripartite motif containing 59 Gene ID: ENSG00000213186	Bisulfite sequencing	187	4
ADS5128-FS2	Human Kruppel-like factor 14 Gene ID: ENSG00000266265	Bisulfite sequencing	278	4

Table (3.4): The CpG Methylation Assays of Five Genes.

Assay ID	CpG Loci	primer	Primer Code	5 mod'
ADS9043	Human ELOVL2-AS1, Intron 1 Transcript ID:ENST00000456190 +902 to +869	PCR F	ADS9043FPB	Biotin
		PCR R	ADS9043RP	-
ADS9043-RS1	CpG+1 to +9	Seq R	ADS9043RS1	-
ADS9042	Human FHL2, Intron 1 Transcript ID:ENST00000393352 -12733 to -12742 From ATG +39255 to +39246 from TSS	PCR F	ADS9042FPB	Biotin
		PCR R	ADS9042RP	-
ADS9042-RS1	CpG -1 to -2	Seq R	ADS9042RS1	-
ADS5739	Human MIR29B2CHG Gene ID: ENSG00000203709 +55252 to+ 55275 from TSS	PCR F	ADS5739FP	
		PCR R	ADS5739RPB	Biotin
ADS5739-FS1	CpG 1 to 4	Seq F	ADS5739FS1	
ADS5740	Human MIR29B2CHG Gene ID: ENSG00000203709 +77078 to +77099 from TSS	PCR F	ADS5740FP	
		PCR R	ADS5740RPB	Biotin
ADS5740-FS2	CpG 4 to 8	Seq F	ADS5740FS2	

ADS5738	Human TRIM59, Promoter Transcript ID:ENST00000309784 -11182 to -11166 from ATG -536 to -520 from TSS	PCR F	ADS5738FP	
		PCR R	ADS5738RPB	Biotin
ADS5738- FS2	CpG -1 to -4	Seq F	ADS5738FS2	
ADS5128- FS2	Human KLF14, Promoter Transcript ID:ENST00000583337 -380-340 to from ATG -352 to -312 from TSS-	PCR F	ADS5128- FPB	
		PCR R	ADS5128-RP	Biotin
ADS5128- FS2	CpG 1to 4	Seq R	ADS5128- FS2	

Table (3.5): Target CpG Sites Coordinates.

Gene	Assay ID	Assay Location	From ATG	From TSS	GRCh38	# of CpG
ELOVL2	ADS9043-RS1	Intron 1	n.a	+ 902 to + 869	Chr6:110446 61-11044628	9
TRIM59	ADS5738-FS2	Promoter	- 11182 to - 11166	- 536 to - 520	Chr3:160450 365- 160450349	4
KLF14	ADS5128-FS2	Promoter	- 380 to - 340	- 352 to - 312	Chr7:130734 413- 130734373	4
FHL2	ADS9042-RS1	Intron 1	- 12733 to - 12812	+ 39255 to + 39176	Chr2:105399 249- 105399328	2
MIR29B2CHG	ADS5739-FS1	Intron 4	N/A	+ 55252 to + 55275	Chr1:207823 845- 207823822	4
MIR29b2CHG	ADS5740-FS2	3- Downstream	N/A	+ 77078 to + 77099	Chr1:207802 019- 207801998	4

Table (3.6): Target Sequences in the Assays.

Assay ID	Genomic Target Sequence	Bisulfite Converted Target Sequence	Pyrosequencing Dispensation order
ADS9043-R	CCAGCCGGCGCCGG TTTCGCGCGGCGGC TCAACGTCCACG	CRTAAACRTTA AACCRCCRCRC RAAACCRACRC CRACTAAA	ACGATGATCGATG AGTCGATCGATCG ATCAAGATGCAG ATCGATCAGACT
ADS5128-FS2	TCC/TGACTGGGGA GTTTCGCTCTGTTA CCATTACCTGGCTC GCCGGCAG G > A rs7808283, LOSS OF CPG	TTYGATTGGGG AGTTTYGTTTTG TTATTATTATTT GGTTYGTGTT AG	GTCTGACTGGAGT TCGCTCTGTACTA TACTGCTCAGTCG CTA
ADS9042-RS1	AGACCCGGAGGGC AC	RTACCCTCCRA ATCT	GATGACTCAGATC T
ADS5738-FS2	CTGAGCGGCGGAG GGCGCAGGCGACA GA	TTGAGYGGYGG AGGGYGTAGGY GATAGATGT	GCTGAGTCAGTCG AGTCGCTAGTCGA T
ADS5739-FS1	CGCAAGGTGGCAC GGGCGGTTCCCGTG GGGA	YGTAAGGTGGT AYGGGYGGTTT TYGTGGGGA	ATCGCTAGTGCTA TCAGTCAGTCTCG TGG
ADS5740-FS2	CCCTGGAGTATTCG GCGTTGATTGCGGG GCACCCGAAATCAG	TTTTGGAGTATT YGGYGTTGATT GYGGGGTATTY GAAATTAG	ATTGAGTGATCAG TCGTGATAGTCGG TGATCGAT

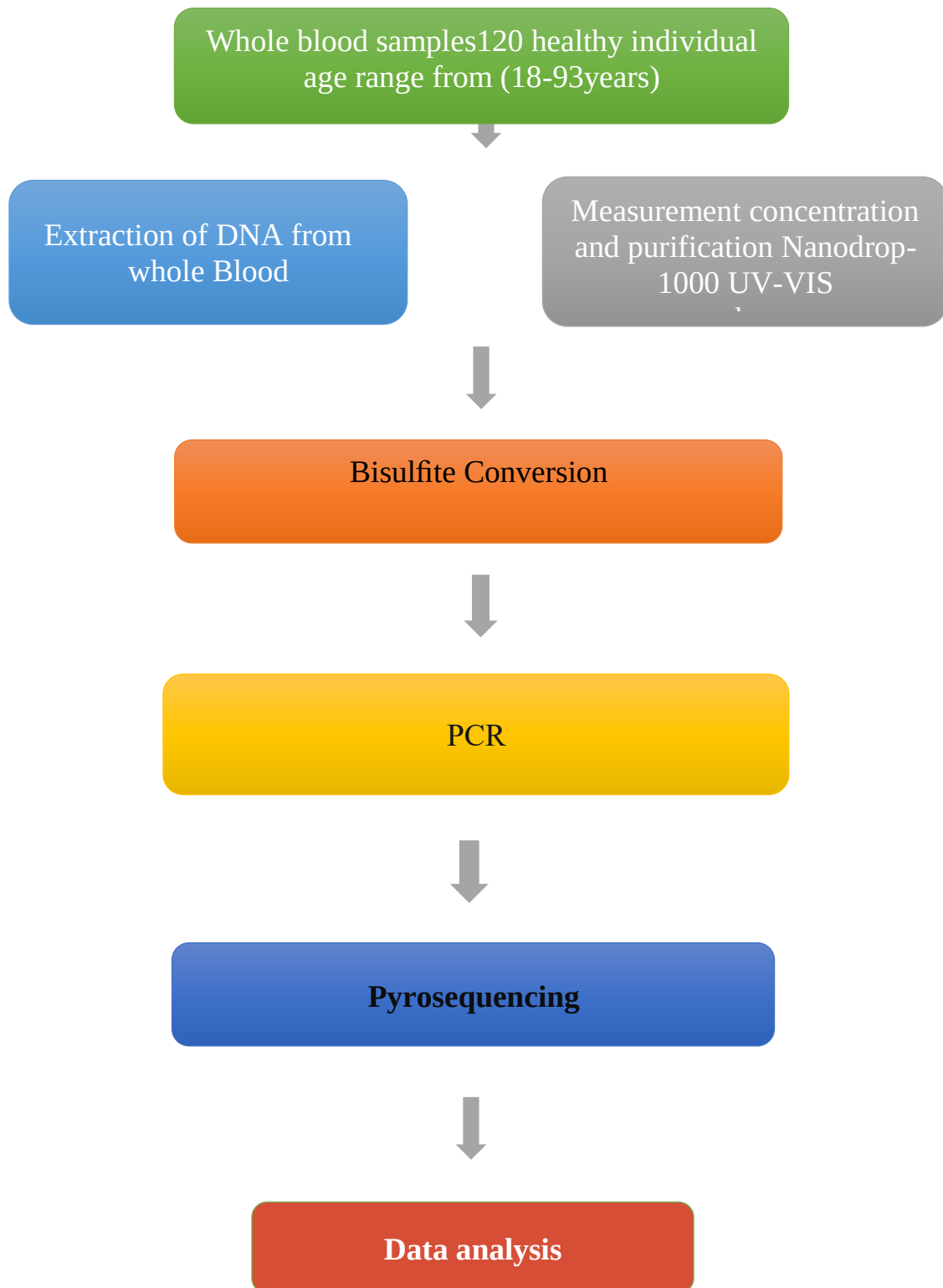
3.1.4 Chemicals

The chemicals that were used in the present study are listed in table (3.7).

Table (3.7): Chemicals and disposable materials used in this study.

NO.	Chemicals and disposable materials	Company	Origin
1	Agarose	US biological	USA
2	Decontamination nucleases spray	Bio-world	USA
3	Ethanol 96% Spray	AMRESCO	USA
4	Ethanol 96%	Scharlab S.L.	Spain
5	Ethidium bromide	Bio-world	USA
6	TE buffer	Biovision	USA
7	EDTA	S.d. fine-Chem	India – Mumbai
8	Nuclease free water	Bio-world	USA
9	Proteinase	DSBIO	USA
10	ME Conversion Reagent	Promega	USA
11	ME Desulphonation Buffer	Promega	USA

3.1.5 Experimental Design



3.2 Method

3.2.1 Preparation of Buffers and Reagents:

3.2.1.1 Preparation of 10X TBE buffer

To prepare 1 liter of 10X TBE dissolve 108g Tris-base, 55 g boric acid and 40 ml 0.5 M EDTA (pH 8.0) in 600 ml deionized water. Adjust final volume to 1 liter with deionized water. *Autoclave was 15 minutes at 121 C° [133].*

3.2.1.2 Preparation of 1% agarose gel

One gram of agarose was added to 100 ml of 1X TBE buffer in flask; dissolved in microwave oven until start the appearance of boiling, removed the flask and swirl the agarose mixture, return the flask to microwave oven until before it boils and no there were flicks. Allow the agarose to cool about 50 °C then added ethidium bromide to the agarose gel 5µl of a 10mg/ml stock solution per 100 ml of agarose gel [133].

3.2.1.3 Prepare 1X (ME) Wash Buffer

- 1- Volume 24 ml of absolute ethanol added to the bottle containing 6 ml of concentrated ME Wash Solution.
- 2- The bottle capped tightly. The reagent is stable at room temperature.

3.2.1.4. Sampling

Blood samples were collected from 120 healthy individual age range from 18-93 years in EDTA tube from different places of Al-Muthanna province during the period from November 2017 to June 2018. All individuals were agreed by signing on consent document of participation. Information were collected from participants included age, gender, family history of genetic diseases, weight, smoking and lifestyle. All individual was healthy. We excluded samples of individuals stated who has a disease, obesity, and smoking. The volume 200µL aliquots were immediately placed in eppendorf tubes and DNA extracted. DNA extracted from blood samples by Quick Blood Genomic DNA Extraction Kit.

3.2.1.5. Sterilization

All plastic ware, glassware and instruments that needed to be in sterile condition were autoclaved at 20 psi for 15 min. Pipette tips and microcenterfuge tubes were sterilized in autoclaved at 1.5 bar for 15 min and then exposed to 70C° for 3-4 hours by electrical oven. The cabinet should be wiped with 70% ethanol and followed by nucleases decontamination spray before begin work to allow the working surface to purge and remove any nuclease activity. In addition, the ultraviolet (UV) germicidal lamp is an important when it continues for more than 20 minutes. The contamination can be avoided by placed all materials and containers in the cabinet should be disinfected in order to avoid the contamination.

3.2.1.6 Preparation of PCR Primers.

The primers were designed to interrogate the DNA methylation status of CpG sites in different five genes (ELOVL2, TRIM59, FHL2, KLF, and MIR29B2CHG). The primers were synthesized by EpigenDX (USA). One primer (opposite sequence primer) is biotin labelled for immobilized to sepharose beads. Lyophilized primer tubes were spin prior to opening to prevent loss of pelleted oligonucleotide, then resuspension of primers by adding free-nuclease water to reaching a final concentration of 100 µM as stock primer solution for each forward and reverse PCR primer and kept at -20 C° for storage. Labeled separate new, sterile, and nuclease-free 1ml microcenterfuge tube for each PCR Primer. For each forward and reverse primer combination was prepared by added nuclease free water to each primer prepare the following mix in the microcenterfuge tube labeled with the appropriate primer number combination by added 90 µl of nuclease free water and 10 µl for each primer to reach the concentration 10 pmole/µl. Gently, pipette the entire volume up and down 10 times to mix thoroughly.

3.2.2 DNA Extraction from Whole Blood

Volume 2 ml of blood samples were collected by standard vein puncture from volunteers. Volume 200 µl aliquots were immediately placed in micro centrifuge tube and

extracted by using Quick Blood Genomic DNA Extraction Kit (DSBIO Company, USA). DNA was extracted of 120 blood samples from unrelated individuals with age ranging 18-93 years old.

- 1- Blood sample (200 μ l) was added to a 1.5ml microcentrifuge tube.
- 2- Proteinase (20 μ l) was added and 220ML solution QS mix well then incubate at 65c for 10-15 min to yield a black homogeneous solution.
- 3- Ethanol 220 μ l (96–100%) Added to the sample and mix by inverting. It is important that the sample and the ethanol are mixed thoroughly and a precipitate may appear.
- 4- The mixture from step 3 was taken by pipette into the spin column placed in a 2 ml collection tube (provided). Centrifuge at 12,000rpm for 1 min then discard flow-through.
- 5- Wash Buffer PS (500 μ l) was added and centrifuge for 1 min at 12,000 rpm then discard flow-through.
- 6- Wash Buffer PE (500 μ l) was added and centrifuge for 1 min at 12,000 rpm then Discard flow-through. Repeat step 6 again.
- 7- For 3 min Centrifuged at 12,000 rpm to dry the column membrane then Discard flow-through and collection tube. It was important to dry the membrane of the spin column since residual ethanol may interfere with subsequent reactions. This centrifugation step ensures that no residual ethanol will be carried over during the following elution, then centrifugation step.
- 8- The spin column carefully removed that the column without contact to the flow-through since this will result in carryover of ethanol. If ethanol carryover occurs empty the collection tube, then reuse it in another centrifugation for 1 min at 12,000 rpm.
- 9- The spin column was placed in a clean 1.5 ml microcentrifuge tube, and added 60 μ l Eluent Buffer AE (pre-warm to 65°C) directly onto the membrane then Incubate at room temperature for 2 minutes and then centrifuge for 2 min at 12,000 rpm to elute bound DNA.
- 10- The tube contains the purified DNA Stored at -20°C for the next processes.

3.2.3 Agarose Gel Electrophoresis

Small DNA molecules migrate through the agarose gel faster than larger molecules. DNA is not visible within an agarose gel. By the use of a dye we will be visualized DNA that binds to DNA were analyzed by gel documentation system according to the following steps:

- 1- Agarose gel 1% was prepared by 1 g of agarose dissolved in 100 ml of 1X TBE and moved the flask to mix the content then put it in microwave for 2 min. after that left to cool at room temperature .
- 2- Volume 5 μ l of ethidium bromide stain were added into agarose gel solution.
- 3- Agarose gel solution poured in tray after fixed comb in proper position after that, left to solidify for 15 minutes at room temperature, then the comb was removed gently from the tray and 6 μ l of extracted DNA were added in to each comb well.
- 4- The gel tray was fixed in electrophoresis chamber and fill by 1X TBE buffer. Then electric current was performed at 120 volt for 15 min.
- 5- Extracted DNA were visualized by using Mthana gel documentation system as shown in figure (3-1).

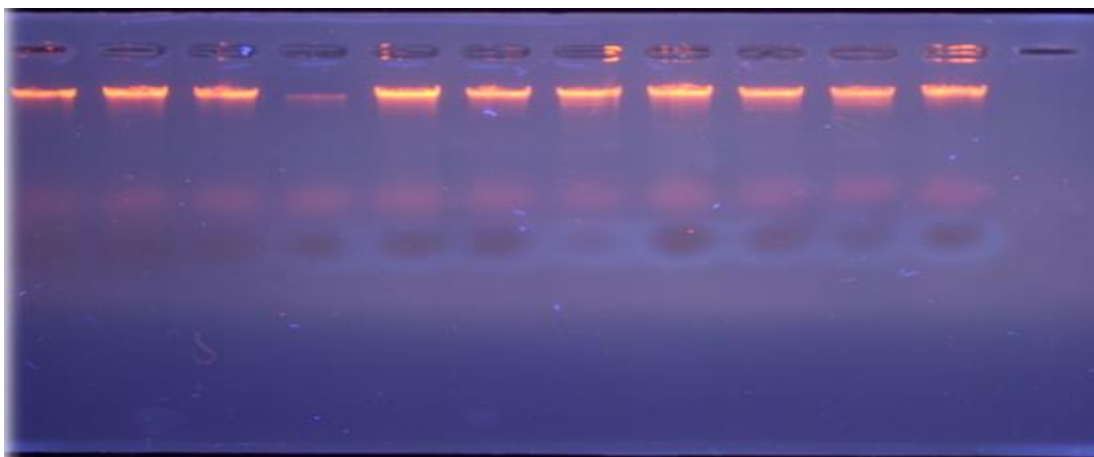


Figure (3.2): Genomic DNA of blood samples shows that electrophonic on agarose gel 1% at 120 volts for 15 minutes

3.2.4 Concentration and Purification of Genomic DNA

DNA concentration was measured with the Nanodrop-1000 UV-VIS spectrophotometer (Thermo Scientific, Loughborough, UK). The instrument was first

blanked with a sample of DNase-free water or elution buffer (the same solvent as the eluted DNA sample) and 1-2 µl of DNA sample used for calculation of concentration. The quantitation of extracted DNA was performed at 260 nm and ratio of the absorbance at 260/280 nm to assess the purity of DNA that was gave normal range of purity. All DNA samples measured by Nanodrop-1000 spectrophotometer showed in Appendixes supplementary data SU1

- ❖ DNA sample (1 – 2 µl) placed directly onto the measurement pedestal.
- ❖ Lower the sampling arm and initiate a spectral measurement using the software on the PC
- ❖ Surface tension is used to hold samples in place between two optical fibers.
- ❖ Light from a xenon flash lamp passes through the top optical fiber down through the liquid column and is detected by the internal spectrometer.
- ❖ When the measurement is complete raise the sampling arm and wipe the sample from both the upper and lower pedestals using a dry lint-free laboratory wipe.

The ratio of absorbance at 260 nm and 280 nm used to assess the purity of DNA and RNA. A ratio of 1.8 is generally accepted value as pure for DNA while a ratio of 2.0 is accepted value as pure for RNA. If the ratio is appreciably lower in either case, it may indicate the presence of protein, phenol or other contaminants that absorb strongly at or near 280 nm [134].

3.2.5 Bisulfite Sodium Conversion

Pre-treatment bisulfite conversion step was used MethylEdge Bisulfite Conversion System (Promega USA) that product has higher efficiency and less bias. The tubes were placed in thermal cycler and the machine program set up on alternative parameters to yield improved result as shown in Table (3-8) below.

Table (3-8): DNA Conversion: Treatment of DNA with sodium bisulfite.

Step	Temp	Time
DNA Denaturation	98 °C	8 min
Extension of conversion	54 °C	60 min
Store	4 °C	Up to 20 h

Procedure

Bisulfite Conversion

- 1- DNA sample (20µl) placed into a 200µl PCR tube. Reaction may also be performed in a 96 well plate.
- 2- Bisulfite ME Conversion Reagent 30µl added to each DNA sample and pipet gently to mix. Centrifuge briefly to collect the sample at the bottom of the tube. If we do not use all the ME Conversion Reagent contained in the tube ensure that the cap is closed tightly before to store the remaining reagent.
- 3- Preheat water baths or heat blocks or Program a thermocycler and incubate the reaction as follows: 8 minutes at 98°C 60 minutes at 54°C Hold at 4°C
- 4- After incubation, the samples can be stored at 4°C or on ice protected from light for up to 20 hours.
- 5- DNA Desulphonation and Cleanup :
 - A. Each sample to be processed place a ME Spin Column into one of the provided Collection Tubes, gloves used when handling the columns and tubes.
 - B. ME Binding Buffer 600µl added to the ME Spin Column. Transfer the entire bisulfite-treated sample to the column. Close the cap and mix by inverting the tube several times.
 - C. At maximum speed, ($\geq 10,000 \times g$) Spin for 30 seconds then discard the flow through and re-insert the ME Spin Column into the same Collection Tube.
 - D. 1X ME Wash Buffer 100µl added (with ethanol added). Spin at maximum speed ($\geq 10,000 \times g$) for 30 seconds.
 - E. ME Desulphonation Buffer 200µl added to each ME Spin Column and Close the caps incubate at room temperature for 15 minutes.
 - F. At maximum speed, ($\geq 10,000 \times g$) Spin for 30 seconds.
 - G. ME Wash Buffer 200µl added (ethanol added), then Spin at maximum speed for 30 seconds. Repeat this wash step once more.
 - H. The ME Spin Column place into a clean 1.5 ml microcentrifuge tube.
 - I. ME Elution Buffer 10µl added and spin at maximum speed ($\geq 10,000 \times g$) for 30 seconds, DNA may be eluted with up to 20µl of ME Elution Buffer if desired. Nuclease-free water or TE buffer ($\geq \text{pH } 6.0$) may be used.

- J. The ME Spin Column removed and discard, Cap the tube and store the eluted DNA at 4°C for use within one week or store the eluted DNA at $\leq -20^{\circ}\text{C}$ for long-term storage. All samples should be stored protected from light.

3.2.6 Sterilization of Working Station

All PCR reactions and primer dilutions were carried out in a UV sterilization cabinet to avoid extra-contamination. The Cabinet was cleaned with nuclease decontamination spray before and after all works. A set of pipettes, laboratory coats, gloves and consumables were designated to each working area so that they were not carried from one place to another, which may cause cross over contamination.

3.2.7 Methylation specific PCR

This assay requires modification of the genomic DNA by sodium bisulfite and two independent primer sets for PCR amplification. One pair designed to recognize the methylated that labeled by Biotin and the other pair the unmethylated versions of the bisulfite – modified sequence. The tubes were placed in programmed thermal cycler. Close the lid and selected run the amplification program follows.

Table (3.9): The components of PCR reaction

Component	30 μl reaction	Final Concentration
10X PCR buffer (15mM MgCl ₂)	3 μl	1X
25mM MgCl ₂	1.8 μl	3.0 mM
mM dNTPs	0.6 μl	200 μM of each
10 μM Fwd primer (ADS9042FPB)	0.6 μl	(6 pmol)
10 μM Rev primer (ADS9042RP)	0.6 μl	(6 pmol)
Hot Star Taq Polymerase	0.15 μl	(0.75 U)
bisulfite treated DNA	1-2 μl	50 $\mu\text{g}\backslash\text{ml}$
Nuclease-free water	Adjust to 30 μl	

Table: (3.10): PCR cycling conditions for ELOVL2, TRIM59, FHL2, MIR29 B2CHG, and KLF14 genes.

PCR step	Temp	Time	Repeat
Initial Denaturation	95°C	15 minutes	1
Denaturation	95°C	30 seconds	45 cycles
Annealing	*	30 seconds	
Extension	68°C	30 seconds	
Final Extension	68°C	5 minutes	1
Hold	4°C	∞	

- * Annealing ELOVL2 (56 °C 30s)
- * Annealing TRIM59 (56 °C 30s)
- * Annealing FHL2 (57 °C 30s)
- * Annealing MIR29B2CHG (60 °C 30s)
- * Annealing KLF14 gene (56 °C 30s)

3.2.8 Pyrosequencing

Pyrosequencing is performed using PSQ 96HS system or PSQ 96HSA system. The systems are equivalent to the Pyro Mark MD system. The Pyrosequencing run as the manufacturer's instruction

3.2.9 Statistical Analysis

In Statistical processes were used the Pearson correlation coefficients for comparison purpose. The linear regression model to check the fitting degree of each CpG site that belongs to certain gene. The correlation between CpG sites and chronological age were analyzed with paired t- test. The model of multiple linear regressions was performed in order to assess the prediction accuracy and fitting of age prediction. All Statistical processes was performed by MiniTap18 Statistical software.

Chapter Four

Results & Discussion

4. Result and Discussion

4.1 Bisulfite Modification

Sodium bisulfite conversion of genomic DNA to differentiate and detect unmethylated versus methylated cytosine is the gold standard for DNA methylation analysis [135]. Bisulfite conversion includes the deamination of unmodified cytosine to uracil, leaving the modified bases 5-mC and 5-hmC. The procedure followed by PCR amplification to reveal the methylation status of every cytosine in gene specific amplification or whole genome amplification. Subsequent PCR reactions are uracil converted to thymine, whereas 5-mC or 5-hmC residues amplified as cytosine. Comparison of sequence information between the reference genome and bisulfite-treated DNA can supply information about cytosine methylation patterns.

Methylation-Specific PCR includes analyzing untreated and bisulfite treated DNA using two different sets of PCR primer pairs: one pair that targets the methylated sequence and the other that targets the converted unmethylated sequence [136].

4.2 Polymerase chain Reaction (PCR)

PCR is a useful tool for qualitative DNA methylation analysis with many advantages, involving ease of design and execution, sensitivity in the ability to expose small quantities of methylated DNA, and the ability to screen a large number of samples. This assay demands modification of the genomic DNA by sodium bisulfite and two independent primer sets for PCR amplification one pair designed to realize the methylated and the other pair the unmethylated versions of the bisulfite-modified sequence [137].

4.3.1 Assays Validation

The assay validation results included quality of the methylation data generated by Pyrosequencing at each CpG site applied to three colors based on score of the data quality. The software compares the pyrogram peaks to the theoretical histogram generated by the sequence to analyze. A match of > 90% of the pyrogram and the histogram results in a blue highlighted percent methylation. A match between 70% and 90% of the pyrogram to the histogram results in a yellow highlighted percent methylation and a red highlighted

methylation result is due to less than a 70% match between the pyrogram and the histogram.

4.3.2 PCR Bias Testing Using Pyrosequencing:

Unmethylated DNA control and in vitro methylated DNA were mixed at different ratios (20%,40%,60%,80% and100%) followed by bisulfite modification, PCR, and Pyrosequencing analysis. The percent methylation obtained from the mixing study should be highly correlated with expected methylation percentages with an R square (r^2) of 0.8 or higher that test doing for all genes and give very significant results as shown of ELOVL2 (Figure 4.1), FHL2 (Figure 4.2), TRIM59 (Figure 4.3), KLF14 (Figure 4.4) and MIR29B2CHG genes (Figure 4.5) in this study.

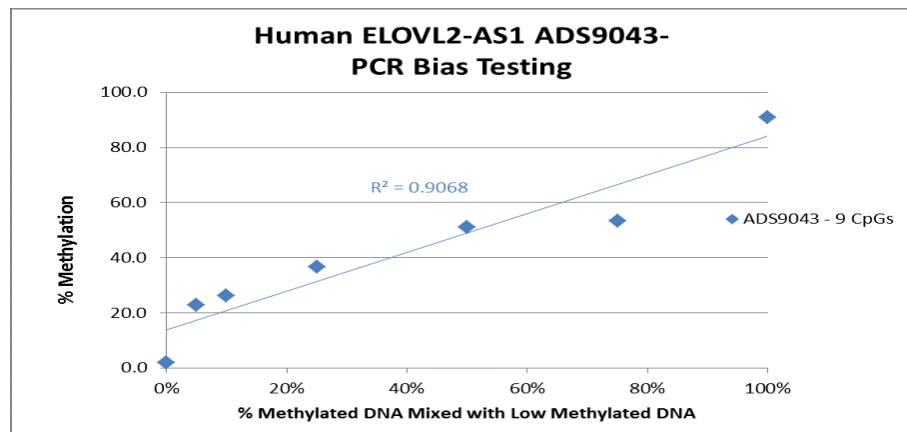


Figure (4.1): PCR Bias Testing Using Pyrosequencing for ELOVL2 gene.

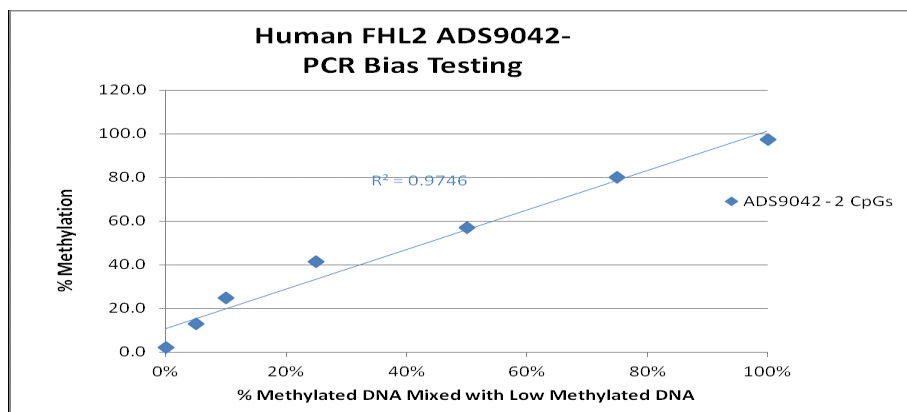


Figure (4.2): PCR Bias Testing Using Pyrosequencing for FHL2 gene.

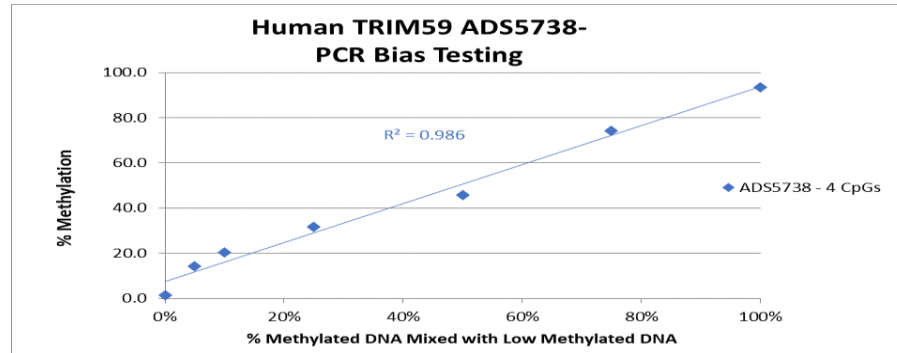


Figure (4.3): PCR Bias Testing Using Pyrosequencing for TRIM59 gene.

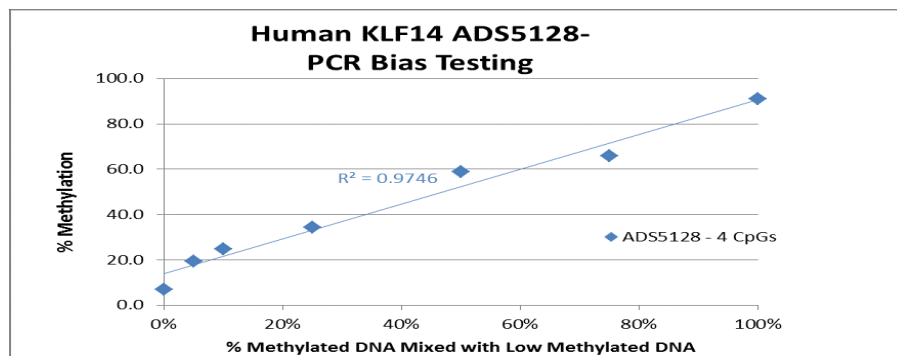


Figure (4.4): PCR Bias Testing Using Pyrosequencing for KLF14 gene.

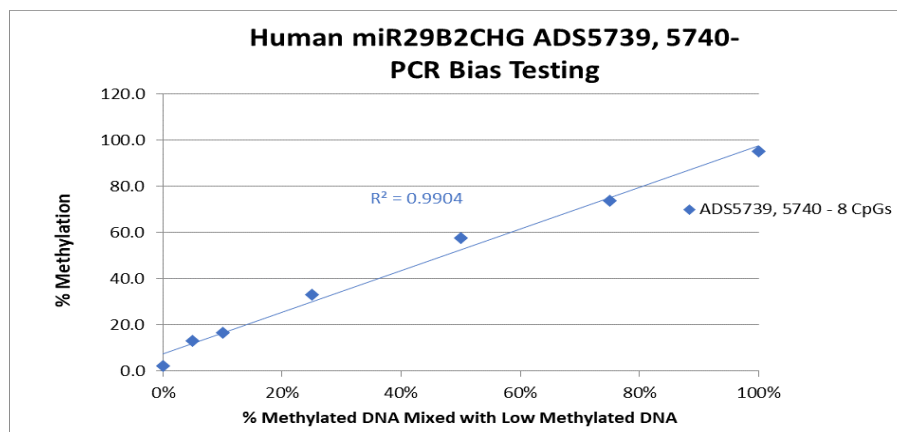


Figure (4.5): PCR Bias Testing Using Pyrosequencing for MIR29B2CHG gene.

4.4 Relationship between CpG Sites of a five Genes and Age

4.4.1 Relationship between CpG Sites of ELOVL2 Gene and Age

The ELOVL2 gene contains nine sites that are CpG1, CpG2, CpG3, CpG4, CpG5, CpG6, CpG7, CpG8 and CpG9. The results shown in table (4.1), it showed that nine CpG sites belonging to ELOVL2 gene have appeared strong significant linear relationship with

age (all P-values < 0.05, 0.01). In addition, the fitting process provided satisfactory correction coefficients as shown in table (4.2) and figure (4.6).

Table (4.1): Results of correlation between the age and CpG sites of ELOVL2

Sites	Pearson Correlation
Age vs. CpG1	0.866**
Age vs. CpG2	0.834**
Age vs. CpG3	0.905**
Age vs. CpG4	0.919**
Age vs. CpG5	0.880**
Age vs. CpG6	0.907**
Age vs. CpG7	0.916**
Age vs. CpG8	0.898**
Age vs. CpG9	0.885**
* Correlation is significant at the 0.05 level (2-tailed).	
** Correlation is significant at the 0.01 level (2-tailed) .	

Table (4.2): Fitting simple linear regression for CpG sites of ELOVL2 gene.

Sites	Regression equation	R-Sq (adj)
CpG1	$\text{CpG1} = 15.34 + 0.4124 \text{ Age}$	73.9%
CpG2	$\text{CpG2} = 13.28 + 0.4084 \text{ Age}$	71.3%
CpG3	$\text{CpG3} = 42.88 + 0.4493 \text{ Age}$	81.5%
CpG4	$\text{CpG4} = 24.98 + 0.4903 \text{ Age}$	82.7%
CpG5	$\text{CpG5} = 11.78 + 0.4530 \text{ Age}$	75.7%
CpG6	$\text{CpG6} = 37.86 + 0.5129 \text{ Age}$	78.9%
CpG7	$\text{CpG7} = 51.36 + 0.4685 \text{ Age}$	82.1%
CpG8	$\text{CpG8} = 15.42 + 0.3398 \text{ Age}$	76.3%
CpG9	$\text{CpG9} = 22.56 + 0.4814 \text{ Ag}$	76.3%

The age prediction model was based on DNA methylation level using bisulfite conversion–pyrosequencing method. The assay of Human ELOVL2 gene is located at Chromosome 6: Chr6:11044661-11044628 forward strand in GRCh38 coordinates. There

are 4 transcriptional variants of the gene; this assay was designed for the analysis of DNA methylation by pyrosequencing to be targeted ELOVL2-AS1 gene encodes ELOVL2 antisense RNA 1 (ncRNA), Intron 1 Ensembl Transcript ID: ENST00000456190 at distance transcription start site (TSS) +902 to +869. In this study of ELOVL2 gene showed nine CpG sites detected at, CpG1 (Chr6:11044628), CpG2 (Chr6:11044631), CpG3 (Chr6:11044634), CpG4 (Chr6:11044640), CpG5 (Chr6:11044642), CpG6 (Chr6:11044644), CpG7 (Chr6:11044647), CpG8 (Chr6:11044655) and CpG9 (Chr6:11044661).

The correlation between DNA methylation level of CpG sites and chronological age were analyzed by linear regression model. The result of each CpG site of ELOVL2 gene reported the Pearson correlation coefficient of CpG4 (R=0.919), CpG7 (R=0.916), CpG 6 (R=0.907) and CpG 3 (R=0.905) were the strong correlation, followed by CpG8 (R=0.898), CpG9 (R=0.885), CpG5 (R=0.880), CpG1 (R=0.866) and CpG2 (R=0.834), showing slightly different among the values, the data shown in table (4.3) and figure (4.6) of nine CpG sites of ELOVL2 gene have appeared significant linear relationship (P-value 0.00) and hypermethylated with age . In addition, the fitting process provided high satisfactory correction coefficients.

One of the most practically important goals for forensic scientists that age prediction. Recently, unprecedented age estimation models were developed by evaluated the methylation degrees of some CpG markers for blood samples [138]. The correlation found between methylation levels of the CpG sites in ELOVL2 gene and Age. The family of ELOVL (elongation of very long chain fatty acids) 1-7 genes in human encoded to enzymes responsible for the elongation of fatty acid chains [96]. Through study of seven genes of ELOVL family identify about 100 CpG sites, ELOVL2 gene (coded for elongase2) was found to be the most closely related with the chronological age prediction due to important roles of elongase2 in the metabolism of polyunsaturated fatty acids in biological integrity of a cell [139]. That PUFAs are involved in crucial biological processes, such as inflammation, energy production, and maintenance of membrane integrity, it is potential that methylation of ELOVL2 is causally linked to the aging process.

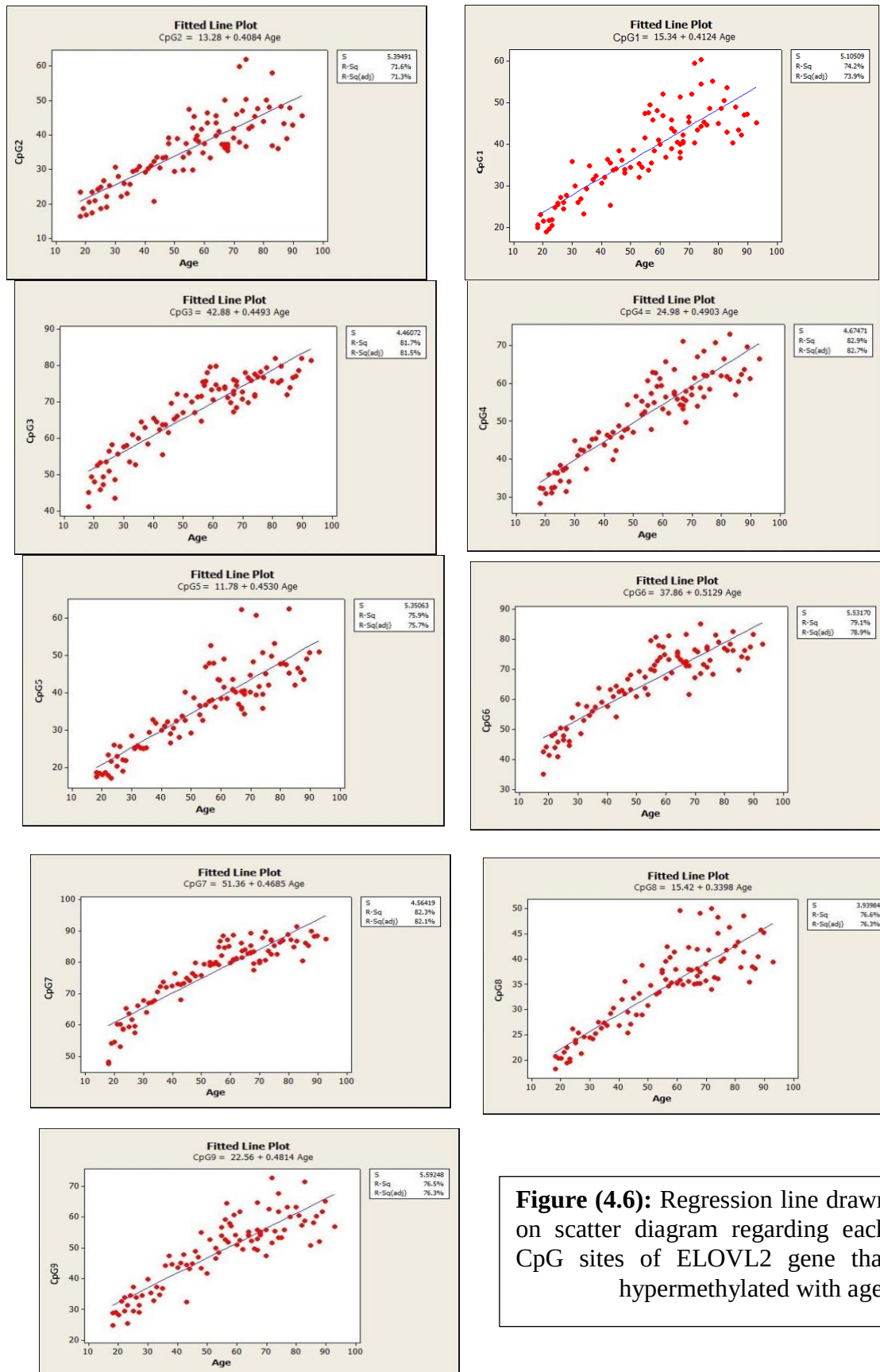


Figure (4.6): Regression line drawn on scatter diagram regarding each CpG sites of ELOVL2 gene that hypermethylated with age.

PUFAs also play important role in the photoaging response of the human skin [140]. The 9765432, which is hypermethylated of previous studies focused on the promoter of certain CpG sites with increase of actual age in blood samples. Human blood have significant sample of design age prediction model by measure the levels methylation because blood sample is stable over a time and has significant methylated DNA markers [141].

The most candidate of age prediction model is ELOVL2 gene because highly significant results of the studies carried out to find the relationship between DNA methylation of CpG sites and chronological age that provide important information about chronological age. Due to the high correlation to age shown by have been identified in the human genome. has been included in all subsequent forensic age- ELOVL2 CpG sites, ELOVL2 gene systems [142]. prediction

The previous study reported spearman correlation coefficient between hypermethylated CpG sites of ELOVL2 gene in blood samples and chronological age was 0.92 [94]. The study of Zbiec was targeted two methylated CpG sites (out of 7 CpG sites) of ELOVL2 gene in blood samples from healthy individuals 2-75 years old, which showed accuracy error of age estimation according to MAD (mean absolute deviation) of ± 5.03 years [143]. Recently, ELOVL2 was again highlighted as the top gene of high-confidence aging-associated CpG sites in blood when comparing nonagenarians with young healthy controls [144].

4.4.2 Relationship between CpG sites of FHL2gene and Age

FHL2 gene contains two sites that are CpG1 and CpG2. From the results shown in table (4.3), two CpG sites belonging to FHL2gene have appeared significant linear relationship with age (all P-values<0.05, 0.01). However, the fitting process has provided somewhat low satisfactory correction coefficients as shown in table (4.4) and figure (4.7). This study was evaluated age associated with methylation for 2CpG sites located in Human four and a half LIM domains 2gene. The assay of FHL2 gene is located at Chromosome 2:105399249-105399258 forward strands in GRCh38 coordinates. The assay was designed for the analysis of DNA methylation by pyrosequencing to be targeted Intron 1 ADS9042-RS1Ensembl Transcript ID: ENSG00000115641 at distance

transcription start site (TSS) +39255 to +39246. FHL2 gene showed two CpG sites detected at CpG1, (Chr2: that age-associated105399249), CpG2 Chr2: Chr2:105399258. Methylation status correlated with age of this two CpG sites methylation level continuous increase with age.

Table (4.3): Results of the correlation between age and CpG sites of FHL2 gene.

Age vs. Sites	Pearson Correlation
Age vs. CpG1	0.461**
Age vs. CpG2	0.579**
* Correlation is significant at the 0.05 level (2-tailed).	
** Correlation is significant at the 0.01 level (2-tailed) .	

Table (4.4): Results of regression analysis of CpG sites of FHL2 gene.

Sites	Regression equation	R-Sq(adj)
CpG1	$\text{CpG1} = 3.609 + 0.07508 \text{ Age}$	29.3%
CpG2	$\text{CpG2} = 7.907 + 0.08128 \text{ Age}$	35.9%

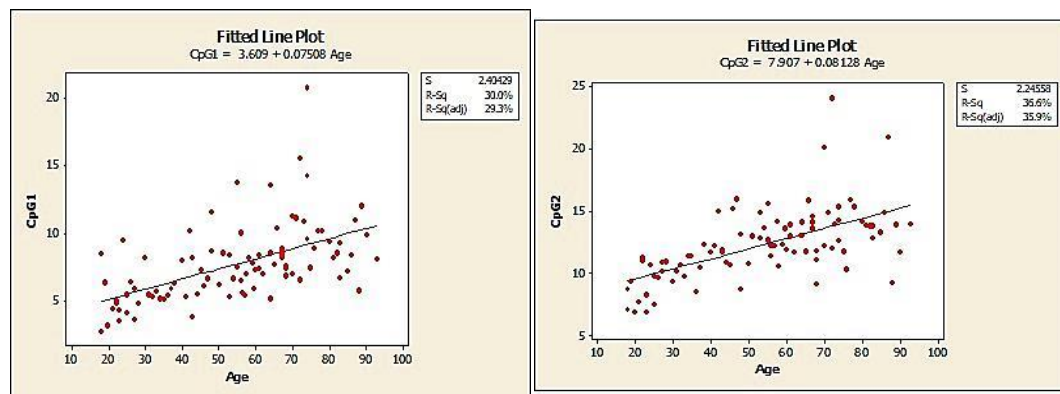


Figure (4.7): Regression line drawn on scatter diagram regarding each CpG site of the FHL2 gene that hypermethylated with age.

The Steengenga study has specified two genomic regions, take place within the CpG islands of the promoters of FHL2 and ELOVL2 genes, whose methylation status was

highly correlated with age. These findings were later assured by similar studies. Both FHL2 and ELOVL2 showed continuous increase in methylation levels with age, with methylation values ranging from 12% to 53% for FHL2 and from 7% to 91% for ELOVL2 [145]. The human FHL2 gene maps to 2q12-q14 and consists of seven exons, the first three of which are non-coding. Computer analysis of the promoter reveals several of putative transcription factor binding sites [146]. The genomic regulation and the promoter region of the *fhl2* gene related with potential transcription factor binding motifs. The actual contribution of particular transcription factors has not been scrutinized, some guide exists that they may be involved in the transcription of the FHL2 gene, which express wild-type p53 that primary myoblasts A33GM possess clearly detectable levels of FHL2 messenger RNA (mRNA), while mutant p53 with rhabdomyosarcoma cells with display very low levels of FHL2 transcripts. Moreover, FHL2 mRNA levels were monitored increase in rhabdomyosarcoma cells stably transfected with an expression vector for a temperature sensitive p53 mutant at a permissive temperature, while no changes were measured in the control cells at both temperatures [146].

The role of FHL2 gene participates in cellular processes such as regulation of gene expression, cytoarchitecture, cell adhesion, cell survival, cell mobility and signal transduction [147] FHL2 was also detected in developing vasculature, including the aortic arch arteries, dorsal aorta, and intersegmental arteries and in all compartments of the heart [148].

4.4.3 Relationship between CpG sites of TRIM59 gene and Age

TRIM59 gene contains four sites that are CpG1, CpG2, CpG3 and CpG4. From the sites belonging to TRIM59 gene results shown in table (4.5), it appeared that all 4 CpG have significant linear relationship with age (all P-values < 0.05, 0.01). The fitting process using the simple regression model that illustrated in table (4.6) and figure (4.8) provided good correction coefficients.

In this study, the assay of Human tripartite motif containing 59 gene is located at Chromosome Chr3:160450365-160450349. This assay was designed to target Promoter ADS5738-FS2 Ensembl Gene ID ENSG00000213186 at distance transcription start site (TSS) -536 to -520. TRIM59 gene showed four CpG sites detected at which are: CpG1,

Chr3:160450349 CpG2, Chr3:160450355 CpG3, Chr3:160450362, CpG4, Chr3:160450365 Four CpG sites have appeared significant linear relationship with age (all P-values<0.05, 0.01) and identified the methylation status was highly correlated with age. Methylation level increased with age.

Table (4.5): Results of correlation between the age and CpG sites of TRIM59 gene

Age vs. Sites	Pearson Correlation
Age vs. CpG1	0.777**
Age vs. CpG2	0.769**
Age vs. CpG3	0.776**
Age vs. CpG4	0.749**
* Correlation is significant at the 0.05 level (2-tailed).	
** Correlation is significant at the 0.01 level (2-tailed)	

Table (4.6): Results of regression analysis of CpG sites of TRIM59 gene.

Sites	Regression equation	R-Sq(adj)
CpG1	$\text{CpG1} = 41.4 + 0.259 \text{ Age}$	50.3%
CpG2	$\text{CpG2} = 48.68 + 0.2591 \text{ Age}$	58.4%
CpG3	$\text{CpG3} = 42.63 + 0.2689 \text{ Age}$	59.2%
CpG4	$\text{CpG4} = 36.67 + 0.2894 \text{ Age}$	54.3%

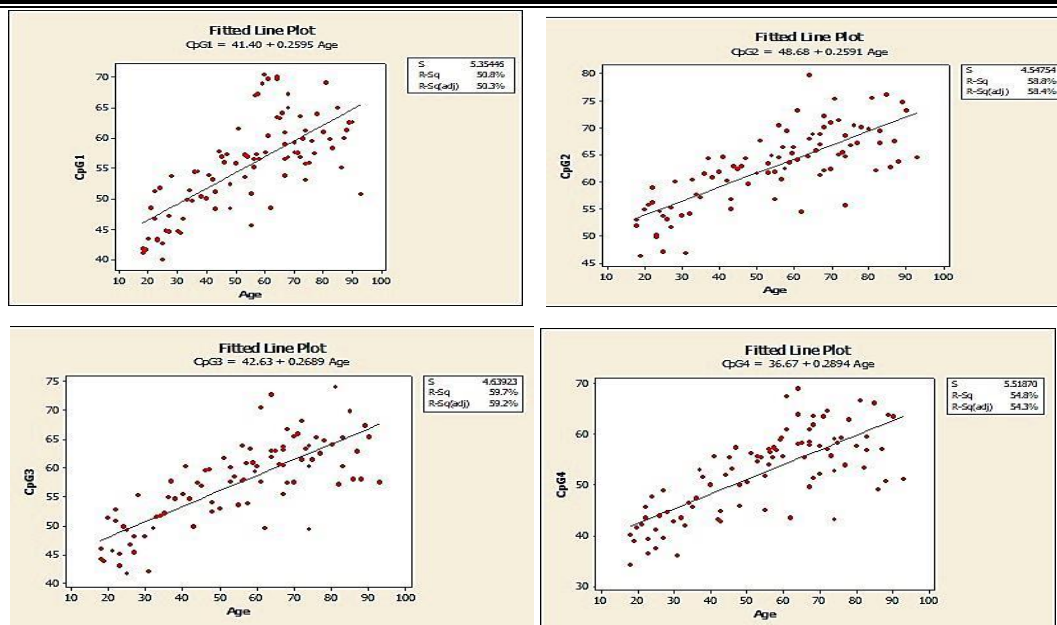


Figure (4.8): Regression line drawn on scatter diagram regarding each CpG site of the TRIM59 gene that hypermethylated with age.

This study compatible with previous study that showed a strong correlation with age and age associated hypermethylation at TRIM59 CpG islands [149]. Tripartite motif-containing 59 (TRIM59), a novel TRIM family member is related to many types of cancers. Zhou et al, reported that TRIM59 expression is markedly increased in may promote gastric carcinogenesis and gastric cancer by degradation of p53 and promoting ubiquitination [103]. Gene family implicated in a number of critical processes including, proliferation, immunity, transcriptional regulation, antiviral, neuro-development, cancer and cell differentiation. However, the function of most TRIM family members is poorly understood and was supposed only based on computational analysis from their RBCC (RING finger, B-box, Coiled-Coil) domains. The domain of RING (Really Interesting New Gene) is frequently involved in proteolysis acting as the ubiquitin-proteasome system and E3 ubiquitin ligases in the regulation of numerous cellular processes including cell cycle regulatory transcription factors, proteins, and signal transducers. Recent studies demonstrate that different TRIM-NHL proteins function as cofactor for the microRNA-induced silencing complex [102].

4.4.4 Relationship between CpG sites of MIR29B2CHG gene and Age:

MIR29B2CHG gene contains eight sites that are CpG1, CpG2, CpG3, CpG4, CpG5, CpG6, CpG7 and CpG8. From the results shown in table (4.7) and figure (4.10), most CpG sites belonging to MIR29B2CHG gene have appeared negative linear relationship with age but not satisfactory. The fitting process has provided not significant correction coefficients as shown in table (4.8) and figure (4.9).

The assay of MIR29B2CHG has two regions one of that is located at 3' downstream Chromosome Chr1:207802019-207801998, that was designed to be targeted 3' downstream ADS5740-FS2 Ensembl Transcript ID ENST00000637970 at distance transcription start site (TSS) +77078 to + 77099 from TSS. This region contains four sites which are: CpG1 Chr1:207801998, CpG2 Chr1:207802007, CpG3 Chr1:207802016, CpG4 Chr1:207802019 The second region has also four sites located at Intron 4, Chr1:207823845-207823822, and ADS5739-FS1 Ensembl Transcript ID: ENST00000637970 which are CpG5 Chr1:207823822, CpG6 Chr1:207823829, CpG7

Chr1:207823833 and CpG8 Chr1:207823845 at distance transcription start site (TSS) +55252 to +55275 from TSS.

Table (4.7): Correlation between the age and CpG sites of MIR29 B2CHG gene.

Sites	Pearson Correlation
Age vs. CpG1	- 0.360**
Age vs. CpG2	- 0.215*
Age vs. CpG3	- 0.209*
Age vs. CpG4	- 0.536**
Age vs. CpG5	- 0.104
Age vs. CpG6	- 0.224*
Age vs. CpG7	0.002
Age vs. CpG8	- 0.177
* Correlation is significant at the 0.05 level (2-tailed).	
** Correlation is significant at the 0.01 level (2-tailed)	

Table (4.8): Fitting simple linear regression for CpG sites of MIR29B2CHG gene

Sites	Regression equation	R-Sq(adj)
CpG1	CpG1 = 17.64 - 0.1069 Age	10.1%
CpG2	CpG2 = 9.246 - 0.04547 Age	2.2%
CpG3	CpG3 = 22.12 - 0.1547 Age	22.4%
CpG4	CpG4 = 7.735 - 0.03245 Age	1.1%
CpG5	CpG5 = 91.28 - 0.01573 Age	0.0%
CpG6	CpG6 = 75.83 - 0.02151 Age	1.8%
CpG7	CpG7 = 93.39 + 0.000941 Age	0.0%
CpG8	CpG8 = 92.99 - 0.03078 Age	1.7%

The results shown in table (4.7) and figure (4.9), MIR29B2CHG gene have appeared negative linear relationship with age. The fitting process has no correction coefficients as shown in table (4.8), that because chose sites different from previous studies.

DNA-based approaches for age prediction in blood showed a correlation between the chronological age and C1orf132 (chromosome1 open reading frame 132) was weak when compared with other genes in the study [150].

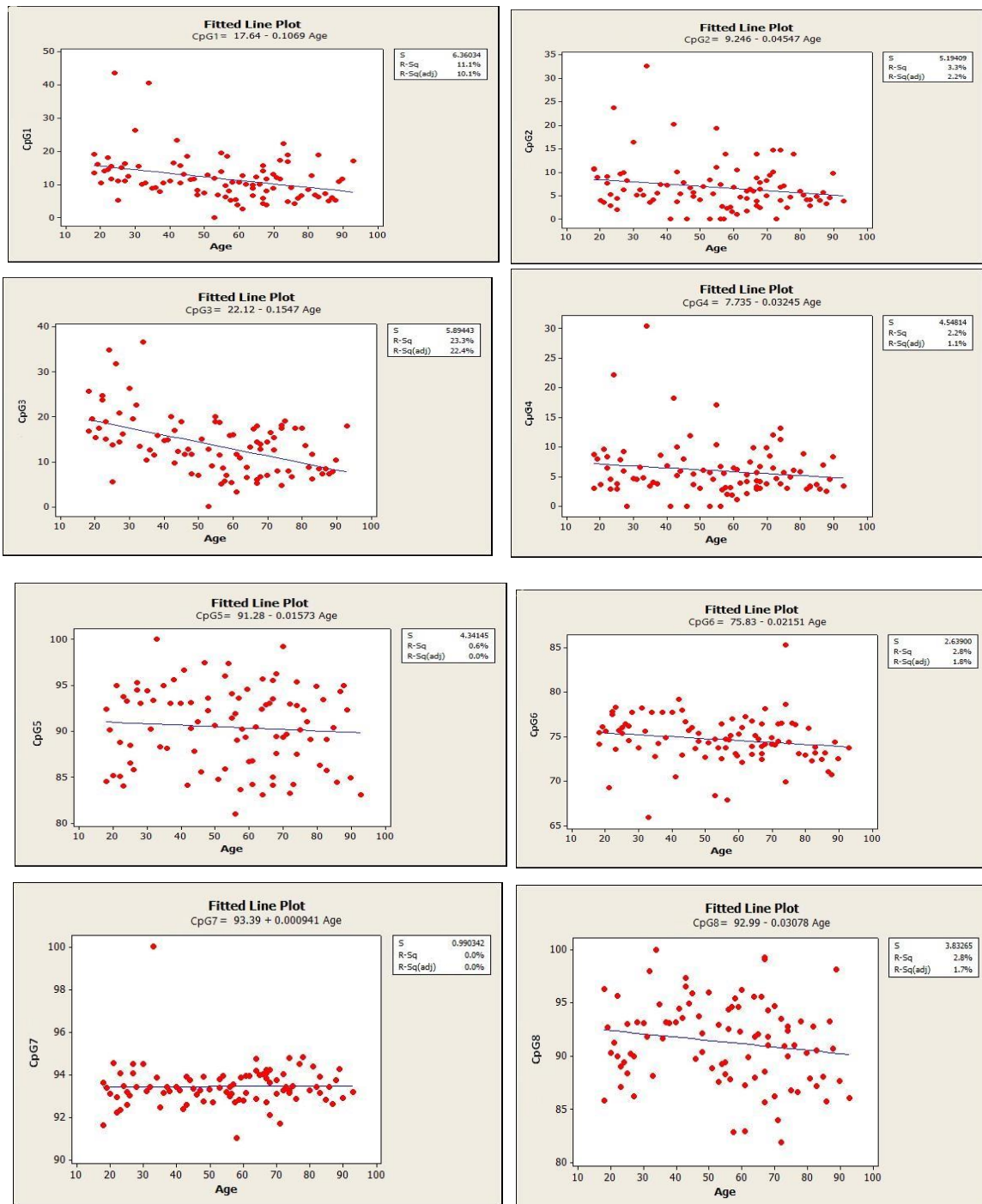


Figure (4.9): Regression line drawn on scatter diagram regarding each CpG site of the MIR29B2CHG gene that hypomethylated with age.

Group of scientists investigated CpG loci in Koreans populations of the ELOVL2, C1orf132, FHL2, and TRIM59 genes showed among the 10 most strongly correlated CpG sites, 4 CpG sites are located in the FHL2 gene, 3 CpG sites are each located in the TRIM59 and ELOVL2 genes. On the other hand, the 10 most correlated CpG sites in the Polish population include three CpG sites in the C1orf132 gene four CpG sites in the ELOVL2 gene, one CpG in the TRIM59 gene and two CpG sites in the FHL2 gene. The age correlation of the C1orf132 gene was stronger in the Polish and the age correlation of DNA methylation in the FHL2 gene was stronger in Koreans the CpG sites that showed the strongest associations with age in each of the C1orf132, KLF14, TRIM59, and FHL2 genes were the same in the Polish and Korean populations. The five CpG sites (C1 in C1orf132, C7 in ELOVL2, C7 in TRIM59, C1 in KLF14, and C2 in FHL2) considered as informative age predictors in blood [149].

4.4.5 Relationship between CpG Sites of KLF14 Gene and Age

KLF14 gene contains four sites that are CpG1, CpG2, CpG3 and CpG4. From the KLF14 gene, the CpG sites belonging to KLF14 gene 11 results shown in table (4.9) and figure (4.9). The CpG sites showed a significant linear relationship with age (all P-values < 0.05, 0.01). The fitting process using the simple regression model illustrated in table (4.10) and figure (4.10) which provided good correction coefficients.

Table (4.9): Results of correlation between the age and CpG sites of KLF14 gene.

Age vs. Sites	Pearson Correlation
Age vs. CpG1	0.510**
Age vs. CpG2	0.728**
Age vs. CpG3	0.685**
Age vs. CpG4	0.724**
* Correlation is significant at the 0.05 level (2-tailed).	
** Correlation is significant at the 0.01 level (2-tailed)	

Table (4.10): Results of regression analysis of CpG sites of KLF14 gene.

Sites	Regression equation	R-Sq(adj)
CpG1	$\text{CpG1} = 2.164 + 0.04931 \text{ Age}$	28.4%
CpG2	$\text{CpG2} = 0.687 + 0.07646 \text{ Age}$	53.1%
CpG3	$\text{CpG3} = - 0.904 + 0.06250 \text{ Age}$	41.9%
CpG4	$\text{CpG4} = - 1.174 + 0.1015 \text{ Age}$	47.8%

The assay of Human Kruppel-like factor 14 ADS5128-FS2 is located at Chromosome Chr7:130734413-130734373 of Promoter Ensembl Transcript ID:ENST00000583337. This assay was designed for the analysis of DNA methylation by pyrosequencing at distance transcription start site (TSS) -352 to -312 from TSS. This gene contains 4 sites which are: CpG1 (Chr7: 130734373), CpG2 (Chr7: 130734376), CpG3 (Chr7: 130734399) and CpG4 (Chr7:130734413). Most of the CpG markers described above show linear correlations with chronological age and CpG sites of KLF14 gene appeared), showed 11 significant linear relationship with age. The results in table (4.9) and figure (4. that all 4 CpG sites belonging to KLF14 gene have appeared significant linear relationship with age (all P-values<0.05, 0.01) and the simple regression model showed median 0).1 correction coefficients, which illustrated in table (4.10) and figure (4.

This result compatible with the Cho study that appeared DNA methylation at four CpG positions of the KLF14 gene was median correlated with chronological age in Koreans. They could only explain age related variance by 20.7 to 36.8% that helped of developing age prediction model for age estimation [150]. Expressed transcription factor KLF14 (encoding Kruppel-like factor 14) are strongly associated with both type 2 diabetes (T2D) and high-density lipoprotein (HDL) cholesterol levels in large-scale genome-wide association studies (GWAS) suggests that KLF14 is a master regulator of gene expression in adipose tissue [151].

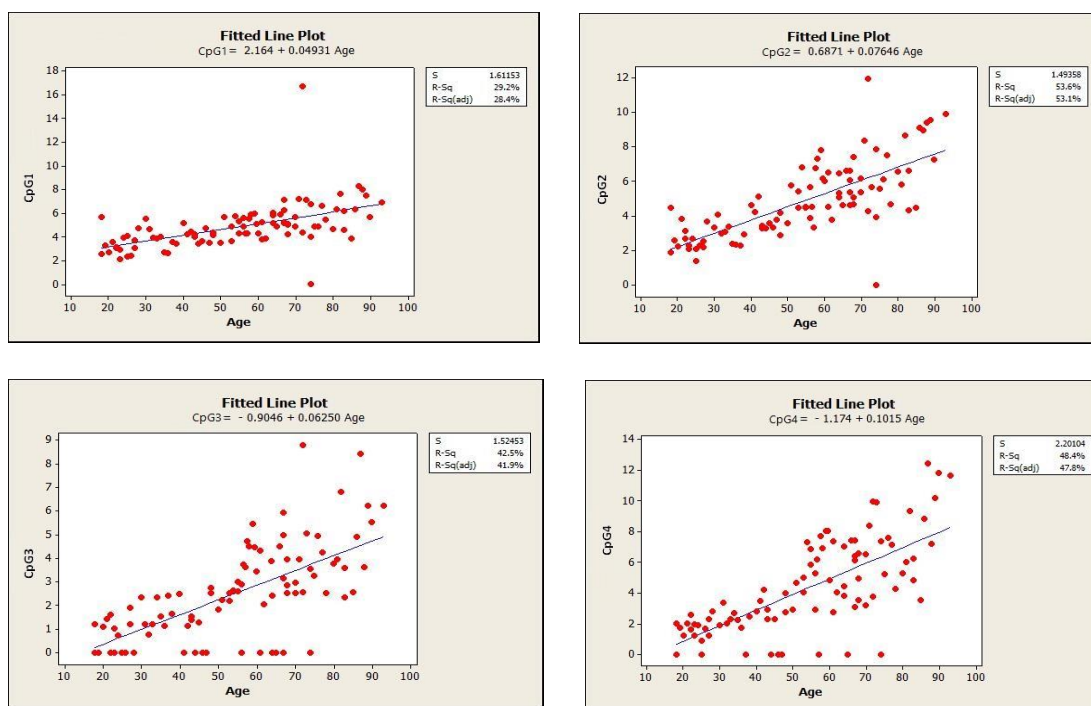


Figure (4.10): Regression line of each CpG site of KLF14 gene hypermethylated with age.

In this study identified single nucleotide polymorphism rs7808283 G>A which caused This SNP mutation not previously the loss of one CpG site of Human KLF14 gene. recorded in Iraq population.

The most common type of such variation is called a single-nucleotide polymorphism, or SNP. SNPs are highly abundant, stable, and distributed throughout the genome. These variations are associated with diversity in the population, individuality, susceptibility to diseases, and individual response to medicine [152]. SNPs can be observed between individuals in a population may influence promoter activity or DNA and pre-mRNA conformation and play a direct or indirect role in phenotypic expression [153].

The genotypic and allelic frequencies of KLF14 rs4731702 SNP between the Mulao and Han populations, whereas difference in the genotypic and allelic frequencies of KLF14 rs4731702 SNP was observed between Han males and females. The association of KLF14 rs4731702 SNP and serum lipid levels is different between the two ethnic groups. The differences in the association of KLF14 rs4731702 SNP and serum lipid

levels between the two ethnic groups might partly result from the differences in gene-environmental interactions [154]. According to the HapMap data, the minor allele frequency of the SNP was 36.7% in Chinese, 30.0% in Japanese, 23.3% in Yoruba, and 45.0% in European population [155].

4.4.6 The Correlation and Regression Model for Five Genes

This section implemented some statistical analysis to investigate the relationship between CpG sites for all genes (all values for CpG sites methylation% of pyrosequencing supplementary data SU2) and the chronological age under method showed in Appendixes First, we checked the correlation between each CpG belonged to gene and the study. chronological age for 92 samples. At the end, we aimed to increase the accuracy age estimation based on DNA methylation markers for the taken samples. For comparison purposes, we first adopted the linear regression model to check the fitting degree of each CpG side that belongs to certain gene using correction coefficient (R^2). Then, we summarize just the sites that give good fitting and correlation to insert in multi regression model in order to assess the prediction accuracy in age.

4.5 Multivariate Regression Model:

In order to obtain the accurate age prediction that represents our aim, we excluded all CpG sites provided not significant indicators according to the correlation coefficients and correction coefficients that have been described in previous section. All rest CpG sites that give good results inserted in a multi regression model in order to find accurate age estimations.

Case (I): Below the multiple regression model includes 15 best age predictors that incorporate in the predicted age.

Age = 12 - 0.239 CpG1 + 0.899 CpG2 + 1.20 CpG3 - 1.17 CpG4 of TRIM59 - 0.521 CpG1 + 0.416 CpG2 OF FHL2 + 0.308 CpG1 - 0.226 CpG2 - 0.055 CpG3 + 1.23 CpG4 - 0.219 CpG5- 0.101 CpG6 + 0.835 CpG7 + 0.711 CpG8 - 0.775 CpG9 OF ELOVL2. The results of applying this model was perfect all P-values were <0.05 and correction coefficient was very high (R^2 = 96.8%) as shown in tables (4.11) and (4.12). The results of general variance (ANOVA table of multi regression model) between the age and all CpG sites mentioned which show a significant difference between those CpG sites and

the age (P-value <0.01, 0.05). The mean absolute difference (MAD) between the predicted 1).1and the chronological age was 6.9262 years as shown in Table (4.12) and Figure (4. **Table (4.11):** Regression statistics of the age prediction model with the 15 best age predictors: CpG1, CpG2, CpG3, CpG4 OF TRIM59, CpG1, CpG2 OF FHL2, CpG1, CpG2, CpG3, CpG4, CpG5, CpG6, CpG7, CpG8 and CpG9 of ELOVL2.

Predictor	P-value
Constant	0.000
CpG1 of TRIM59	0.042
CpG2 of TRIM59	0.042
CpG3 of TRIM59	0.028
CpG4 of TRIM59	0.049
CpG1 of FHL2	0.047
CpG2 of FHL2	0.037
CpG1 of ELOVL2	0.032
CpG2 of ELOVL2	0.010
CpG3 of ELOVL2	0.009
CpG4 of ELOVL2	0.024
CpG5 of ELOVL2	0.034
CpG6 of ELOVL2	0.013
CpG7 of ELOVL2	0.025
CpG8 of ELOVL2	0.043
CpG9 of ELOVL2	0.017
S = 3.888 MAD= 6.9262 R-Sq(adj) = 96.8%	

Table (4.12): Variance analysis of multi regression model.

Source	DF	SS	MS	F	P-value
Regression	15	35286.4	2352.4	41.17	0.000
Residual Error	76	4342.7	57.1		
Total	91	39629.1			

Several methylation markers that supply independent information about chronological age have been particular in the human genome. These loci provide a good option for age prediction modeling. This study choose (ELOVL2, TRIM59, FHL2, KLF, MIR29B2CHG) genes and inserted all best CpG sites that gave good results in a multi regression model in order to determine accurate age estimation. The study assessed age-associated methylation from 15 CpG sites located in five genes that most highly associated with chronological age and therefore put in a model to predict the age of 92 blood samples. The resulting multivariate regression model was able to predict individuals age with a mean error of 6.92 years and all P-values were <0.05 and correction coefficient was very high ($R^2= 96.8\%$) that give good fitting and correlation in order to assess accuracy of age prediction.

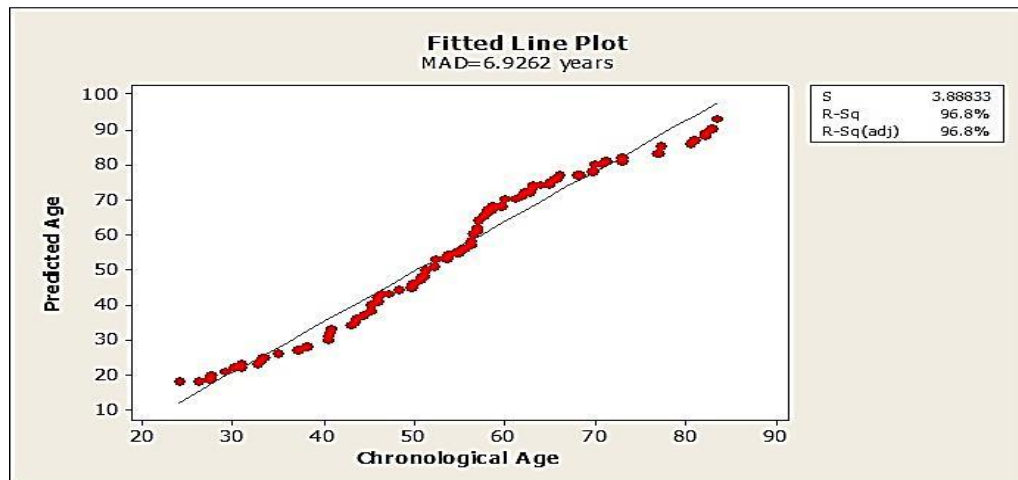


Figure (4.11): Chronological age versus predicted age for CpG sites selected with dentin samples with an MAD of 6.9262 years.

DNA methylation status of the ELOVL2 marker showed 27 CpG sites located in the ELOVL2 gene and they optimized an assay based on bisulfite conversion and pyrosequencing. Blood samples collected from individuals aged 2 to 75 years and examination of 303 allowed selection of the most informative site, explaining 83% of variation in age. Two CpG sites in ELOVL2 give linear regression model and enabled age prediction with $R^2 = 0.859$, prediction error = 6.85 and mean absolute deviation MAD =

5.03 years. [140]. The Zbiec-Piekarska reported second assay where they included different other CpG markers next to ELOVL2. In this assay ELOVL2, C1orf132, TRIM59, KLF14, and FHL2 appeared age predictions with a MAD of 3.9 years [149]. The Bekaerts study selected 4 age-associated genes (ASPA, PDE4C, ELOVL2, and EDARADD) and determined methylation levels for CpG site from 206 blood samples of both living and deceased individuals (age range: 0–91 years). The data used to compare prediction accuracy with both non-linear and linear regression models. A regression model in which the methylation levels of ELOVL2 were squared appeared the highest accuracy with a Mean Absolute Deviation (MAD) between predicted age and chronological age of 3.75 years and detected R^2 of 0.95. Samples was appeared no difference in accuracy obtained either from living and deceased individuals or between the 2 genders. In addition, 29 teeth were analyzed from different individuals (age range: 19–70 years) using the same set of markers resulting in a MAD of 4.86 years and detected R^2 of 0.74. Cross validation of the results obtained from blood samples showed the strongest and reproducibility of the assay. The set of four CpG DNA methylation markers is qualified of producing highly accurate age predictions for blood samples from living and deceased individuals [156]. Most of the CpG markers used in the age-association studies described above show linear correlations with chronological age.

Case (II): Below the multiple regression model which includes only the CpG sites of ELOVL2 gene that incorporate in accurate predicted age.

$$\text{Age} = -61.064 + 0.255 \text{ CpG1} - 0.080 \text{ CpG2} + 0.665 \text{ CpG3} + 0.663 \text{ CpG4} + 0.207 \text{ CpG5} - 0.288 \text{ CpG6} + 0.664 \text{ CpG7} + 0.497 \text{ CpG8} - 0.551 \text{ CpG9 of ELOVL2}.$$

The application of multiple regression model by choosing more strongest CpG sites comparing to the case (I). All P-values were <0.05 and correction coefficient was very high ($R^2 = 98.0\%$) as shown in table (4.13). The results in table (4.14) showed general variance (ANOVA table of multi regression model) between the age and all CpG of ELOVL2 which refer to exist a significant difference between those CpG sites and the age (P-value $<0.01, 0.05$). The mean absolute difference (MAD) between the predicted and 2). the chronological age was MAD= 2.8939 years as shown in Table (4.13) and Fig. (4.

Table (4.13): Regression statistics of the age prediction model with the 9 best age predictors of ELOVL2: CpG1, CpG2, CpG3, CpG4, CpG5, CpG6, CpG7, CpG8 and CpG9.

Predictor	P-value
Constant	0.000
CpG1	0.002
CpG2	0.000
CpG3	0.001
CpG4	0.020
CpG5	0.001
CpG6	0.018
CpG7	0.004
CpG8	0.008
CpG9	0.001
S = 3.0296 MAD= 2.8939 R-Sq(adj) = 98.0%	

Table (4.14): Results of variance analysis of multi regression model.

Source	DF	SS	MS	F	P-value
Regression	1	33890.9	33890.9	531.55	0.000
Residual Error	90	5738.2	63.8		
Total	91	39629.1			

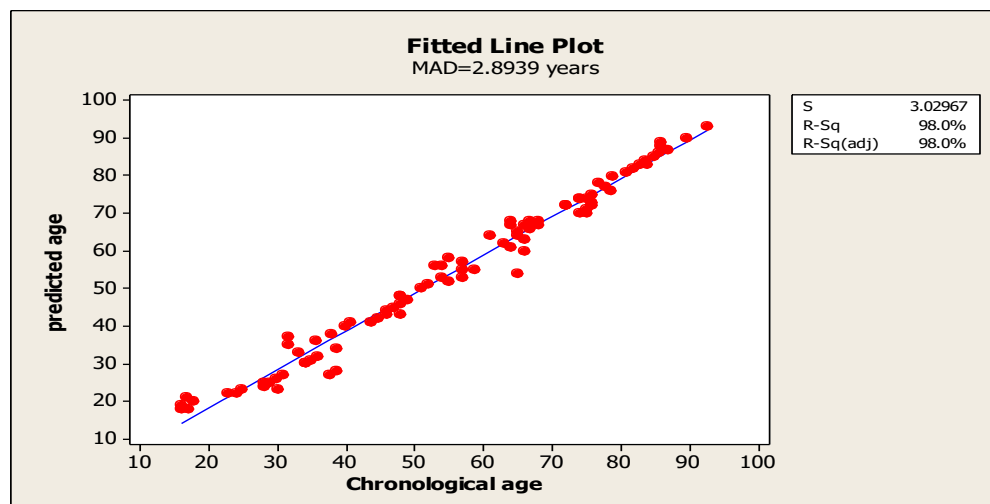


Figure (4.12): Chronological age versus predicted age for the CpGs sites of ELOVL2 gene with an MAD of 2.8939 years.

4.6 Relationship Between Methylation Values and Gender

This section presents the results of multistage variance analysis to check the relationship between the methylation values of five genes under study and the gender. Tables (4.15), (4.17), (4.19), (4.21) and (4.23) present the descriptive statistics between the gender and methylation of the genes: TRIM59, KLF4, FHL2, MIR29B2CHG and ELOVL2 respectively. The results of variance analysis suggest that there is no significant difference between the methylation values and the male or the female (P-values >0.05) for all genes as shown in tables (4.16), (4.18), (4.20), (4.22) and (4.24)

Table (4.15): Descriptive statistics of the CpG sites of TRIM59 gene classified according to the gender.

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval	
						for Mean	
						Lower Bound	Upper Bound
CpG1	Female	48	53.7178	7.60820	1.13416	51.4320	56.0035
	Male	44	55.5756	6.07152	.94821	53.6592	57.4920
	Total	92	54.6035	6.94136	.74851	53.1153	56.0917
CpG2	Female	48	61.9267	7.95640	1.18607	59.5363	64.3170
	Male	44	63.6512	6.43930	1.00565	61.6187	65.6837
	Total	92	62.7488	7.28235	.78528	61.1875	64.3102
CpG3	Female	48	56.3822	7.95728	1.18620	53.9916	58.7729
	Male	44	58.0634	6.89785	1.07726	55.8862	60.2406
	Total	92	57.1837	7.47534	.80609	55.5810	58.7864
CpG4	Female	48	50.9844	8.78824	1.31007	48.3442	53.6247
	Male	44	53.0049	7.44570	1.16282	50.6547	55.3550
	Total	92	51.9477	8.19137	.88330	50.1914	53.7039

Table (4.16): Results of variance analysis for difference between the CpG sites of
TRIM59 gene and gender

		Sum of Squares	df	Mean Square	F	P-value
Cp G1	Between Groups	74.048	1	74.048	1.547	.217
	Within Groups	4021.461	90	47.875		
	Total	4095.509	91			
Cp G2	Between Groups	63.804	1	63.804	1.206	.275
	Within Groups	4443.970	90	52.904		
	Total	4507.775	91			
Cp G3	Between Groups	60.636	1	60.636	1.086	.300
	Within Groups	4689.221	90	55.824		
	Total	4749.857	91			
Cp G4	Between Groups	87.576	1	87.576	1.310	.256
	Within Groups	5615.798	90	66.855		
	Total	5703.375	91			

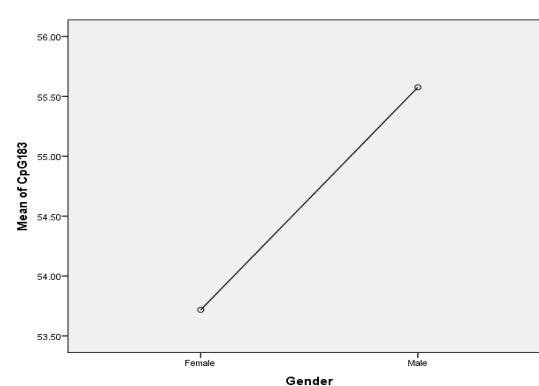
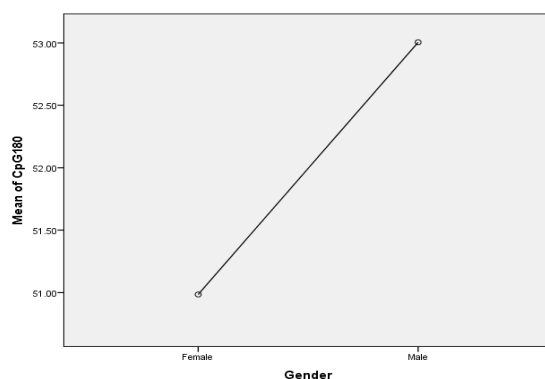
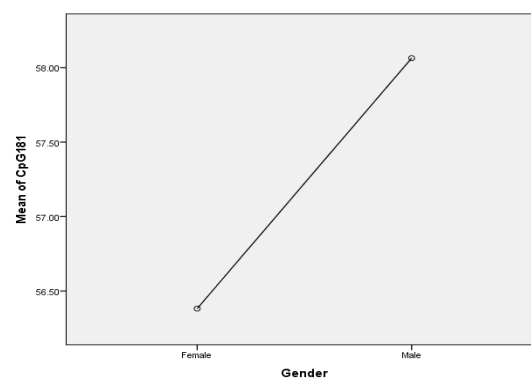
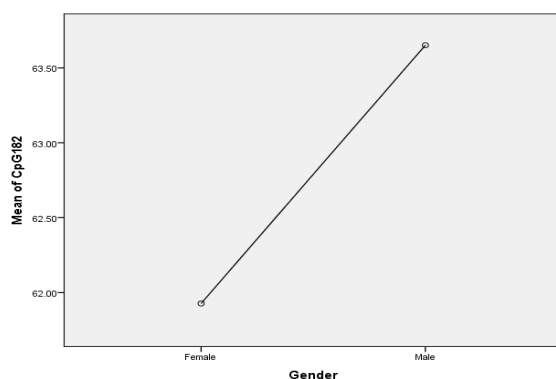


Figure (4.13): The CpG sites of TRIM59 gene and gender show that there is no significant difference between them.

Analysis of variance (ANOVA), linear regression model and the results of the multi stage variance analysis check the relationship between the methylation values of TRIM59 gene and gender. The results of variance analysis show that there is no significant difference between the methylation values of TRIM59 gene and the gender because of the fact that P-value for CpG1 0.217, CpG2 0.275, CpG3 0.300 and CpG4 0.256 this means no relationship.

Table (4.17): Descriptive statistics of the CpG sites of FHL2 gene classified according to the gender.

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean	
						Lower Bound	Upper Bound
CpG1	Female	48	7.4396	3.12197	.45062	6.5331	8.3461
	Male	44	7.8105	2.88583	.46814	6.8620	8.7591
	Total	92	7.6035	3.00812	.32437	6.9585	8.2484
CpG2	Female	48	11.7708	3.10120	.44762	10.8703	12.6713
	Male	44	12.6579	3.19794	.51877	11.6068	13.7090
	Total	92	12.1628	3.15688	.34042	11.4860	12.8396

Table (4.18): Results of variance analysis for difference between the CpG sites of FHL2 gene and gender.

		Sum of Squares	df	Mean Square	F	P-value
CpG1	Between Groups	2.918	1	2.918	.320	.573
	Within Groups	766.231	90	9.122		
	Total	769.149	91			

CpG2	Between Groups	16.689	1	16.689	1.68	.197
	Within Groups	830.412	90	9.886	8	
	Total	847.101	91			

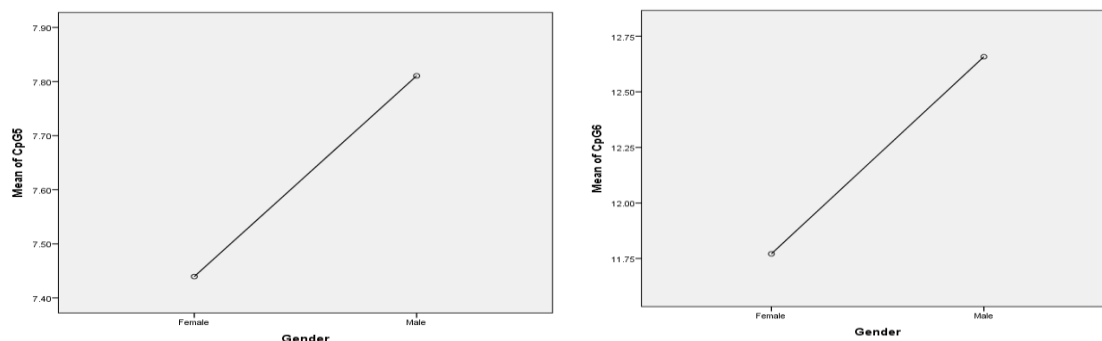


Figure (4.14): The CpG sites of FHL2gene and gender show that there is no significant difference between them.

Analysis of variance (ANOVA), linear regression model and the results of the multi stage variance analysis used to check the relationship between the methylation values of FHL2 gene and gender. The results of variance analysis show that there was no significant difference between the methylation values of FHL2 and the gender because of the P-value for CpG1 0.573 and CpG2 0.197 this means no relationship.

Table (4.19): Descriptive statistics of the CpG sites of MIR29B2CHG gene classified according to the gender.

		N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean	
						Lower Bound	Upper Bound
CpG1	Female	48	12.0875	7.01208	1.01211	10.0514	14.1236
	Male	44	12.1632	6.56310	1.06468	10.0059	14.3204
	Total	92	12.1209	6.77784	.73087	10.6678	13.5741
CpG2	Female	48	6.4235	4.97542	.71814	4.9788	7.8683
	Male	44	7.3776	5.67477	.92057	5.5124	9.2429
	Total	92	6.8451	5.28516	.56991	5.7120	7.9783
CpG3	Female	48	13.8204	6.40542	.92454	11.9605	15.6804
	Male	44	14.6003	6.98267	1.13274	12.3051	16.8954
	Total	92	14.1650	6.63796	.71579	12.7418	15.5882

CpG4	Female	48	6.1094	4.44597	.64172	4.8184	7.4004
	Male	44	6.4013	4.86593	.78936	4.8019	8.0007
	Total	92	6.2384	4.61060	.49717	5.2499	7.2269
CpG5	Female	48	90.3352	4.41153	.63675	89.0542	91.6162
	Male	44	90.8350	4.33638	.70345	89.4097	92.2603
	Total	92	90.5560	4.35991	.47014	89.6213	91.4908
CpG6	Female	48	74.4852	3.16949	.45748	73.5649	75.4055
	Male	44	75.0271	1.80186	.29230	74.4348	75.6194
	Total	92	74.7247	2.65353	.28614	74.1557	75.2936
CpG7	Female	48	93.4781	1.16263	.16781	93.1405	93.8157
	Male	44	93.3903	.76415	.12396	93.1391	93.6414
	Total	92	93.4393	1.00176	.10802	93.2245	93.6541
CpG8	Female	48	91.3252	3.67931	.53106	90.2568	92.3936
	Male	44	91.3847	3.77062	.61167	90.1454	92.6241
	Total	92	91.3515	3.69798	.39876	90.5587	92.1444

Table (4.20): Results of variance analysis for difference between the CpG sites of MIR29B2CHG gene and gender

;p.-10		Sum of Squares	df	Mean Square	F	P-value
CpG1	Between Groups	.121	1	.121	.003	.959
	Within Groups	3904.701	90	46.485		
	Total	3904.822	91			
CpG2	Between Groups	19.307	1	19.307	.689	.409
	Within Groups	2354.987	90	28.036		
	Total	2374.294	91			
CpG3	Between Groups	12.899	1	12.899	.290	.591
	Within Groups	3732.414	90	44.433		
	Total	3745.312	91			
CpG4	Between Groups	1.808	1	1.808	.084	.773
	Within Groups	1805.091	90	21.489		
	Total	1806.899	91			
CpG5	Between Groups	5.298	1	5.298	.276	.600
	Within Groups	1610.452	90	19.172		
	Total	1615.750	91			
CpG6	Between Groups	6.228	1	6.228	.883	.350
	Within Groups	592.275	90	7.051		
	Total	598.503	91			
CpG7	Between Groups	.164	1	.164	.162	.689
	Within Groups	85.136	90	1.014		
	Total	85.300	91			
CpG8	Between Groups	.075	1	.075	.005	.941
	Within Groups	1162.303	90	13.837		
	Total	1162.378	91			

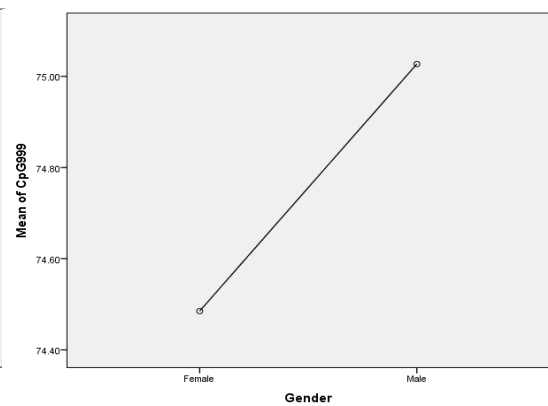
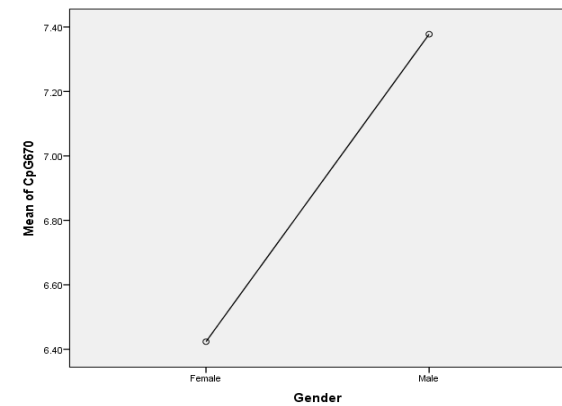
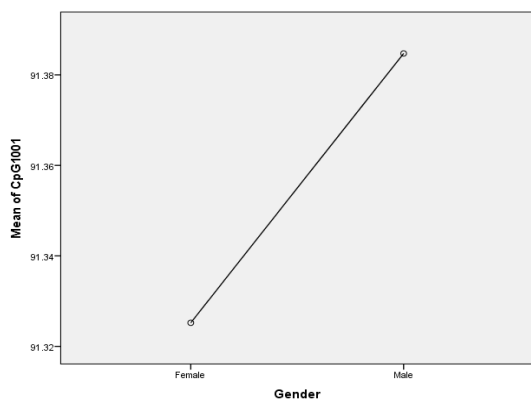
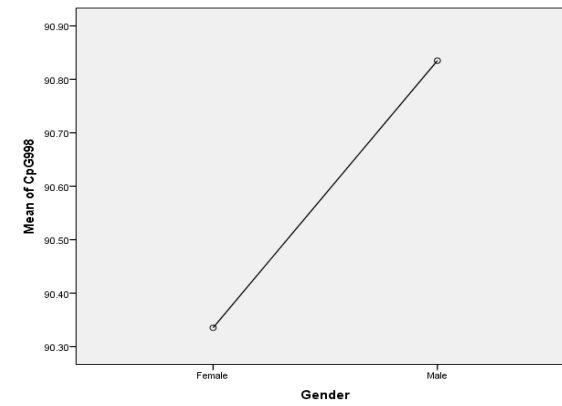
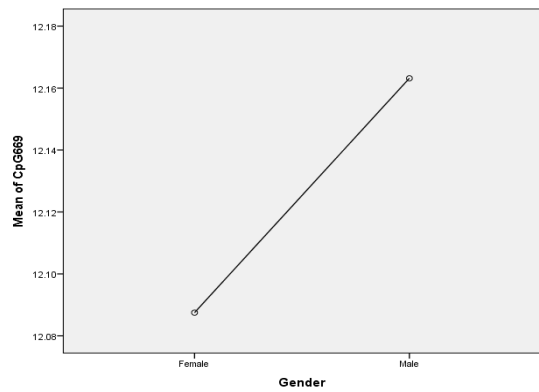
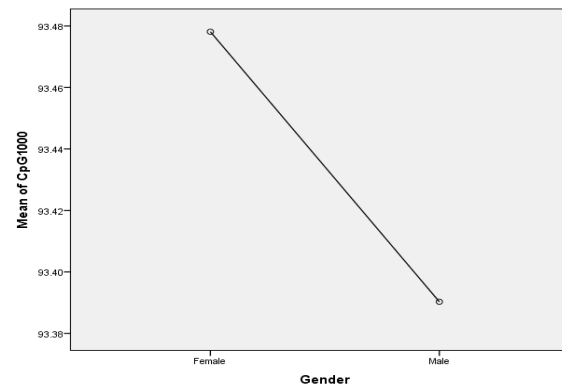
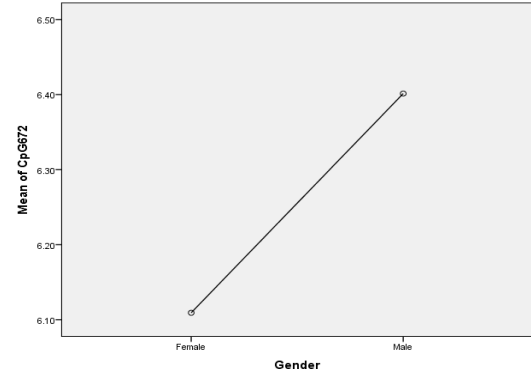
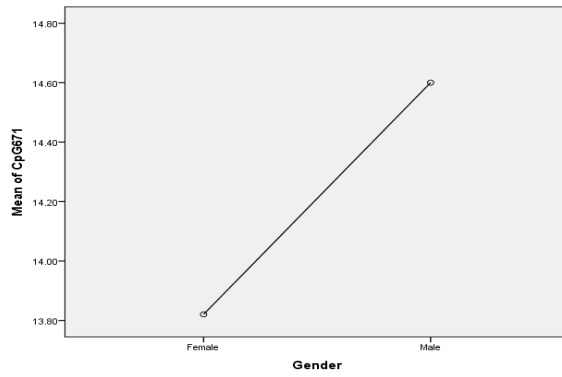


Figure (4.15): The CpG sites of MIR29B2CHG gene and gender show that there is no significant difference between them.

The linear regression model, analysis of variance (ANOVA) and the results of the multistage variance analysis included checking the relationship between the methylation values of MIR29B2CHG gene and gender. The results of variance analysis appeared that there is no significant difference between the methylation values of MIR29B2CHG and the gender because of the P-value for CpG1 0.959, CpG2 0.409, CpG3 0.591, CpG4 0.773, CpG5 0.600, CpG6 0.350, CpG7.689 and CpG8 0.941 this means no relationship.

Table (4.21): Descriptive statistics of the CpG sites of ELOVL2 gene classified according to the gender

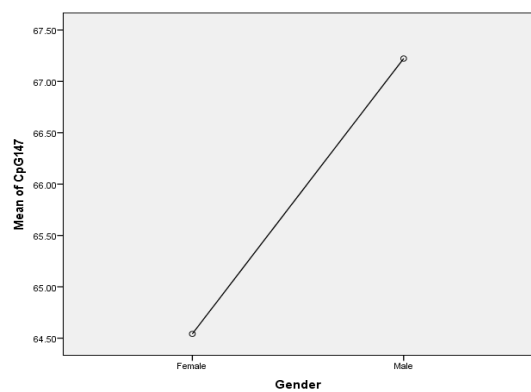
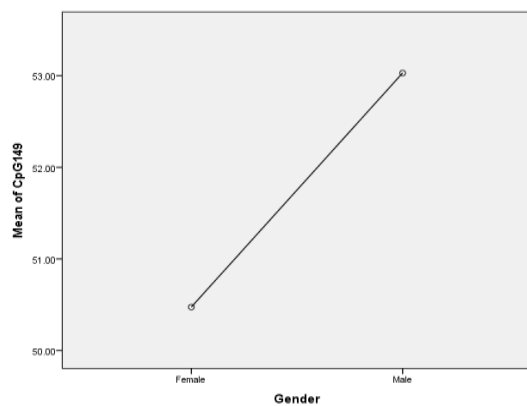
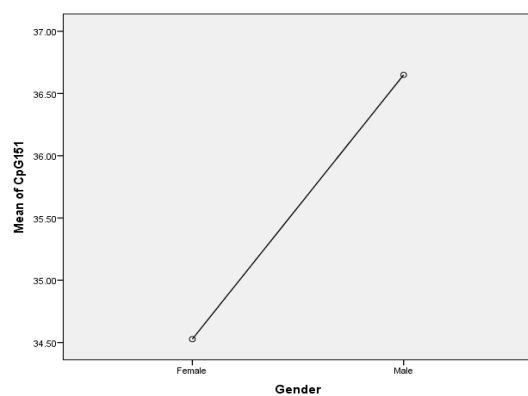
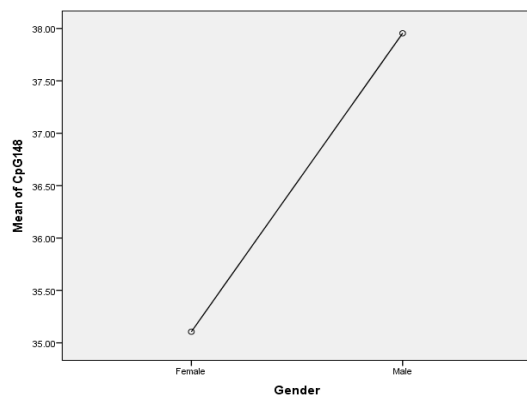
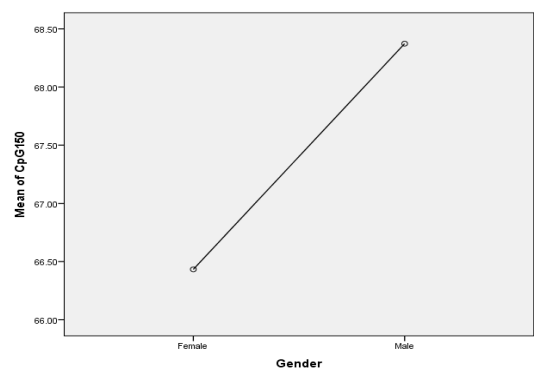
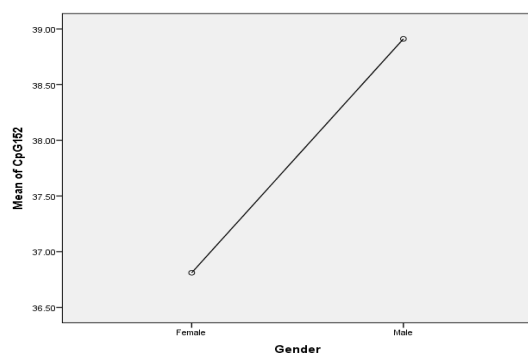
Sites	Gender	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean	
						Lower Bound	Upper Bound
CpG1	Female	48	36.8104	9.99992	1.44336	33.9067	39.7141
	Male	44	38.9114	9.98509	1.50531	35.8756	41.9471
	Total	92	37.8152	9.99365	1.04191	35.7456	39.8848
CpG2	Female	48	34.5271	9.99553	1.44273	31.6247	37.4295
	Male	44	36.6500	10.14842	1.52993	33.5646	39.7354
	Total	92	35.5424	10.07000	1.04987	33.4570	37.6278
CpG3	Female	48	66.4333	10.11187	1.45952	63.4972	69.3695
	Male	44	68.3727	10.67650	1.60954	65.1268	71.6187
	Total	92	67.3609	10.37408	1.08157	65.2125	69.5093
CpG4	Female	48	50.4750	10.95477	1.58119	47.2941	53.6559
	Male	44	53.0295	11.51257	1.73559	49.5294	56.5297
	Total	92	51.6967	11.23639	1.17147	49.3697	54.0237
CpG5	Female	48	35.1063	10.32900	1.49086	32.1070	38.1055
	Male	44	37.9545	11.32526	1.70735	34.5114	41.3977
	Total	92	36.4685	10.85157	1.13135	34.2212	38.7158
CpG6	Female	48	64.5417	11.92469	1.72118	61.0791	68.0042
	Male	44	67.2227	12.14737	1.83128	63.5296	70.9159
	Total	92	65.8239	12.04084	1.25534	63.3303	68.3175
CpG7	Female	48	75.8938	10.98743	1.58590	72.7033	79.0842
	Male	44	77.9750	10.55987	1.59196	74.7645	81.1855
	Total	92	76.8891	10.77666	1.12354	74.6573	79.1209

CpG8	Female	48	32.7021	7.31011	1.05512	30.5794	34.8247
	Male	44	35.2932	8.76519	1.32140	32.6283	37.9580
	Total	92	33.9413	8.09919	.84440	32.2640	35.6186
CpG9	Female	48	47.3604	10.96503	1.58267	44.1765	50.5443
	Male	44	50.3591	11.95317	1.80201	46.7250	53.9932
	Total	92	48.7946	11.48391	1.19728	46.4163	51.1728

Table (4.22): Results of variance analysis for difference between CpG sites of ELOVL2 gene and gender.

Sites	Sources	Sum of Squares	df	Mean Square	F	P-value
CpG1	Between Groups	101.330	1	101.330	1.015	.316
	Within Groups	8987.109	90	99.857		
	Total	9088.439	91			
CpG2	Between Groups	103.460	1	103.460	1.020	.315
	Within Groups	9124.385	90	101.382		
	Total	9227.845	91			
CpG3	Between Groups	86.345	1	86.345	.801	.373
	Within Groups	9707.214	90	107.858		
	Total	9793.559	91			
CpG4	Between Groups	149.807	1	149.807	1.189	.278
	Within Groups	11339.522	90	125.995		
	Total	11489.329	91			
CpG5	Between Groups	186.241	1	186.241	1.592	.210
	Within Groups	10529.597	90	116.996		
	Total	10715.839	91			
CpG6	Between Groups	165.013	1	165.013	1.140	.289
	Within Groups	13028.334	90	144.759		
	Total	13193.347	91			
CpG7	Between Groups	99.439	1	99.439	.855	.358
	Within Groups	10468.971	90	116.322		
	Total	10568.409	91			

CpG8	Between Groups	154.125	1	154.125	2.385	.126
	Within Groups	5815.198	90	64.613		
	Total	5969.323	91			
CpG9	Between Groups	206.426	1	206.426	1.575	.213
	Within Groups	11794.661	90	131.052		
	Total	12001.087	91			



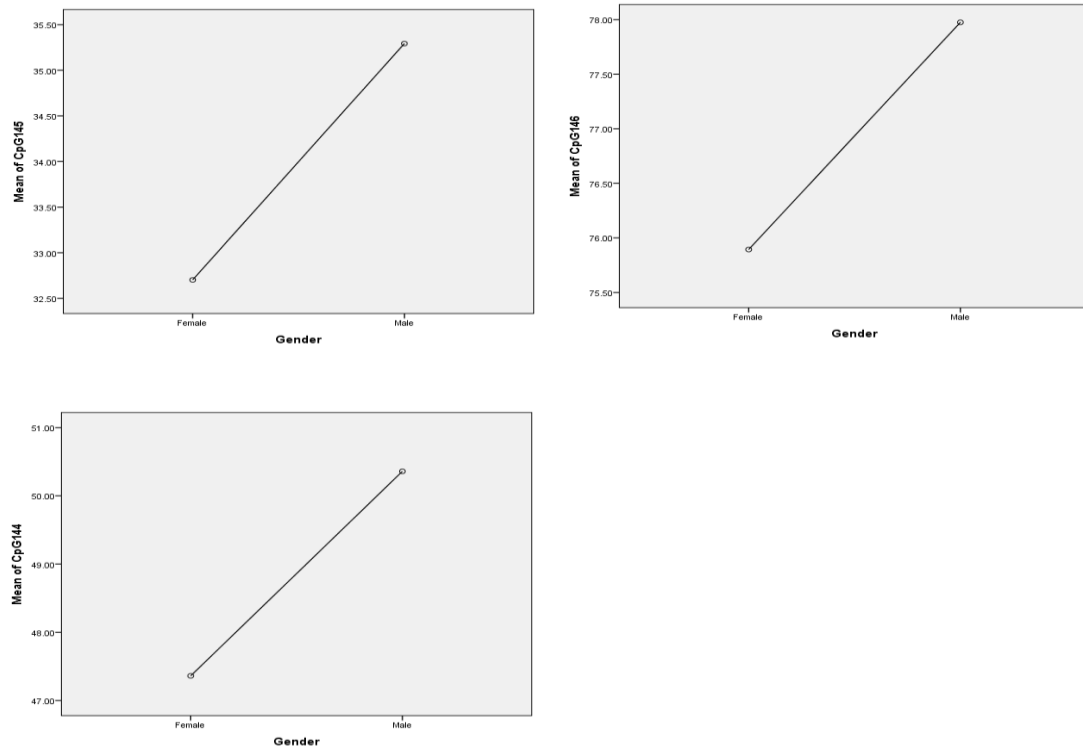


Figure (4.16): The CpG sites of ELOVL2 gene and gender appeared that there is no significant difference between them.

The linear regression model, analysis of variance (ANOVA) and the results of the multistage variance analysis check the relationship between the methylation values of ELOVL2 gene and gender. The results of variance analysis appeared that there is no significant difference between the methylation values of ELOVL2 and the gender because of the P-value for CpG1 0.316, CpG2 0.315, CpG3 0.373, CpG4 0.278, CpG5 0.210, CpG6 0.289, CpG7 0.358, CpG8 0.126 and CpG9 0.213 this means no relationship.

Table (4.23): Descriptive statistics of the CpG sites of KLF14 gene classified according to the gender.

		N	Mean	Std. Dev.	Std. Error	95 % Confidence Interval		Minim	Maxi
						Lower Bound	Upper Bound		

GpC1	Female	48	4.6250	1.46904	.21204	4.1984	5.0516	.00	8.30
	Male	44	5.1045	2.27422	.34285	4.4131	5.7960	2.10	16.70
	Total	92	4.8543	1.90173	.19827	4.4605	5.2482	.00	16.70
GpC2	Female	48	4.6333	2.04963	.29584	4.0382	5.2285	.00	9.90
	Male	44	5.1136	2.30829	.34799	4.4119	5.8154	2.10	11.90
	Total	92	4.8630	2.17845	.22712	4.4119	5.3142	.00	11.90
GpC3	Female	48	2.3042	2.04876	.29571	1.7093	2.8991	.00	8.40
	Male	44	2.7091	1.94731	.29357	2.1171	3.3011	.00	8.80
	Total	92	2.4978	2.00027	.20854	2.0836	2.9121	.00	8.80
GpC4	Female	48	4.1604	3.13937	.45313	3.2488	5.0720	.00	12.50
	Male	44	4.5795	2.97349	.44827	3.6755	5.4836	.00	11.90
	Total	92	4.3609	3.05164	.31816	3.7289	4.9928	.00	12.50

Table (4.24): Results of variance analysis for difference between the CpG sites of KLF14 gene and gender

Sites	Sources	Sum of Squares	df	Mean Square	F	P-value
GpC1	Between Groups	5.279	1	5.279	1.467	.229
	Within Groups	323.829	90	3.598		
	Total	329.108	91			
GpC2	Between Groups	5.296	1	5.296	1.117	.293
	Within Groups	426.558	90	4.740		
	Total	431.854	91			
CpG3	Between Groups	3.764	1	3.764	.940	.335
	Within Groups	360.336	90	4.004		
	Total	364.100	91			
CpG4	Between Groups	4.033	1	4.033	.430	.513
	Within Groups	843.406	90	9.371		
	Total	847.439	91			

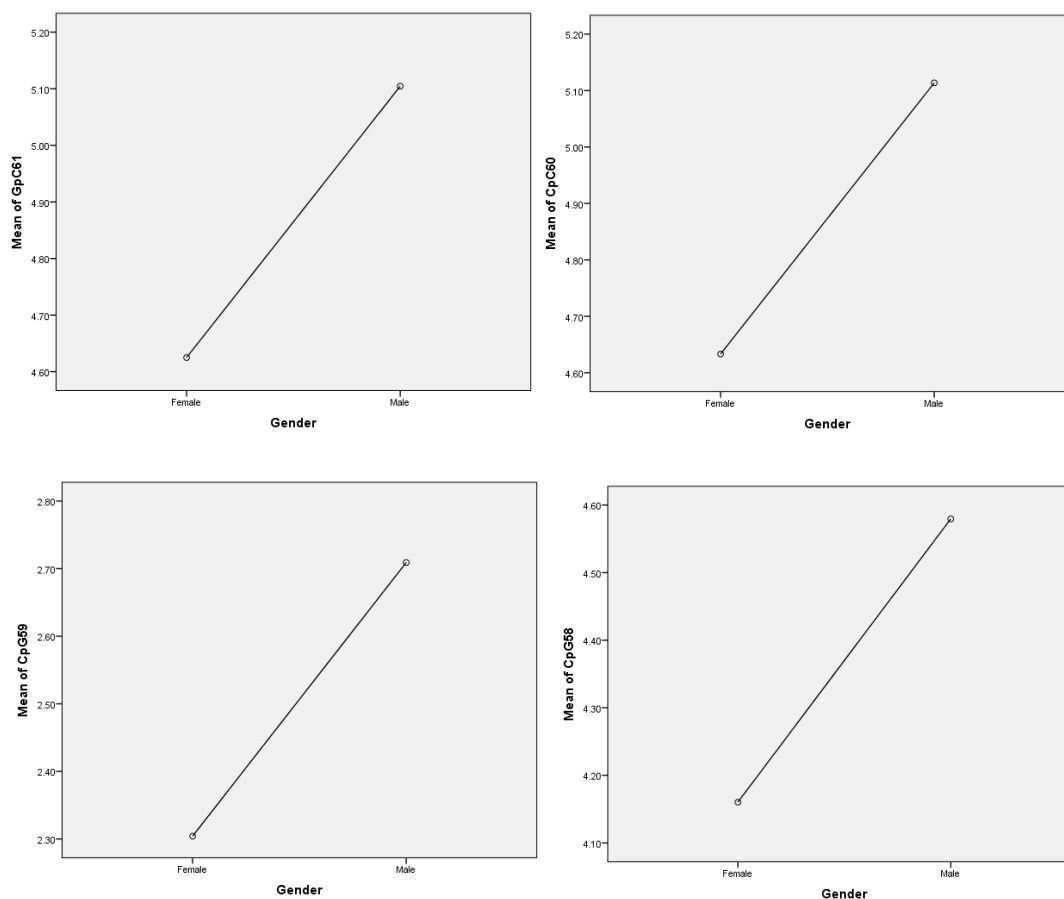


Figure (4.17): The CpG sites of KLF14 gene and gender appeared that there is no significant difference between them.

To check the relationship between the methylation values of KLF14 gene and gender used linear regression model, analysis of variance (ANOVA) and the results of the multi

stage variance analysis. The results of variance analysis appeared that there is no significant difference between the methylation values of KLF14 gene and the gender because of the P-value for CpG1.229, CpG2 .293, CpG3.335 and CpG4.513 this means no relationship.

The DNA methylation level compared between women and men using analysis of variance (ANOVA) and linear regression model and the results of the multi stage variance analysis to determine the extent of relationship between the methylation values of (TRIM59, KLF14, FHL2, MIR29B2CHG and ELOVL2) genes under study and the

gender. Tables (4.15), (4.17), (4.19), (4.21) and (4.23) present the descriptive statistics with respect to the gender and methylation of the genes: (TRIM59 KLF4, FHL2, MIR29B2CHG and ELOVL2). The results of variance analysis suggest that there is no significant difference between the methylation values and the male and the female (P-values >0.05) for all genes as shown in tables (4.16), (4.18), (4.20), (4.22) and (4.24), and no difference appeared as shown of figures (4.13), (4.14), (4.15), (4.16), (4.17). The result of this study did not detect significant differences in methylation levels between women and men of any sites of all genes, with these results can understand that gender does not take part a significant role in the age associated methylation.

The study methylation levels of the genes NPTX2, TRIM58, GRIA2 and KCNQ1DN, and detection no significant differences in methylation levels between women and men for any CpG marker and conclude that gender does not play a big role in the age-associated methylation [157]. Bekaert investigated the relationship between gender and age-associated methylation selected 4 age associated genes (ASPA, PDE4C, ELOVL2, and EDARADD) resulting no significant differences or correlation between methylation and gender were observed for any of the selected CpG sites between men and women. In addition, the MAD between predicted and chronological age was not significantly different (t-test P-value > 0.8) between females (MAD > 3.95 years, r > 0.97) and males of CpG methylation reported at specific [156]. The degree (MAD > 3.53 years, r > 0.98) with a number of phenotypes namely, age show association sites in an Arab population and gender but they chose some genes different from that of this study [158].

Chapter Five

Conclusions and recommendations

5.1. Conclusion

- 1- DNA methylation is actually considering promising age predictive biomarker in the Iraq population.
- 2- Twenty seven methylated CpG sites detected of ELOVL2, TRIM59, FHL2, KLF14, and MIR29B2CHG genes.
- 3- The fifteen CpG markers of five genes (ELOVL2, TRIM59, and FHL2) showed linear correlations with chronological age estimation with a mean error of ± 6.92 years and correction coefficient was ($R^2 = 96.8\%$).
- 4- The nine CpG sites of ELOVL2 gene have appeared strong linear relationship with actual age. The Mean Absolute Deviation (MAD) between predicted and real chronological age was ± 2.893 years.
- 5- Detection of novel single nucleotide polymorphism rs7808283 G>A associated with the loss of one CpG site of human KLF14 gene.
- 6- There was no significant difference between the methylation values and the male and the female (P-values >0.05) for all genes.
- 7- Pyrosequencing method considered as a golden method for DNA methylation level prediction and give further results for age estimation.

5.2. Recommendation

- 1- ELOVL2 can use as more significant biomarker for age estimation in forensic field and must remember that the other biomarkers of this study also appeared significant relationship with age.
- 2- DNA methylation levels of more multiple biomarker can be studying and from various tissues for investigation in relation of them with ageing.
- 3- DNA methylation changes with ageing that can use with medical field to finding solutions for ageing and age associated diseases by controlling of DNA methylation level.

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الخلاصة

تقدير العمر مفيد لتقليل عدد الاشخاص المشتبهين بسبب امكانية تجهيز بعض المميزات العامة لاجل تحديد المظهر. واسمات فوق الجينية تعد طريقه واعدة في تقدير العمر. مثيلة الدنا لها دور مهم جدا في تجهيز معلومات شخصيه لتقدير العمر الزمني. افادت الدراسات الحديثه لكون المحتوى المثيلي لمواقع متعدده في جينوم الانسان لها علاقه وثيقه مع العمر. لذلك المحتوى المثيلي في جينات خاصة يجهز مفاتيح الحلول في التحقيقات الجنائيه مثل تقدير العمر. في هذه الدراسه تم جمع 120 عينة دم من اشخاص أصحاء في المجتمع العربي العراقي معدل اعمارهم بين 18-93 سنة. عينة جينوم الدم تم تحويلها باستخدام املاح البايوسولفيت وبعدها استخدمت تقنيه تفاعل البلمرة المتسلسله لتضخيم الجينات وبعدها تم استخدام طريقه البايروسكونس لتحديد نسبه المحتوى المثيلي. النتائج تضمنت 92 عينة من اصل 120 وتم الحصول على 27 موقع CpG يحتوي عل المثل حيث حددت في الجينات التالية, ELOVL2, TRIM59, FHL2, KLF14 and MIR29B2CH التحليل الاحصائي استخدم تحليل الانحدار متعدد المتغيرات لتحديد نسبه معامل الارتباط ونسبة الخطأ بين مستوى المثل والعمر. خمس عشر موقع CpG من الجينات ELOVL2, TRIM59, FHL2 اظهرت علاقة خطية مع العمر الزمني تمكنا من خلالها تقدير العمر للاشخاص بفارق 6.92 سنة عن العمر الحقيقي ونسبة الارتباط كانت % 96.8 لاكثر ايجابية احصائيا تم اختيار فقط تسعة مواقع لها ارتباط بين العمر والمحتوى المثيلي في جين ELOVL2 والتي تتضمن ارتباط قوي وتتطابق جيد ونسبه الارتباط 98% مع محتوى المثل. نموذج تقدير العمر اعتمد على موديل تحليل متعدد المتغيرات لتحديد دقة التقدير. المواقع التسعة في جين ELOVL2 اظهرت علاقة خطية قوية مع العمر $P\text{-values} < 0.05, 0.01$. الفرق المعنوي بين العمر التقديري و العمر الزمني الحقيقي كان ± 2.893 سنة. بشكل ملحوظ, مستوى المثل في جين ELOVL2 اقترح وبقوة كمؤشر لتقدير العمر في هذا العمل. وفي هذه الدراسة تم تحديد متعدد الاشكال لنيوكلوئيد مفرد جديدة في المجتمع العراقي مرتبطة مع خسارة موقع واحد CpG في جين KLF14 الذي يقع على الكروموسوم (Chr7:130734413-130734373) في محفز الجين.



واسمات مثيلة الدنيا لتقدير العمر

هذه الرسالة مقدمة كجزء من متطلبات نيل درجة الماجستير في
علوم الحياة / علم الحيوان

إعداد الطالبة

هبة سلمان جعيل الغانمي

بإشراف

أ.م.د. نهاد عيال مطر الراشدي

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