NEAR EAST UNIVERSITY
INSTITUTE OF GRADUATE STUDIES
DEPARTMENT OF MEDICAL BIOLOGY AND GENETICS

INVESTIGATION OF THE RELATIONSHIP BETWEEN METHYLATION OF CIRCADIAN RHYTHM GENES AND MENOPAUSE

M.Sc. THESIS

Günay KUŞAF

Nicosia
January, 2022

GÜNAY- KUŞAF
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Supervisor
Prof. Dr. Rasime KALKAN

Nicosia
January, 2022

Approval

We certify that we have read the thesis submitted by Günay Kuşaf titled **"Investigation of the Relationship Between Methylation of Circadian Rhythm Genes and Menopause**" and that in our combined opinion it is fully adequate, in scope and in quality, as a thesis for the degree of Master of Science.

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Declaration

I hereby declare that all information, documents, analysis and results in this thesis have been collected and presented according to the academic rules and ethical guidelines of Institute of Graduate Studies, Near East University. I also declare that as required by these rules and conduct, I have fully cited and referenced information and data that are not original to this study.

Günay Kuşaf

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Abstract

The Investigation Of The Relationship Between Methylation Of Circadian Rhythm Genes And Menopause

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The hormones of females decrease gradually, and the follicular activity of the ovary starts to be insufficient with increased age. As the oocyte number is not infinite, the ovarian reserve fall with age. After a while, the condition of menopause which is defined as twelve months of amenorrhea occurs. In the occurrence of menopause, participation of both genetic and epigenetic factors is demonstrated by several studies. The genetic and epigenetic factors play a role in the timing of menopause. The effects of environmental factors and circadian rhythms on the occurrence of menopause, and timing of menopause can not be ignored.

The aim of study is based on the identification of the methylation status of the *ARNTL* and *CLOCK* genes in post-menopausal women. DNA samples were extracted from 56 postmenopausal women and 48 premenopausal women for DNA testing. The Methylation Specific High-Resolution Melting (MS-HRM) technique was applied for determination of the methylation pattern of the *ARNTL* and *CLOCK* promoter regions. There was no statistically significant association between the methylation status of the ARNTL gene or CLOCK gene and menopause. Furthermore, no statistically significant interaction was observed between the ARNTL and CLOCK genes.

Keywords: menopause, arntl, clock, ms-hrm, methylation

Özet

Sirkadiyen Ritim Genlerinin Metilasyonu İle Menopoz Arasındaki İlişkinin Araştırılması

Kuşaf Günay

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Danışman: Prof. Dr. Rasime Kalkan

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Artan yaş ile kadınların hormanları giderek azalmaya başladığı gibi, yumurtalığın foliküler activiteside yetersiz kalmaya başlar ve ardından on iki ay boyunca adet görememe olayı olarak tanımlanan menopoz durumu ortaya çıkmaktadır. Epigenetik ve genetik faktörlerin, menopoza etki ettiği birçok araştırmalar tarafından gösterilmiştir. Menopoz yaşının tanımlanmasında genetik ve epigenetik faktörler rol oynamasına rağmen, yapılan aile çalışmalarında görülüyor ki menopoz yaşı kadından kadına farklılık göstermektedir. Bu durumdan ötürü, menopoz yaşının belirlenmesinde çevresel faktörlerin ve sirkadiyen ritminin de önemli rol oynadığı göz ardı edilemez.

Bu çalışmada, menopoz sonrası dönemde olan kadınlarda *ARNTL* geninin ve *CLOCK* geninin metilasyon durumunun saptanması amaçlanmıştır. Çalışmaya dahil edilen, 56 postmenopozal kadından ve 48 premenopozal katılımcıdan DNA izolasyonu yapılmıştır. *ARNTL* ve *CLOCK* promotor bölgelerinin metilasyon paterninin analizi için Metilasyona Özgü Yüksek Çözürnüklü Erime Eğrisi (MS-HRM) analizi uygulanmıştır. *ARNTL* geninin veya *CLOCK* geninin metilasyonu ile menopoz arasında istatiksel olarak anlamlı bir ilişkisi bulunamamıştır. Ayrıca, *ARNTL* ve *CLOCK* genlerinin metilasyonlarının birbiri ile istatiksel olarak ilişkili olmadığı saptanmıştır.

Anahtar kelimeler: menopoz, arntl, clock, ms-hrm, metilasyon

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List of Abbreviation

DNA:	Deoxyribonucleic Acid
RNA:	Ribonucleic Acid
***ARNTL*:**	Aryl Hydrocarbon Receptor Nuclear Translocator Like
***BMAL1*:**	Brain and Muscle ARNTL-Like 1
***CLOCK*:**	Circadian Locomoter Output Cycles Protein Kaput
***PER*:**	Period
***CRY*:**	Cryptochorome
ncRNA:	Non-Coding RNA
CpGs:	Cytosine Phosphate Guanine Dinucleotides
5mC:	5-Methylcytosines
DNMTs:	DNA Methyltransferases
H2A(X):	H2A Histone Family Member X
siRNAs:	Small Interfering RNAs
miRNAs:	MicroRNAs
lncRNAs:	Long Non-Coding RNAs
3'-UTR:	Three Prime Untranslated Region
mRNAs:	Messenger RNAs
ATP:	Adenosine Triphosphate
SWI/SNF:	Switch/Sucrose Non-Fermentable
HPG:	Hypothalamic Pituitary Gonadal
ERG:	Retinal Electroretinogram Responses
DSPS:	Delayed Sleep Phase Syndrome
ASPS:	Advanced Sleep Phase Syndrome
SCN:	Suprachiasmatic Nucleus
TTFL:	Transcription and Translation Feedback Loop
CKs:	Choline Kinases
PPs:	Protein Phosphatase
***RORE*:**	Retinoic Acid Related Orphan Receptor Response Element
***RAR-BZIP*:**	Proline and Acidic Amino Acid-Rich Basic Leucine Zipper
DBP:	D-Box Binding Protein
TEF:	Thyrotroph Embryonic Factor
***HLF*:**	Hepatic Leukemia Factor

***NFIL3*:**	Nuclear Factor Interleukin – 3 – Regulated Protein
HDACs:	Histone Deacetylases
H3K9:	Histone H3 Lysine 9
SNPs:	Single Nucleotide Polymorphism
BMI:	Body Mass Index
MI:	Myocardial Infarction
SAD:	Seasonal Affective Disorder
BD:	Bipolar Disorder
PTMs:	Posttranslational Modification
H3S10:	Histone H3 Serine 10
HAT:	Histone Acetyltransferase
H3K14:	Histone H3 Lysine 14
CCGs:	Clock Control Genes
NAD(+):	Nicotamine Adenine Nucleotide
***SIRT1*:**	NAD(+) – Dependent Deactylase Sirtuin 1
***NCOR*:**	Nuclear Receptor Corepressor
***SMRT*:**	Nuclear Receptor Corepressor 2
***NURD*:**	Nucleosome Remodeling and Deacetylase
***CHD4*:**	Chromodomain Helicase DNA Binding Protein 4
H3K4:	Histone H3 Lysine 4
H3K27:	Histone H3 Lysine 27
H3K36:	Histone H3 Lysine 36
HMTs:	Histone Methyltransferases
HDMs:	Histone Demethylases
***MLL1*:**	Mixed Lineage Leukemia 1
H3K9me2:	Histone H3 Lysine 9 Di-Methylation
HP1:	Heterochromatin Protein 1 Family
***LSD1*:**	Lysine Specific Demethylase
***JARID1A*:**	Jumonic, and Arid Domain Including Histone Demethylase 1A
H3K4me3:	Histone H3 Lysine 4 Tri-Methylation
GnRH:	Gonadotropin Releasing Hormone
LH:	Luteinizing Hormone
FSH:	Follicule Stimulating Hormone
MS-HRM:	Methylation Sensitive – High Resolution Melting

PCR: Polymerase Chain Reaction

dsDNA: Double Stranded DNA

ssDNA: Single Stranded DNA

CHAPTER I

Introduction

Epigenetics is explained as inheritable modifications which take place in gene expression while it does not lead to variations to occur in DNA (deoxyribonucleic acid) sequence (Zovkic, 2021). In other words, modifications act as heritably and stably across to DNA rather than the sequence of the gene. The gene expressions are regulated by epigenetic modifications via chemical alterations of DNA and histone proteins (Aboud, Tupper and Jialal, 2021). Epigenetic modifications are divided into four main groups, these are; non-coding RNA (ribonucleic acid), DNA methylation, RNA modifications and histone modifications. Furthermore, epigenetics is essential to maintaining cellular identity and performance (Lei *et al.*, 2020).

The epigenetic modifications can be affected by various factors such as; pathologic and physiologic stimulants, and also environmental ones which are age, eating habits, stress, physical activeness, consumption of smoke and alcohol (Pagiatakis *et al.*, 2021). Many studies indicate that epigenetic processes play an important role during the development of various pathological mechanisms such as ageing, coronary artery disease, diabetes mellitus, and high blood pressure, and the activity of these processes has been monitored as contributing to the occurrence of various diseases in the human body (Lei *et al.*, 2020).

The hormones of females play a major role during their lifetime. Puberty, the opportunity to experience being a mother, and the ability to play cardioprotective roles are all influenced by female hormones. On the other hand, physical, psychological and sentimental disruptions are figured out at the age of afterward of the mid-forties of almost all females, regardless of the history of their cultures and healthfulness status. These conditions are caused by the gradual decrease in female hormones, estrogen and progesterone, followed by menopause (Hajj *et al.*, 2020).

Menopausal condition is described as a lasting cessation of menses the consequence of deficiency of ovarian follicular activity. According to clinical findings, menopause is reported as occurring afterward twelve consecutive months of amenorrhea; no other pathologic or physiologic reason can be encountered (Rozenberg *et al.*, 2020).

In the period of menopause; women face difficulty in an exact description of physical, psychological or sexual disturbances, hot flashes, jitters, depression, sleeplessness, and tiredness. These are the main findings which are reported about the consequences of menopause (Hajj *et al.*, 2020). Researchers determined that

epigenetic age plays a significant role in menopause timing. It was concluded that earlier menopause correlates with raised epigenetic age due to reason of higher levels of DNA methylation than the expected rate (Levine *et al.*, 2016).

The intrinsic clock oscillator takes place in every organism and acts to provide adaptation to destructive conditions; it is called the circadian clock (Shao *et al.*, 2021). Furthermore, the circadian rhythm is defined as all of the biological processing which is monitored between 24 hours, and these rhythms that happen within 24 hours are established by the circadian clock (Pines, 2016). The circadian rhythms provide organisms with the capability of coordinating suitable physiological responses to the light-dark changes that are related to the rotation of the earth (Goldstein and Smith, 2016). In mammals, metabolism and physiological processes are under the consideration of the circadian clock, and the appearance of different kinds of diseases can be encountered such as metabolic syndrome and cancer predisposition if a deterioration happened in the control of these processes (Pines, 2016). The circadian system acts in whole levels of a woman's life as a cornerstone: from follicle generation to arrangement of hormonal balance; and the process from embryo implantation to birth (Shao *et al.*, 2021). The circadian rhythms take place in the body of the organisms. They have produced the transcriptional translation feedback loop; and *ARNTL* (aryl hydrocarbon receptor nuclear translocator like) which is also called *BMAL1* (Brain and Muscle ARNT-like 1), *CLOCK* (Circadian locomoter output cycles protein kaput), *PER* (Period), and *CRY* (Cryptochrome) genes have a significant function in this cycle. When *CLOCK* and *BMAL1* bind each other leads to activation of transcription of *PER* and *CRY* genes, that create a heterodimer structure in the cytoplasm and then turn back into the nucleus for being able to repress their transcription activity by suppressing the *CLOCK*– *BMAL*1 complex. This process occurs in nearly 24 hours (Goldstein and Smith, 2016). The circadian clock genes are not only factors that play a part, but also genetic and epigenetic factors also importantly participate in the modulation of the circadian system. The rhythmical gene expression is modulated by peripheral factors, while these factors are mediated by epigenetic processes (Saad *et al.*, 2021).

The aim of study the molecular mechanism of methylation of *ARNTL*, and *CLOCK* genes is to:

(a) determine the methylation levels of related genes

(b) investigate the effect of methylation pattern of these genes on menopause

(c) identify the methylation differences of the relevant genes in pre and postmenopausal women.

In view of the study's purpose of investigating the relationship between methylation of circadian rhythm genes and menopause, the research questions are:

(a) is there any relationship between the methylation status of the *ARNTL* gene and menopause

(b) is there any relationship between the methylation status of the *CLOCK* gene and menopause.

In literature, the possible effects of methylation pattern of *ARNTL* (*BMAL1*), and *CLOCK* genes during menopause; and the correlation of the methylation pattern of the *ARNTL* and *CLOCK* genes with age have not been shown yet. This study will be the first to show the relationship between the methylation status of the *ARNTL* and *CLOCK* genes with menopause.

CHAPTER II

General Information

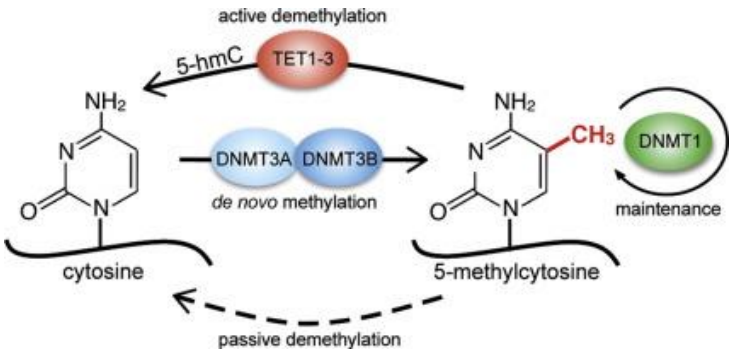
2.1. Epigenetics

Epigenetics is heritable and/or acquired alteration in the gene expression level which do not lead to the occurrence of any alterations in DNA sequence (Strachan and Read, 2010). Heritable changes in gene expression are inherited via mitosis as generally, but transmission can sometimes occur by meiosis as well. Therefore, a genetic constitution can cause different phenotypic constitution formation, dependent on how gene(s) expression(s) happened in a locus or loci (Radcliffe, 1900). Gene expression during the transcription or post-transcription level, and translation or post-translational level can be influenced by epigenetic processes (Moosavi and Ardekani, 2016). Epigenetic processes play a crucial role in the formation of every biological condition in our body system, from the implantation of the embryo to the demise of the individual (Moosavi and Ardekani, 2016).

DNA methylation, chromatin remodeling, non-coding RNA (ncRNA) associated gene silencing, and histone modifications are categorized as epigenetic mechanisms (Lei *et al.*, 2020). DNA methylation is the most commonly known epigenetic process (Weinhold, 2006), which plays a role in the regulation of gene expression and it is the process that methyl group is added to the C5 carbon of cytosine phosphate guanine dinucleotides (CpGs) (Gardini, 2020). The cytosines are converted to 5-methylcytosines (5mC) by DNA methyltransferases (DNMTs) enzymes. Three DNMTs enzymes are found in humans; these are DNMT1, DNMT3A, and DNMT3B (Loaeza-Loaeza, Beltran and Hernández-Sotelo, 2020). The DNMT1 enzyme maintains the methylation of DNA, while DNMT3A and DNMT3B enzymes maintain the de novo methylation (Figure 1) (Gardini, 2020).

Figure 1:

DNA Methylation (Ambrosi, Manzo and Baubec, 2017)



In DNA methylation the epigenetic alterations are straightly taking place on DNA, or on histone proteins which DNA is packaged into nucleosomes (Zovkic, 2021). The interaction of histone post-translational modifications and DNA methylation figured out by non-coding RNAs and regulatory proteins. These modifications are the cornerstone processes in epigenetic which chromatin can rearrange into euchromatin, heterochromatin, and nuclear compartmentalisation (Moosavi and Ardekani, 2016). Methylation of DNA is related to gene silencing. The prominent factor in most types of cancer is that tumor suppressor genes are hypermethylated and proto-oncogenes are hypomethylated. The controlling of gene expression, genomic imprinting, and the inactivation of the X chromosome are the processes that DNA methylation is figured in their occurrence.

The post-translational modification to histone proteins is the other epigenetic mechanism (Aboud, Tupper and Jialal, 2021). The methylation, acetylation, phosphorylation, ubiquitylation, etc… are given as an example of epigenetic modifications (Weinhold, 2006) that plays a role in the alteration of DNA-histone interplays in the nucleosome. Histone acetylation consists of lysine residue which is charged as positive, which leads to weakness in the interplay of DNA and histone, therefore the structure of chromatin is opened and transcription is activated. By histone methylation, 1-3 methyl groups are added to lysine while 1-2 methyl groups are joined to arginine while the charge of histone protein remains the same; the transcription can be activated or repressed by histone methylation. The negative phosphate group is joined to histone tails by histone phosphorylation, and also H2A(X) phosphorylation acts a part in the repair of DNA damage. The ubiquitin group is added to the lysine residue by histone ubiquitylation, and it plays a role in both gene silencing and transcription.

Non-coding RNA associated gene silencing is the third mechanism of epigenetics. The ncRNA is a functional RNA molecule; while transcription of the ncRNA occur, translation to the proteins does not happen (Aboud, Tupper and Jialal, 2021). The classification of ncRNAs is based on their length. Small interfering RNAs (siRNAs) and microRNAs (miRNAs) are categorized as small non-coding RNAs due to reason of they are smaller than 200 nucleotides. On the other hand, long non-coding RNAs (lncRNAs) are bigger than 200 nucleotides. The proteins' expression during the transcription and translation processes is regulated by non-coding RNA. Studying long non-coding RNAs is difficult because of the deficiency of short preservation of

long non-coding RNAs; temporal and spatial expression of lncRNAs play a major role in how chromatin structure is regulated, and how factors can be recruited to the transcription system; and gene expression can also be understood in the absence of long non-coding RNAs. However, the genes' expression is inhibited by the joining of miRNAs to the untranslated area of (3'- UTR) messenger RNAs (mRNAs), resulting in deterioration of target messenger RNA and suppression of subsequent protein translation processes (Pagiatakis *et al.*, 2021).

The ATP (adenosine triphosphate) dependant chromatin remodeling is one of the epigenetic mechanisms. It is the complexes that a lot of protein takes apart, and which gene expression is regulated by modification of nucleosome organization of DNA utilizing the energy produced from ATP hydrolysis. The proteins of these complexes act as a transcription factors to activate transcription by helping the open and reachable structure of chromatin to form, and various proteins are recruited to be included in the transcription process. For instance, SWI/SNF complexes support the generation of this structure via a mechanism including sliding nucleosomes, evicting the H2A:H2B heterodimers or carrying away the histone protein octamers from DNA. The chromatin remodelling factors lead to the gene being silenced by the structure of chromatin is immensely packed through organising the nucleosomes on the DNA, therefore access to the transcription proteins is prevented (Pagiatakis *et al.*, 2021). The chemical modifications of histone proteins are interacted with DNA for being promoted gene expression or repressed the gene transcription (Aboud, Tupper and Jialal, 2021).

The link between epigenetics and the occurrence of various diseases has been proven. For instance: cancer, autoimmune disorders, obesity, metabolic disorders, neurological diseases such as Huntington’s Disease, Alzheimer’s, Fragile X Syndrome (Moosavi and Ardekani, 2016).

2.2. Circadian Clock

The HPG (hypothalamic-pituitary-gonadal) axis is governed by circadian rhythm, as well as the timing of ovulation and releasing of reproductive hormones are affected by circadian rhythm activity (LJ *et al.*, 2014). The word circadian originates from ‘circa’ that comes from the word ‘approximately’ while ‘dies’ comes from the ‘day’(ED and JS, 2013). All of the biological activities which are taken place in endogenic releasing between 24 hours are under the consideration of the circadian system (Pines, 2016). The environmental light-dark changes that were happened

during the rotary motion of the earth can give rise to different physiological reactions and these reactions can be coordinated in a suitable way via circadian rhythms (Goldstein and Smith, 2016). The circadian rhythms occurring in a day are managed by circadian clocks. The circadian clocks are encountered in plants and animals as commonly, and also it can be seen in fungi and cyanobacteria (Pines, 2016). The metabolism, blood pressure, retinal electroretinogram responses (ERG), body temperature, and circulating hormones can be shown as examples of rhythms that happen during a day. In mammalians, the circadian system has an important function in the regulation of metabolism and physiological systems. Different kinds of consequences or diseases such as metabolic syndrome and cancer susceptibility are raised in the case of degradation of the circadian clock (Pines, 2016). Furthermore, the advanced sleep phase syndrome (ASPS), and delayed sleep phase syndrome (DSPS) that are sleeplessness or hypersomnia when someone's internal time and requested rest plan are misaligned can be resulted by malfunctioning of circadian clocks (ED and JS, 2013). Also, circadian rhythms play an important role at all levels throughout a woman's life. The processes from follicle generation to internal secretion controlling, also from fertilized egg implantation to birth are examples of these rhythms (Shao *et al.*, 2021).

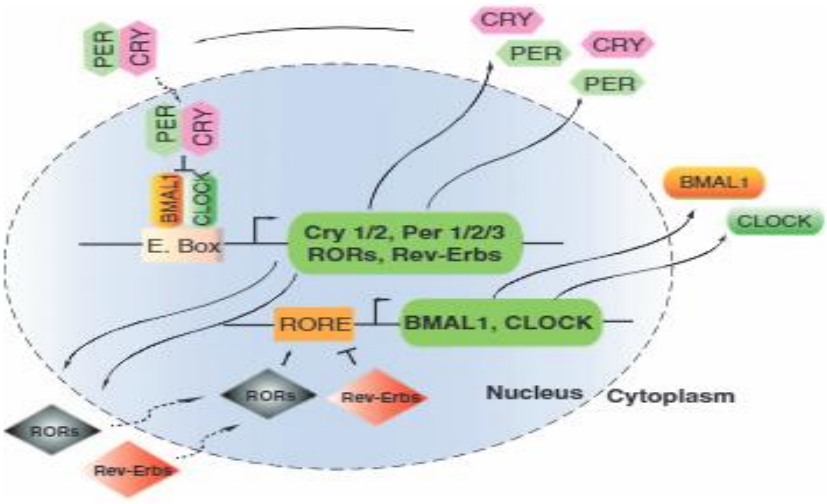
2.2.1. Circadian Gene Networks

The regular responsibilities of the circadian system are related with a hierarchical central network and peripheral clocks. In general, the central clock is situated in the suprachiasmatic nucleus (SCN) in the hypothalamus. The rhythmical knowledge is maintained and transferred to downstream clocks in peripheral tissues and organs by circadian clock. In basis, the circadian clock is a rhythmical transcription and translation feedback loop (TTFL) (Shao *et al.*, 2021). This hierarchical system is very complicated, and it is managed by clock genes via a master circadian pacemaker placed in the SCN in the hypothalamus (Pines, 2016). The TTFL is conducted by four clock proteins: *CLOCK* and *BMAL1* which are initiators, while *PER* and *CRY* are inhibitors of transcription. Moreover, in the localization and stabilization of these four proteins; kinases (CKIα, CKIε, and CKIδ) and phosphatases (PP1, and PP5) play a role as well (Partch, Green and Takahashi, 2014). The protein that is produced by *BMAL1* makes a heterodimer structure with *CLOCK* (ED and JS, 2013). The *CLOCK* and *BMAL1* are aggregated in the nucleus. The *CLOCK* and *BMAL1* make a heterodimer structure then bind to the E-boxes including enhancers or promoters of

the transcription depressor genes *PER* and *CRY* (Shao *et al.*, 2021). These two inhibitors are relocated into the nucleus and interact with transcription initiators. By using ubiquitin-dependant pathways, levels of two inhibitor proteins were reduced. Consequently, the suppression of *BMAL1:CLOCK* is alleviated and a new cycle starts approximately in a day. The intrinsic interval of clock is determined by casein kinases CKIδ and CKIε; and it is controlled by the degradation or insertion of *PER:CRY* heterodimers to the nucleus. The activity of *PER:CRY* complex is either antagonized or modulated by phosphatases PP1 and PP5, respectively. The clock's intrinsical process is established by *PER:CRY* levels while reduction or insertion of the nucleus and casein kinases take part as well (Partch, Green and Takahashi, 2014). Additionally, the next loop after the first loop is composed by E-box interceded transcription of orphan nuclear receptor genes *REVERBα*, *REVERBβ* and *RORα*, *RORβ*. The competition is made between *REVERB* and *ROR* receptor genes for being able to bind to the Retinoic acid related Orphan receptor Response Element (*RORE*) whose binding sites are situated in the promoter region of the *BMAL1* gene (ED and JS, 2013). These receptors take a part in the inhibition and initiation of transcription respectively (Shao *et al.*, 2021). In other words, transcription is repressed by *REVERBβ* or *REVERBα*, while it is activated by *RORα* or *RORβ* (Figure 2) (ED and JS, 2013).

Figure 2:

Trascription and Translation Feedback Loop (Lee and Kim, 2013)



Further patterns comprised in the circadian clock network are D-box elements. The D-box elements are recognized by *RAR-bZip* (proline and acidic amino acid-rich

basic leucine zipper) transcriptional initiation factors DBP (D-box binding protein), *TEF* (thyrotroph embryonic factor), and *HLF* (hepatic leukemia factor); while *NFIL3* (nuclear factor interleukin-3-regulated protein which called as E4BP4) is a transcriptional repressor. The expressiveness of *RAR-bZip* initiators is guided by core clock TTFL via joining to E-boxes in their promoter regions; meanwhile depressor *NFIL3* is regulated by *ROREs*. The expressiveness of initiators and inhibitors were separated temporally, during which rhythmicity affected additional genes. The circadian transcription of the type of cells is determined by the rhythmical epigenome, which is acts as a central. Oscillation on biological mechanisms which temporarily separate metabolism within 24 hours is imposed by rhythmical epigenome. For instance, circadian lipid and glucose metabolic processes in mouse muscle and liver are synchronized by histone deacetylase HDAC3, and HDAC3 rhythmically recruited within a suppressor complex which is driven by the circadian protein *REVERBα*. Rhythmical recruitment of HDAC3 conducts oscillations in H3K9 acetylation and chromatin condensation at lipid biosynthetic genes, a molecular mechanism underlying the daily rhythm examined in hepatic lipogenesis (Pacheco-Bernal, Becerril-Pérez and Aguilar-Arnal, 2019).

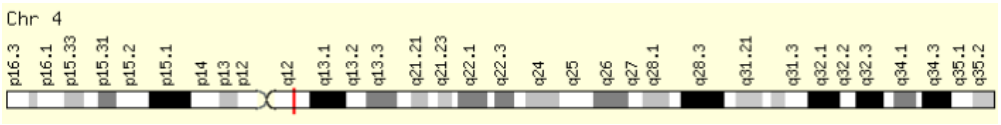
2.2.2. *CLOCK* Gene

The *CLOCK* gene is located on chromosome 4q12 (Figure 3). The protein that is produced by the *CLOCK* gene significantly participates in the regularization of rhythms of the circadian system. This protein is a transcription initiator and includes DNA-binding histone acetyltransferase activity. The expression of this gene is monitored in the pancreas, thymus, prostate, testis, ovary, skeletal muscle, kidney, leukocytes, lung, brain, placenta, heart, liver, small intestine, colon, and spleen (*CLOCK Gene - GeneCards | CLOCK Protein | CLOCK Antibody*). There are 1411 diseases (*MalaCards: Search Human Diseases - CLOCK*) which are related to this gene such as delayed sleep phase disorder, major depressive disorder, etc… In some populations, obesity, metabolic syndrome, and behavioural alterations are encountered in the case of the *CLOCK* gene polymorphisms. (*CLOCK Gene - GeneCards | CLOCK Protein | CLOCK Antibody*). The single nucleotide polymorphisms (SNPs) in the *CLOCK* gene are associated to sleep diminution, the concentration of adipocytokine, body mass index (BMI), and uptake of energy. Reduction of sleep time in carriers of the T allele of the rs68533192 was concluded by Riestra and colleagues. Moreover, the *CLOCK* variant of rs11726609 was

demonstrated as related to increased BMI; while rs6820823 and rs3792603 variants were reported as correlate to reduced BMI (Riestra *et al.*, 2017). The variant of *CLOCK* 3111T/C (T → C (rs1801260)) interrelates with bipolar mania in bipolar disorder patients. Especially, this SNP correlates to raised actimetric and sleep disruptions and occurrence of manic episodes in patients who have bipolar disorder. The C allele carriers who are obese or overweight encounter with difficultness of weight loss compared to TT homozygous (Ozburn *et al.*, 2016). According to the findings of Pagliai and colleagues, SNP of rs4580704 is associated to high level of triglyceride and LDL-cholesterol (Pagliai *et al.*, 2019).

Figure 3:

Genomic Location of CLOCK Gene (CLOCK Gene - GeneCards | CLOCK Protein | CLOCK Antibody)



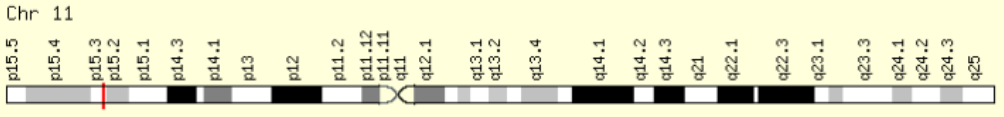
2.2.3. *ARNTL* (*BMAL1*) Gene

The aryl hydrocarbon receptor nuclear translocator like (*ARNTL*) is also known as *BMAL1* (brain and muscle ARNT-like 1). The location of the *ARNTL* gene is on chromosome 11p15.3 (Figure 4). This gene is a transcription initiator and an important element of the circadian clock system. It is greatly expressed in the brain, skeletal muscle, and heart (*ARNTL Gene - GeneCards | BMAL1 Protein | BMAL1 Antibody*). There are 142 diseases which are related to this gene (*MalaCards: Search Human Diseases - ARNTL*); such as advanced sleep phase disorder, delayed sleep phase disorder, etc… (*ARNTL Gene - GeneCards | BMAL1 Protein | BMAL1 Antibody*). The rs3789327 and rs12363415 variants of the *ARNTL* gene were reported as related to type 2 diabetes mellitus in myocardial infarction (MI) patients. Furthermore, the association between these variants in myocardial infarction patients and high blood pressure were concluded by Škrlec, Milić and Steiner. Diurnal alterations of blood pressure level were analyzed as correlated with rs12363415. The SNPs of *ARNTL* gene were seem as responsible in the occurrence of high blood pressure, diabetes mellitus, and metabolic diseases which lead to rise the risk of being myocardial infarction (Škrlec, Milić and Steiner, 2020). The relation of SNPs of the *ARNTL* gene to seasonal affective disorder (SAD) and bipolar disorders (BD) was documented by various articles (Rajendran and Janakarajan, 2016). According to

Min and colleagues' conclusions, major distinctions were found between samples of control and bipolar disorder patients in the *ARNTL* variants of rs10832022 and rs11022765 (Min *et al.*, 2021).

Figure 4:

Genomic Location of ARNTL Gene (ARNTL Gene - GeneCards | BMAL1 Protein | BMAL1 Antibody)



2.2.4. Epigenetic Regulations of Circadian Gene Network

The classic regulation of circadian system is governed by core clock genes, several genetic and epigenetic factors have participated in the controlling of circadian transcription. Due to all investigations about human behavioural features and diseases are not possible to be related to being explained by TTFL. Therefore, the epigenetic processes are known as vital intermediaries of environmental elements which regulate rhythmic gene expression (Saad *et al.*, 2021). In the fibre of chromatin, transcription loops are guided by a molecular clock. The necessity of a circadian mechanism for chromatin modifications to be able to regulate circadian rhythms is undoubtedly verified conceptionally (Pacheco-Bernal, Becerril-Pérez and Aguilar-Arnal, 2019). The maintenance of transcription is provided by controlling the chromatin and genome structure coordinately. The nucleosome is the fundamental packaging unit of chromatin, and genetic information is packed into chromatin. The conditions of chromatin are specified by histone posttranslational modifications (PTMs). The histone modifications are considered cornerstones of the circadian alterations (Tomita and Onishi, 2018). The fine adjustment of the circadian clock system is controlled by epigenetics, consequently, histone modifications have been indicated as a complementary component of the TTFL (Robinson and Reddy, 2014). The light-stimulated phosphorylation of histone H3 at Serine 10 (H3S10) in the core clock is accepted as a first proof of implication of epigenetic mechanism in the regulation of circadian rhythm. Conspicuously, rhythmical epigenetic activity in various tissues has been analyzed by high yielded analysis. For instance, high plasticity in reaction to light-dark loop lengthiness in neural network traits in SCN is monitored. The adaptation of SCN to different light intervals is provided by DNA methylation expression at certain genes which are interrelated with neurotransmitter

receptors, ion channels that potassium, calcium, and GABA channels are included (Pacheco-Bernal, Becerril-Pérez and Aguilar-Arnal, 2019).

The gene expression' oscillations regularised by correlation of transcription of circadian rhythm and alterations of chromatin. By the connection of histone acetyltransferase (HAT) p300 and intrinsical clock histone acetyltransferase action rhythmical acetylation of histone (H3K14, H3K9) was established on promoter areas of clock control genes (CCGs). The second one was occurred by deactivation of the acetylation of non-histones and histones by NAD(+)-dependant deacetylase sirtuin 1 (*SIRT1*). Histone acetylation and deacetylation used by *CLOCK* for being able to regulation of circadian rhythms. The histone H3 acetylation at *PER* and *CRY* promoters is governed by working of HAT p300 and *CLOCK:BMAL1* heterodimer structure for affecting their expression. Moreover, *CLOCK* activates the HAT and the *CRY1* is recruited by acetylated *BMAL1* to the *CLOCK:BMAL1* structure for repression of transcription. The acetylation of histone H3 in the promoter region of *PER1, PER2,* and *CRY1* were documented while it has been reached to peak along active state of transcription (Saad *et al.*, 2021). The histone acetylation levels are maintained by HAT, and histone-deacetylases (HDACs) (Tomita and Onishi, 2018). The HDACs are as crucial as HATs in the regulation of circadian transcriptions, dependence-associated phenomena, memory generation, and metabolic process (Saad *et al.*, 2021). For instance, transcription is repressed by recrution of *NCOR* and/or *SMRT* to *ROREs* by *REVERBα* (Tomita and Onishi, 2018). In other words, HDAC3 is recruited by nuclear receptor corepressor 1 (*NCOR*) and leads to suppression of *BMAL1* activity. Therefore, the circadian rhythms are influenced. By the fluctuation of HDAC3 recruitment, HDAC3/*REVERBα*/*NCoR* complex is formed. Many genes can be transcribed by either histone alteration which is related with HDAC3 fluctuation or signalling pathways from HDAC3/*REVERBα/NCOR* complex. However, acetylation of H3 is increased by HDAC suppressors, and expression of *PER2* is affected. *SIRT1* is NAD(+)-dependant histone deacetylase, it is under the direct interaction with CCG by joining the CLOCK:BMAL1 complex, stimulating deacetylation and deterioration of PER2 in mice. The *SIRT1* is a sensor that binds to NAD+ and is responsible for enzymatic interactions associated with the circadian system. Additionally, the role of *SIRT1* in brain functions such as aging, neurodegeneration, synaptic plasticity, and memory generation can not be ignored (Saad *et al.*, 2021). One of the important supression of transcription mechanism is

intermediated by *CRY* and *PER* which HDAC1 and HDAC2 are recruited by Sin3 complex. *NURD* is other co-suppressor complex that HDAC1 and HDAC2 subunits are included, it joints to *PER:CRY* complex and nearby histones are deacetylated; by this way, CCG are repressed (Tomita and Onishi, 2018). The chromatin remodelers comprised in controlling the circadian system contain the *NURD* that ATP-dependent nucleosome remodelling *CHD4* (chromodomain helicase DNA binding protein 4) is included. Therefore, *NURD* co-suppressor interplays and succours *PER* complexes to be able to silence the transcription (Pacheco-Bernal, Becerril-Pérez and Aguilar-Arnal, 2019).

The circadian related gene expression is also regulated by the participation of histone methylation and demethylation. Rhythmical methylation of histones at H3K4, H3K9, H3K27, and H3K36 are catalyzed by various histone methyltransferases (HMTs) and histone demethylases (HDMs) (Saad *et al.*, 2021). The HMTs mixed lineage leukemia 1 (*MLL1*) interplays with the *CLOCK:BMAL1* complex, oscillating pattern are imposed on the H3K4 initiating epigenetic alteration at the promoters of *DBP* and *PER1*. The circadian enzymatic activeness of *MLL1* seems that it is driven by post-translational modifications of rhythmical acetylation at K1130 and K1133 (Pacheco-Bernal, Becerril-Pérez and Aguilar-Arnal, 2019). The *CLOCK:BMAL1* complex is recruited to the E-boxes of CCG promoter regions by *SUV3*9 methyltransferase which is one of the vital governors of rhythmical H3K9 di-methylation (H3K9me2). Presumptively, via the connection of *SUV39H* with *PER2*, rhythmical optional heterochromatin is organized by H3K9me2 HP1 binding at DBP, *PER1*, and *PER2* while repression is happened. The lysine specific demethylase (*LSD1*), *JUMONJIC*, and *ARID* domain including histone lysine demethylase 1a (*JARID1A*) are important joining partners of *CLOCK:BMAL1*, in this way transcription is enhanced by *CLOCK:BMAL1*. Removing of methyl groups from H3K9 and H3K4 which are linked with *CLOCK* and *BMAL1* is catalyzed by *LSD1*. *LSD1* has been documented as a keystone element of the circadian machine and a regulator of CCG expression. The *JARID1A* is directly interacted with *CLOCK:BMAL1* complex enable the circadian system to be controlled and circadian gene expression to be regulated. While demethylase independent function is monitored by *JARID1A*, H3K4me3 is not affected; however, it is acted on and suppressing HDAC1 recruitment (Saad *et al.*, 2021).

2.3. Menopause

The woman's reproductive system includes various secretory organs which participate in the secretion of hormones, generate female properties, and also provide fertility.The system of female is regulated by hypothalamic-pituitary gonadal (HPG) axis (Shao *et al.*, 2021). The ovulation cycle is managed by gonadotropin releasing hormone (GnRH) which is secreted from the hypothalamus. The pituitary induces the synthesizing and secreting of the luteinizing hormone (LH) and follicule stimulating hormone (FSH) by GnRH (Goldstein and Smith, 2016).

When 12 months pass without menstrual cycle from the last period it is described as menopause (Dalal and Agarwal, 2015). The stage of menopause, endocrinological, biological and clinical characteristics of switching ovary function have been documented. During the menstrual period, estrogen, androgen and progesterone hormones are produced by ovaries in a cyclical pattern under the management of FSH and LH that are secreted by the pituitary gland (Rozenberg *et al.*, 2020). Consequently, the response to the pituitary hormones, which are FSH and LH can not be capable to be done by the ovary. According to this reason, the production of oestrogen and androgen is arrested by ovarian in the phase of menopause (Dalal and Agarwal, 2015).

The menopausal transition which is also called perimenopause is the stage that irregular menstrual cycles started to be monitored till the recent menstruation and it is the last phase before the occurrence of menopause. The fluctuation in reproductive hormone levels is seen in the perimenopause stage. In the early menopausal period; by the increasing age, the ovarian reserve begins to be decreased due to reason of the number of oocyte is not infinite, and the level of estrogen decrease which causes to rise in FSH and LH levels (Rozenberg *et al.*, 2020). During the perimenopause stage; insomnia, hot flashes, dryness of vagina, sexual dysfunction, mood disruptions, anxiousness, unrest (Jehan *et al.*, 2017), osseous loss, corruption of lean body mass, and raised fat mass are figured out as some of the characteristics of menopause indications. According to consequences analysis of menopause; acceleration of most of the women’s aging is detected due to reason of deleterious effect of menopause on the cardiovascular and mucsculoskeletal system (Vaccaro *et al.*, 2021).

2.3.1. Menopause and Circadian Rhythms

The natural age of menopause varies but the causes remain unclear. While genetic factors influence the timing of menopause and can not be ignored, in family studies, inheritable conditions of the timing of menopause are shown to be variable.

Therefore, factors from the environment at the timing of menopause are considered as considerably contributing. Consumption of smoking, eating habits, body fat composition has been shown as modifiable risk elements of menopause age. The reproductive outcomes of women who are night workers are affected in a negative way involving alteration in menstrual cycle, elevated risk of premature birth and miscarriage, and age of menopause is influenced from night work. The negative impacts on ovulation and fecundity are suspected by circadian rhythm deterioration (Stock *et al.*, 2019). The time of menopause may be affected via irregularity of the gonadal role due to the reason of desynchronisation from the signs of environmental circadian marks. The timing of day-night pineal melatonin release is suppressed and altered by exposure to unnatural light sources at night. Findings which follicle entirety is protected by melatonin, and proof of the significance of synchronised endocrinal signals and expression of CCG among central and peripheral points on mammal activity of ovary, night shift workers may face with earlier menopause compare to counterparts whose works are not night due to reason of night work linked deterioration of circadian rhythm (Stock *et al.*, 2019).

The GnRH and LH are affected by daily neural stimulant, and hence, age-related impairments of normal activity can cause irregularity in the menstrual cycles or amenorrhoea. Disruptions of sleep are proven as one of the keystone symptoms of menopause and that is associated with the deviation of normal awake-sleep circadian model. Different articles and studies have examined and documented poor sleep quality as being a common problem in middle age and in women going through menopause, it has been linked with disorders such as anxiety, depression, sleep apnea, and sexual dysfunction. According to a Hispanic cohort study of multinational women from Latin America (6000 women, average age 50); more than %50 of the participants were documented as complained about the sleep problems that had been investigated as were linked with advanced age, conditions which faced after the menopause, intensiveness of vasomotor indications, and specific somatic diseases such as chronic obstructive lung disease, obesity, high blood pressure, and diabetes. This information leads to the perspective of females who are in the stage of post menopause are more suffer from sleep problems compared to females who are in pre-menopause stage. The circadian rhythm of melatonin from the epiphysis cerebi (pineal gland), which is greatly synchronised withal customary time of sleep within a day, it was proven as the underlying mechanism of these indications. Intrinsic

releasing of melatonin declines withal ageing in both sexes, among females, menopause is related withal a considerable decrease of melatonin rates which lead to sleep being affected (Pines, 2016).

The intrinsical circadian period of men is established as longer than women. Even the circadian period is already defined as has consistent quality of circadian rhythm in genetics, this period of system can be altered as a result of exposures from environment and chemical substances. The findings of women's shorter circadian period against men's can be associated with women's excessive oestrogen rates. Due to, uninterrupted implementation of oestradiol benzoate lead to important decreasing of period to be happened in hamsters who are blind and ovaries were ectomized. A shorter circadian period in females can be explained by an excessive release of oestrogen (Duffy *et al.*, 2011).

The role of genetics in the circadian system is robustly verified. Moreover, various circadian rhythm's patterns may be revealed due to reason of a single nucleotide polymorphism (SNP). The deterioration of the circadian system and increment in sleep abnormalities in women who are in post-menopause stage compared to counterparts who are not in the stage of menopause has been concluded by different studies (Pines, 2016).

CHAPTER III

Methodology

3.1. Study Case

A total 56 postmenopause and 48 premenopause women were enrolled in this study. The blood specimens of participants were congregated by the Department of Obstetrics and Gynecology in Cengiz Topel Government Hospital.

Participants were attentively inspected about their bone mass density (BMD), and if they have been exposed to exogenic hormones or not such as birth control medications and hormone replacement therapy during the stage of post-menopause, also habitude of cigarette, alcohol, and physical activeness.

The criterions of inclusion and exclusion were evaluated as;

Inclusion criterions: Every woman who is in the stage of post-menopause should be in the stage for at least one year.

Exclusion criterions: Women who are in the period of menopause unnaturally, women who medicaments such as antianxiety, antidepressant, exogenic hormones are taken, women who have serious illness or mental retardation.

The form of informed consent was confirmed by all of the participants and the study was permitted by the Scientific Research Ethics Committee of the Near East University (Project No: YDU/2021/93-1830). The reports of clinics and biochemists were attentively inspected and compared.

3.2. Materials

The list of equipments and kits has been shown in table (Table 1).

Table 1 (Continued):

Equipments and Kits

Equipments		**Kits**
Micropipettes and Micropipette Tips (2-20-100-1000 µl)	Eppendorf Tubes (1.5 ml)	The AllPrep DNA/RNA/Protein Mini Kit – QIAGEN
PCR Tubes (0.2 mL)	Water Bath	The EpiTect Bisulfite Kit – QIAGEN
Micro-Centrifuge	Vortex Mixers	The EpiTect HRM PCR Kit - QIAGEN
Deep-Freeze	NanoDrop	

	Spectrophotometer
Thermal Cycler	Real-Time PCR System (Rotor Gene)

3.3. Methods

3.3.1. DNA Extraction

The protocol of the AllPrep DNA/RNA/Protein isolation kit (Qiagen GmbH, Hilden, Germany) was used for the method of DNA extraction from blood specimens of participants. The specimens are initially lysed and homogenized via greatly denaturing guanidine isothiocyanate including buffer, that DNases, RNases and proteases are directly inactivated for being ensured isolation of intact DNA, RNA, and proteins. Afterward, lysate is passed from spin column which permits joining of genomic DNA as selectively and efficiently by conjunction of high salt buffer. The column is washed and pure to be eluted.

1. Blood samples were incubated into the erythrocyte lysis (EL) buffer at 4 ^{0}C for 15 minutes. After incubation process, samples were centrifuged at 1000 x g, 4 ^{0}C for 15 minutes.
2. 5 ml EL buffer was put to the pellet and centrifuged at 1000 x g at 4 ^{0}C for 10 minutes for discarding the supernatant.
3. Pellet (lysis cells) were dried after centrifugation process which supernatant was discarded.
4. The cells were disrupted by addition of 350-600 µl buffer RLT, then were vortexed.
5. The lysate was pipetted into a spin column which is located in a 2 ml collection tube and then centrifuged at 14 000 rpm (full speed) for 3 minutes.
6. 500 µl AW1 buffer (wash buffer) was put to the collection tube. The tubes centrifuged at 8000 x g (10 000 rpm) as 15 seconds. After the centrifugation period is done collection tubes should be checked and all of the filtrate should be in the collection tubes; if there is some in the spin column centrifugation step should be repeated. The process of discarding collection tubes and transferring spin columns to clean collection tubes was carried out.

7. Afterward 500 µl AW2 was put in each tube, lids are closed and centrifuged at 20 000 x g (14 000 rpm) which is full speed as 2 minutes.
8. The DNA spin columns were transferred to 1.5 microlitre collection tubes. 100 µl EB buffer to the each spin column and lids should be closed. Then, incubation step of samples was carried out at room temperature as 2 minutes. At the end of incubation step, samples were centrifuged at 8000 x g (10 000 rpm) as 1 minute for being eluted the DNA.

When the process of DNA extraction has been completed, the concentration and purity of DNA were evaluated by using NanoDrop ND-1000 Spectrophotometer (Thermo Fisher Scientific).

3.3.2. Bisulfite Modification

The sodium bisulfite conversion is evaluated as a best method for determination of methylation conditions of DNA sequence. When sodium bisulfite conversion method is applied to the target DNA, the unmethylated cytosine are converted to uracil while methylated cytosines remain same. In other words, afterward the sodium bisulfite treatment conversion of cytosine to uracil is done by removing the amino groups (deamination) from cytosine which leads to cytosine turn to uracil sulfonate and it is followed by conversion of sulfonate uracil to uracil by desulfonation (Figure 5). Consequently, two different DNA sequences are revealed as methylated and unmethylated.

Figure 5:

Methylated and Umnetyhlated DNA Sequences After Bisulfite Modification (Qiagen, 2014)

	Original sequence	**Bisulfite modificated sequence**
Unmethylated Version	A-C-G-T-C-G-T-C-G-A	A-U-G-T-U-G-T-U-G-A
Methylated Version	A-**C**-G-T-**C**-G-T-**C**-G-A	A-**C**-G-T-**C**-G-T-**C**-G-A

For being able to reaching accurate results of methylation status all of the unmethylated cytosines should be converted. Therefore, DNA is incubated in high sodium bisulfate concentration at high temperature and low pH. These hard circumstances mostly conclude with high rate of DNA fragmentation and following DNA loss in purification step. The purification step is used for removing of bisulfite salts and chemics applied in conversion method which suppress the sequencing processes. The bisulfite modification method was applied by the protocols of EpiTect

Bisulfite Kit (Qiagen) according to the Unmethylated Cytosine's in DNA from Low Concentration Solutions. By this Kit, DNA fragmentation is obstructed by unique DNA Protect Buffer, that includes a pH-indicator dye as a mixing control in reaction install, permitting verification of the right pH value for conversion of cytosines. This involes following steps:

1. DNA which will be utilized in bisulfite conversion method was dissolved. Required number of aliquots of Bisulfite Mix was thawed by addition of 800 µl RNase free water to every aliquot. Bisulfite Mix was vortexed until dissolving as completely.

2. Bisulfite reactions were prepared in 200 µl PCR tubes accordance to given table below (Table 2). The mixed volume of DNA solution and RNase free water should be 40 µl as totally.

Table 2:

Bisulfite Reaction Components (Qiagen, 2014)

Component	**Volume per reaction (µl)**
DNA Solution (1-500 ng)	Variable (maximum 40 µl)
RNase Free Water	Variable
Dissolved Bisulfite Mix	85
DNA Protect Buffer	15
Total	140

3. The tubes were closed and bisulfite reactions were completely mixed at room temperature until DNA Protect Buffer turn green to blue afterward the annexing of DNA-Bisulfite Mix. Turning buffer from green to blue is the indication of mixing is adequate and pH is correct for being converted of bisulfite reaction.

4. Bisulfite DNA was converted by thermal cycler according the program of thermal cycler which is demonstrated in table (Table 3).

Table 3:

Thermal Cycler Conditions (Qiagen, 2014)

Denaturation	**Incubation**	**Denaturation**	**Incubation**	**Denaturation**	**Incubation**	**Hold**
95 ^{0}C	60 ^{0}C	95 ^{0}C	60 ^{0}C	95 ^{0}C	60 ^{0}C	20 ^{0}C
5 min	25 min	5 min	85 min	5 min	175 min	∞

5. For the step of incubation, the bisulfite reaction included PCR tubes were located into the thermal cycler. The PCR tubes were used due to reason of they can be tightly closed.

Cleaning the bisulfite modificated DNA

6. When bisulfite conversion has been finished, the brief centrifugation process of bisulfite reactions included PCR tubes and transferring bisulfite reactions to the clean 1.5 microliter microcentrifuge tubes was done.

7. 560 µl Buffer BL including 10 µg/ml carrier RNA was added to each sample. Solution was mixed by vortexing and then centrifuge shortly.

8. Mixtures were transfused to EpiTect spin columns. The centrifugation of spin columns was carried out at full speed as 1 minute, afterward the process of discarding flow through and transferring spin columns back to collection tubes was carried out.

9. 500 µl Buffer BW (wash buffer) was added to each spin column then centrifuge at full speed for 1 minute, afterward the process of discarding flow through and transferring spin columns back to collection tubes was carried out.

10. 500 µl Buffer BD (desulfonation buffer) was put to every spin column, then incubation process happened at 15-25 °C (room temperature) as 15 minutes. After the incubation step has been completed, spin columns were centrifuged at full speed for 1 minute, afterward the process of discarding flow through and transferring spin columns back to collection tubes was carried out.

11. 500 µl Buffer BW was put to every spin column, then the process of centrifugation at full speed as 1 minute, afterward the process of discarding flow through and transferring spin columns back to collection tubes was carried out.

12. Spin columns were located in new 2 ml collection tubes, for removing of having any risk of liquid residue centrifugation process at full speed as 1 minute was applied.

13. Spin columns were located in new 1.5 ml microcentrifuge tubes. 20 µl Buffer EB was put to each tube. And purified DNA was eluted by centrifugation at almost 15 000 x g (12 000 rpm) for 1 minute.

EpiTect PCR Control DNA Set was used as a universal methylated and unmethylated control.

The NanoDrop Spectrometer was applied for controlling the concentration and purity of the bisulfite treated specimens

3.3.3. Methylation Sensitive High Resolution Melting Analysis

Detecting and quantificating the bisulfite modificated methyl DNA, Rotor Gene Q for methylation sensitive high resolution melting (MS-HRM) analysis was used

while primers have been designed properly to the protocols of EpiTect HRM PCR (HRM polymerase chain reaction) Handbook (Qiagen GmbH). By the HRM analysis, DNA samples about their melting behaviour via PCR amplification were characterized. In other words, DNA samples were qualified based on their separation from double – stranded DNA (dsDNA) to single – stranded DNA (ssDNA) through rising temperature. The HotStarTaq Plus DNA Polymerase, EpiTect HRM PCR Buffer, RNase free water, EvaGreen, and dNTPs are included as components of EpiTect HRM PCR Master Mix.

1. The EpiTect HRM PCR Master Mix, primer solutions, RNase free water, template DNAs, and control DNAs were dissolved.

2. The reaction mix was prepared according to table (Table 4).

Table 4:

Reaction Components Using for EpiTect HRM PCR Master Mix (Qiagen, 2009)

Component	**Volume per 10 µl reaction**	**Final concentration**
2X EpiTect Master Mix	5 µl	1x
10 µM (each) primer mix	0.75 µl	0.75 µM forward primer 0.75 µM reverse primer
RNase-free water	Variable	-
Template DNA	Variable	5-10 ng/reaction
Total volume per reaction	10 µl	

3. The reaction mix extensively and deploy convenient volumes into PCR tubes. Then template DNA of patients was added to the PCR tubes and mixed.

4. Cycling protocol was optimized for HRM analysis on the Rotor Gene Q by following the steps in the given table (Table 5).

Table 5 (Continued):

Cycling Conditions for HRM Analysis (Qiagen, 2009)

Initial PCR Activation Step	**5 min**	**95 ºC**
3-step cycling		
Denaturation	10s	95 ºC
Annealing	30s	55 ºC
Elongation	10s	72 ºC
Number of cycles 40-45		
Denaturation	30s	95 ºC

Pre-hold	30s	50 °C
HRM Analysis for Rotor-Gene Q	2s	65-95 °C
		0.1°C increments

5. The PCR tubes were located into the real time cycler, PCR cycling program was started, and followed by HRM analysis.

3.4. Statistical Analysis

The chi-square test and two-tailed Fisher's exact test were used for statistical analysis and their relationships with characteristics of participants. The SPSS 15.0 software (SPSS, Chicago, IL, USA) was implemented for calculations, with a statistical significance of $P < 0.05$.

CHAPTER IV

This study was performed on 48 women who are in the premenopausal stage (control) and 56 postmenopausal women. Menopause was determined by the latest information from the World Health Organization's criterion which is defined as at least one year of amenorrhoea. Either steroid hormone nor biological therapies were not taken by participants.

The mean age of 48 participants who are in the stage of perimenopause was 33.4 years (mean ± Std. Deviation, 33.4 ± 6.8), and the mean age of 56 participants who are in the stage of menopause was 56.6 (mean ± Std. Deviation, 56.6± 4.8).

4.1. Methylation Pattern of *CLOCK* Gene

To identify the methylation pattern of our specimens, universal methylated and unmethylated control DNA (EpiTect Control DNA Set) was used as a control (Figure 6).

In total; the *CLOCK* promoter was methylated in 42 (41.2%) samples, while 60 (58.8%) samples were unmethylated (Table 6).

In control group; 28 (58.3%) samples were unmethylated and 20 (41.7%) were methylated (Figure 7) (Table 6).

In post-menopausal women; 32 (59.3 %) samples were unmethylated, while 22 (40.7 %) samples were methylated (Table 6).

The significant relationship between the methylation pattern of the *CLOCK* gene and menopause could not be found ($p > 0.05$) (Table 6).

Table 6 (Continued):

Methylation status of CLOCK gene in Pre-Menopause and Post-Menopause Subjects

CLOCK		**Menopause Status**			
		Pre-menopause	**Post-menopause**	**Total**	**p value**
Unmethylated	Observed	28	32	60	p > 0.05
	% within column	58.3%	59.3 %	58.8 %	
Methylated	Observed	20	22	42	

	% within column	41.7%	40.7 %	41.2 %
Total	Observed	48	54	102
	% within column	100.0%	100.0 %	100.0 %

Figure 6:

Universal Methylated Control and Universal Unmethylated Control for CLOCK Gene

The universal unmethylated control of the *CLOCK* gene was shown as purple, while universal methylated control the *CLOCK* gene was shown as blue. In Y axis fluorescence (dF/dT) is shown, while temperature (^{0}C) is shown in X axis

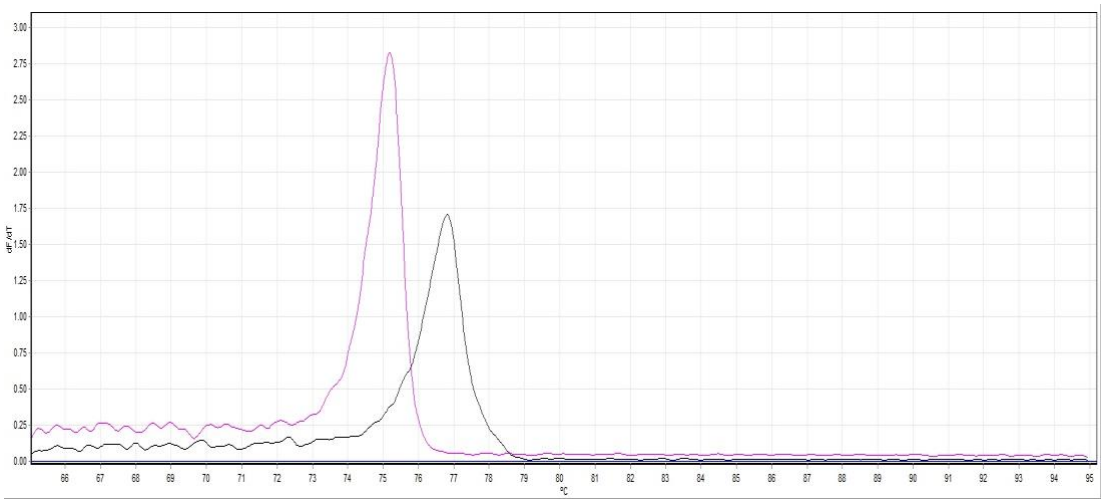
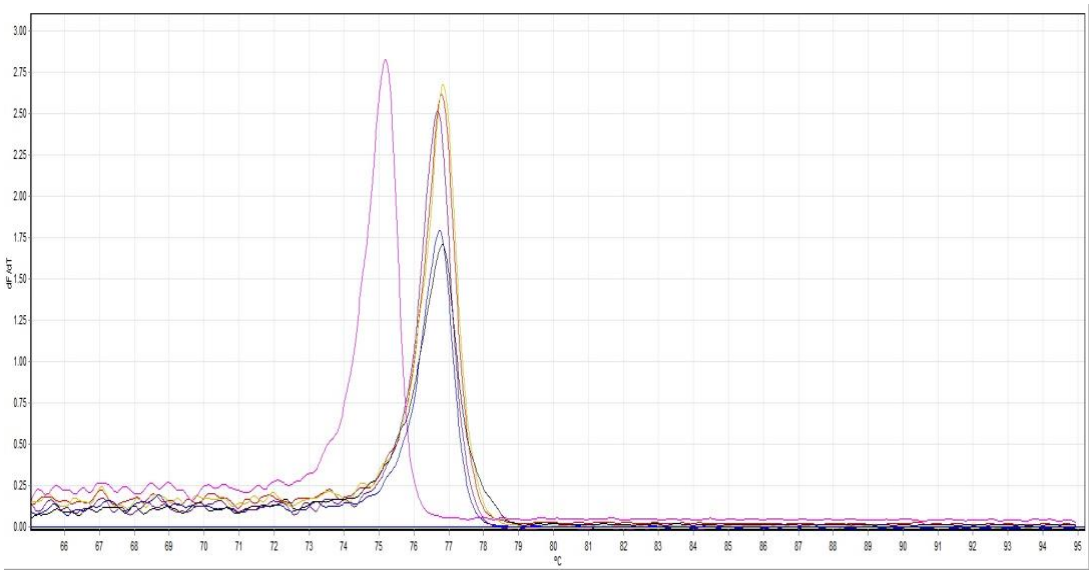


Figure 7 (Continued):

Methylated CLOCK Patients

The *CLOCK* unmethylated contol is indicated as pink, while methylated control is indicated as blue. Patients number 26, 27, 28, and 56 were methylated.



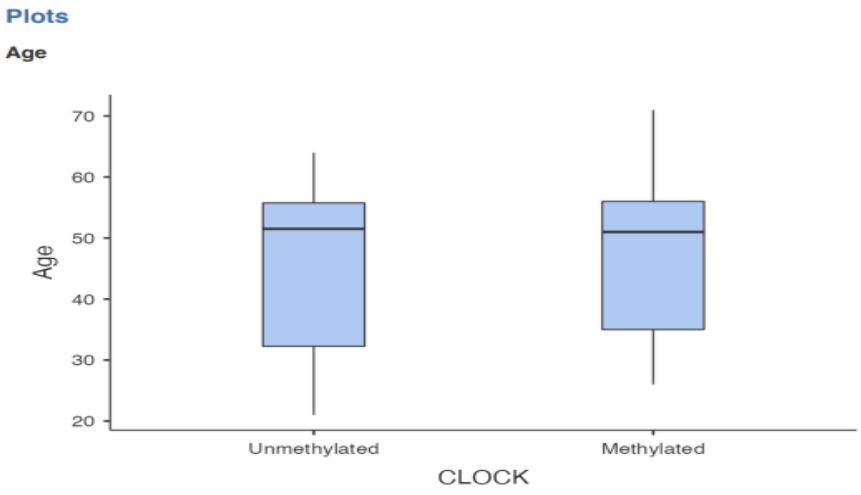
4.1.1. Interaction Between Age and *CLOCK* Methylation

The mean age and standard deviation of 60 unmethylated samples were 44.2 and 13.6 respectively. While the mean age and standard deviation of 42 methylated samples were 46.7 and 12.2 respectively (Graphic 1).

The significant relationship between the methylation pattern of the *CLOCK* gene and age could not be found ($p > 0.05$) (Table 6).

Graphic 1:

Plot Graphic of Age and CLOCK Methylation



4.2. Methylation Pattern of *ARNTL* Gene

As sum; 56 (56.0%) methylation and 44 (44.0%) unmethylation was detected in the *ARNTL* gene analysis (Table 7).

In control group (pre-menopause); 22 (50.0%) samples were methylated and 22 (50.0%) samples were unmethylated (Figure 8) (Table 7).

In post-menopausal women; 34 (60.7%) samples were methylated, while 22 (39.3%) samples were unmethylated (Figure 9) (Table 7).

The statistically significant correlation between the methylation pattern of the *ARNTL* gene and menopause could not be detected ($p > 0.05$) (Table 7).

Table 7:

Methylation Status of ARNTL Gene in Pre-Menopause and Post-Menopause Subjects

ARNTL		**Menopause Status**			
		Pre-menopause	**Post-menopause**	**Total**	**p value**
Unmethylated	Observed	22	22	44	$p > 0.05$
	% within column	50.0%	39.3 %	44.0 %	
Methylated	Observed	22	34	56	
	% within column	50.0%	60.7 %	56.0 %	
Total	Observed	44	56	100	
	% within column	100.0%	100.0 %	100.0 %	

Figure 8:

Unmethylated ARNTL Patient

ARNTL unmethylated control is shown pink, while methylated control is shown purple. Unmethylated *ARNTL* gene was detected in the patient number 50.

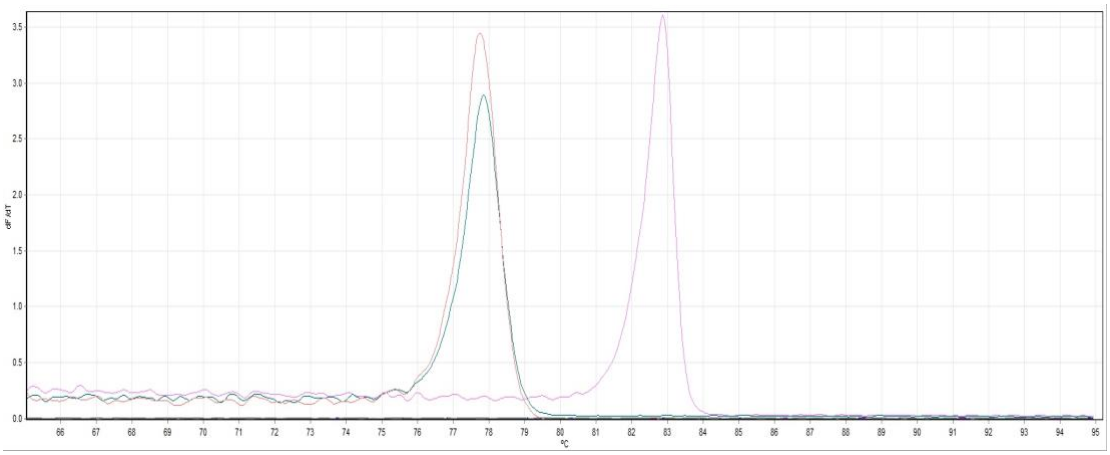
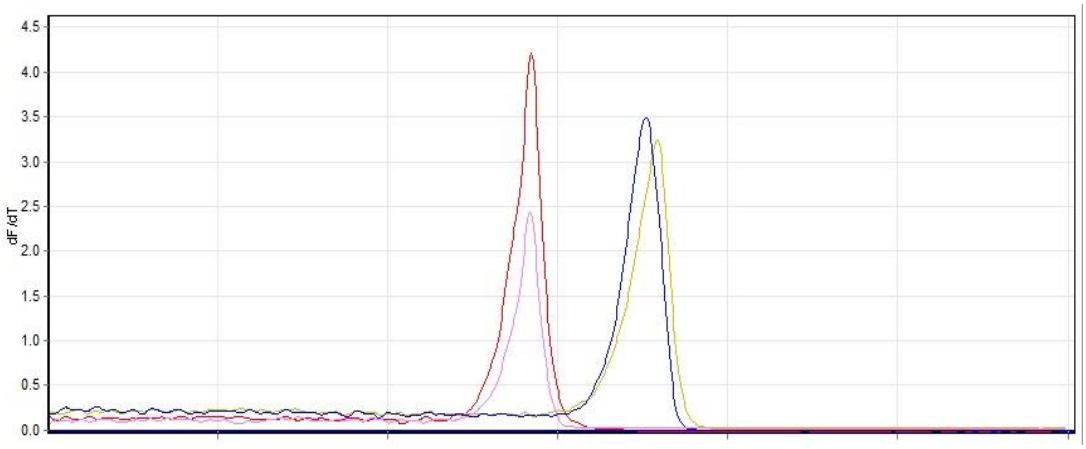


Figure 9:

Unmethylated and Methylated ARNTL Patients

The *ARNTL* unmethylated control was monitored as pink, while blue was monitored as universal *ARNTL* methylated control. Patient number 15 was unmethylated which shown as purple peak, while patient number 52 was methylated which shown as yellow.



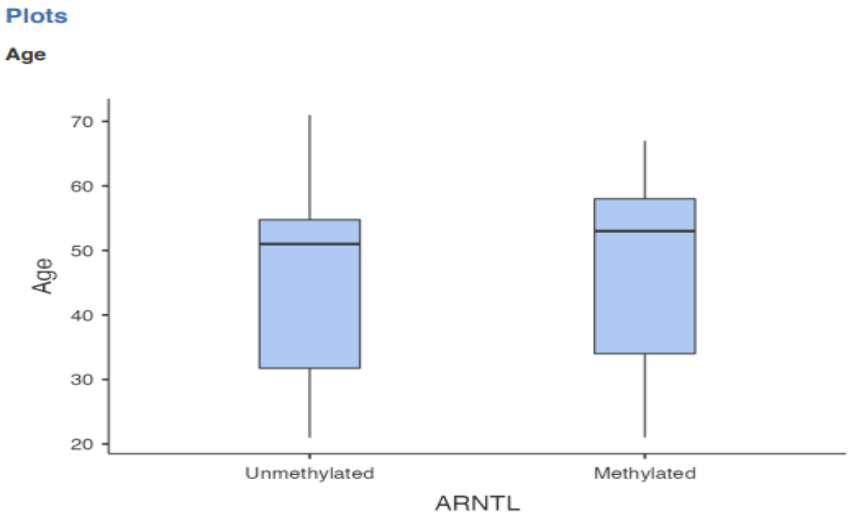
4.2.1. Interaction Between Age and *ARNTL* Methylation

The mean age and standard deviation of 44 unmethylated samples were 44.0 and 14.7 respectively. While the mean age and standard deviation of 56 methylated samples were 47.9 and 13.0 respectively (Graphic 2).

The statistically significant correlation between the methylation pattern of the *ARNTL* gene and age could not be monitored ($p > 0.05$) (Table 7).

Graphic 2:

Plot Graphic of Age and ARNTL Methylation



4.3.Correlation of Methylation Pattern of *CLOCK*, and *ARNTL* on Menopause

The association of the *ARNTL* and *CLOCK* genes was studied. Analyzed both two genes were unmethylated in 28 (63.6%) specimens, while methylated in 22 (45.8%) specimens (Table 8).

The *ARNTL* gene was methylated, while the *CLOCK* gene was unmethylated in 26 (54.2%) samples (Table 8).

The *ARNTL* gene was unmethylated, while the *CLOCK* gene was methylated in 16 (36.4%) samples (Table 8).

The significant relationship between the methylation pattern of *CLOCK* and *ARNTL* genes was not analyzed ($p > 0.05$) (Table 8).

Table 8:

The Interaction Between ARNTL and CLOCK Genes

ARNTL		CLOCK			
		Unmetylated	**Methylated**	**Total**	**p value**
Unmethylated	Observed	28	16	44	p > 0.05
	% within column	63.6%	36.4 %	100.0 %	
Methylated	Observed	26	22	48	
	% within column	54.2%	45.8 %	100.0 %	
Total	Observed	54	38	92	
	% within column	58.7%	41.3 %	100.0 %	

CHAPTER V

Discussion

The function of methylation of DNA in gene regulation was supported by different researchers. Currently, DNA methylation is the most known epigenetic mechanism that the activeness of genes is regulated (Moore, Le and Fan, 2012). The involvement of DNA methylation in imprinting disorders and cancer is demonstrated by several studies (Jin and Liu, 2018). Furthermore, according to recent studies DNA methylation take a part in autoimmune disorders, metabolic diseases, psychological diseases, obesity and ageing (Kalkan and Becer, 2019).

Epigenetic bio-markers of ageing based on methylation levels are reported in various articles, illustrating the reflectance of chronological age on DNA methylation levels. Levine and colleagues concluded that the age of menopause was substantially related to epigenetic age acceleration. In other words, earlier menopause correlates with raised epigenetic age due to reason of higher level of DNA methylation rate than the expected rate (Levine *et al.*, 2016). In our study, the association between the mean age of participants and their methylation and unmethylation status of the *ARNTL* and CLOCK genes was studied. The mean age of unmethylated *ARNTL* gene was 44.0 (mean ± Std. Deviation, 44.0 ± 14.7), and the mean age of methylated *ARNTL* gene was 47.9 (mean ± Std. Deviation, 47.9± 13.0). The mean age of unmethylated *CLOCK* gene was 44.2 (mean ± Std. Deviation, 44.2 ± 13.6), and the mean age of methylated *CLOCK* gene was 46.7 (mean ± Std. Deviation, 46.7± 12.2). According to the result of statistical analysis, there were no significant associations between the age of participants and their methylation status of the *ARNTL* and *CLOCK* gene were detected ($p > 0.05$).

The relation of the age of menopause and night shift workers was investigated by Stock and colleagues. The night worker women are at higher risk of menopause at an earlier age was concluded by Stock and colleagues. The risk of menopause at an earlier age is more apparent for females younger than 45 years old due to the fact that they face exposure to night shift work at current time and during their lives. The raised risk of being menopause an earlier age among night labourers is not supported as it is netley affected by the stimulating impact of circadian system deterioration on ovulation (Stock *et al.*, 2019). In our study group, neither the control group nor the menopause group has a night worker. According to this reason, we could not

investigate the relationship between the methylation status of the *CLOCK* and *ARNTL* genes with night workers who are in the stage of menopause.

The circadian rhythms are controlled by *BMAL1*, *CLOCK*, *PER*, and *CRY* genes. Epigenetic and genetic factors participate in the regulation of the circadian clock system (Saad *et al.*, 2021). Due to circadian rhythm's role in metabolism and physiological mechanisms, diseases such as cancer and metabolic syndrome can be detected when there is a deterioration in the system (Pines, 2016). As far as, the polymorphisms of the *BMAL1* and *CLOCK* genes and their actions on menopause have been analyzed by various researches. Semenov and colleagues analyzed (*CLOCK*) 3111T/C gene polymorphism in the participants who are in the stage of menopause and control group. They did not find any differences in *CLOCK* 3111T/C genotypes or allele frequency between the control group and main groups (Semenova *et al.*, 2020).

Melatonin concentrations decline with age as a result of decreased pineal gland function, that is the result of alterations occurring in the pineal gland and other parts of the circadian system during physiological aging (Tan *et al.*, 2018). The melatonin level, at the beginning of the day of Caucasian women who with *CLOCK* 3111C allele was identified as lower compared to women who are the carrier of the *CLOCK* 3111T allele (Semenova *et al.*, 2018).

With age, the circadian clock-controlled genes which act in the regulation of the circadian system lose their responsiveness. Due to this reason, impairment in homeostasis is encountered and it is exacerbated in females who are in the menopausal transition stage. Therefore, José Hernandez-Morante and colleagues studied to investigate if the expression of circadian genes in adipose tissue is affected by the consequence of menopause and relationship between the modification of circadian genes expression on metabolic syndrome. In subcutaneous adipose tissue, the *PER3* expression level of women who are in menopause is 42% higher than their counterparts who are in the premenopausal stage was concluded by José Hernandez-Morante and colleagues. Furthermore, the *PER2* expression was detected as 21% higher in the women who are in the stage of post-menopause compared to counterparts who in the pre-menopause stage (José Hernandez-Morante *et al.*, 2012). While the role of circadian gene polymorphisms on breast cancer was analyzed by Se Truong and colleagues, the significant relation with breast cancer in post-menopausal women and rs11932595 polymorphism of the *CLOCK* gene was reported. According

to further observations relation of this polymorphism and premenopausal women was not monitored (Se Truong *et al.*,). The raised risk of breast cancer on the women whose work shift is night and have *BMAL1* rs2290035, rs969485 SNPs was concluded by Zienolddiny and colleagues. Furthermore, the *CLOCK* rs111133373 SNP was demonstrated as causing to the risk of breast cancer to be increased on night worker women (Zienolddiny *et al.*, 2013).

In our study, the methylation status of the *CLOCK* and *ARNTL* genes in both premenopausal and postmenopausal women were identified. We detected the *ARNTL* promoter methylation in 50.0% premenopausal women, and 60.7% in postmenopausal women. *ARNLT* gene was unmethylated 50.0% of premenopausal patients, and 39.3% of postmenopausal patients. We determined the *CLOCK* promoter methylation in 41.7% of women who in the period of perimenopause, and 40.7% of women who in the period of postmenopause. On the other hand, the unmethylation of the *CLOCK* gene was analyzed in 58.3% in control samples, and 59.3% in menopause samples. Both of the genes were unmethylated in 63.6% of participants, while methylated in 45.8% of participants. The *ARNTL* gene was unmethylated, while the *CLOCK* gene was methylated in 36.4% of patients. On the other hand, the *ARNTL* gene was methylated, the *CLOCK* gene was unmethylated in 54.2% of patients. The correlation of the methylation pattern of analyzed two genes and menopause, and also an important relationship between the methylation pattern of the analyzed two genes could not be found statistically ($p > 0.05$).

CHAPTER VI

Conclusion and Recommendations

The circadian rhythm and epigenetic modifications have an important effect on the life of women. The involvement of epigenetics and circadian rhythms in menopause was shown in various articles. In this study, the relationship between the methylation patterns of the *ARNTL* and *CLOCK* genes which are circadian clock genes, and menopausal conditions could not be encountered. Moreover, the correlation of the methylation pattern of the *ARNTL* and *CLOCK* genes with age could not be detected. Furthermore, the correlation of methylation pattern of the *ARNTL* gene and the *CLOCK* gene's methylation pattern could not be monitored.

The circadian rhythm takes part in the regulation of metabolism and physiological system. In the malfunctioning of circadian rhythm; disrupted sleep-wake cycles, depression, and mood disorders are encountered. Physical disturbances, depression, sleeplessness, raised fat mass, corruption of the lean body, etc… are the discomforts are encountered in the stage of menopause. According to these findings, the consequences of menopause and malfunction of circadian rhythms are almost observed identical. Our study is the first study in which the association of the methylation status of the *ARNTL* and *CLOCK* genes in menopause was investigated. This study will be the basis for future studies and will shed light. In future, we will examine the relationship between the methylation status of other circadian system genes. In addition to methylation analysis, polymorphisms of circadian system genes are planned.

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APPENDICES

YAKIN DOĞU ÜNİVERSİTESİ
BİLİMSEL ARAŞTIRMALAR ETİK KURULU

ARAŞTIRMA PROJESİ DEĞERLENDİRME RAPORU

Toplantı Tarihi : 29.07.2021
Toplantı No **:** 2021/93
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Yakın Doğu Üniversitesi Tıp Fakültesi öğretim üyelerinden Doç. Dr. Rasime Kalkan'ın sorumlu araştırmacısı olduğu, YDU/2021/93-1380 proje numaralı ve **"Sirkadyen ritim genlerinin metilasyonu ve menopoz ile arasındaki ilişkinin araştırılması"** başlıklı proje önerisi kurulumuzca online toplantıda değerlendirilmiş olup, etik olarak uygun bulunmuştur.

Y

Prof. Dr. Rüştü Onur
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February 2020-Current	Master	Near East University	Medical Biology and Genetics