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

INVESTIGATION OF THE RELATIONSHIP BETWEEN THE METHYLATION OF TRIM3 GENE AND MENOPAUSE

M.Sc THESIS

Zuhal Alfadil Osman SAAD

Nicosia
June, 2022

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Supervisor
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Nicosia
June, 2022

Approval

We certify that we have read the thesis submitted by Zuhal Alfadil Osman Saad titled "**Investigation of the Relationship Between Methylation of *TRIM3* Gene 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.

Zuhal Alfadil Osman Saad

16/06/2022

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Abstract

The Investigation of the Relationship Between Methylation of *TRIM3* Gene and Menopause

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Age-related hormone declines in women cause the follicular activity of their ovaries to slow down and becomes insufficient as they age. The ovarian reserve declines with age because the number of oocytes are finite and cannot be increased. After a while, the condition of menopause which is defined as twelve months of amenorrhea occurs. Numerous studies have shown that genetics and epigenetics are both involved in the menopause process.

The objective of the study is to determine the methylation status of the *TRIM3* gene in postmenopausal women. A total of 60 postmenopausal women and 54 premenopausal (control) women provided DNA samples. Methylation Specific High-Resolution Melting (MS-HRM) was used to determine the pattern of methylation in the *TRIM3* promoter region. In this study, no statistically significant interaction between *TRIM3* gene methylation status and menopause was observed.

Keywords: epigenetics, menopause, trim3, methylation

Özet

TRIM3 Metilasyonu ile Menopoz Arasındaki İlişkinin İncelenmesi

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Kadınlarda yaşa bağlı hormon düşüşleri, yumurtalıklarının foliküler aktivitesinin yavaşlamasına ve yaşlandıkça yetersiz kalmasına neden olmaktadır. Yumurtalık rezervi yaşla birlikte azalmaktadır. Bu sürenin sonunda on iki aylık adet görmeme olarak tanımlanan menopoz durumu ortaya çıkmaktadır. Çok sayıda çalışma, hem genetik hem de epigenetik değişikliklerin menopoz sürecinde yer aldığını göstermiştir.

Bu çalışmada, menopoz sonrası dönemde olan kadınlarda *TRIM3* geninin metilasyon durumunun saptanması amaçlanmıştır. Çalışmaya dahil edilen, 60 postmenopozal kadından ve 54 premenopozal katılımcıdan DNA izolasyonu yapılmıştır. TRIM3 promotör bölgesindeki metilasyon paternini belirlemek için Metilasyona Özgü Yüksek Çözünürlüklü Erime Eğrisi analizi (MS-HRM) kullanılmıştır. Bu çalışmada, *TRIM3* gen metilasyon durumu ile menopoz arasında istatistiksel olarak ilişkili olmadığı saptanmıştır.

Anahtar kelimeler: epigenetik, menopoz, trim3, metilasyon.

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List of Abbreviations

DNA: Deoxyribonucleic Acid

RNA: Ribonucleic Acid

ncRNA: Non-Coding RNA

***TRIM*:** Tripartite Motif-Containing Protein

***TRIM3*:** Tripartite Motif-Containing Protein

5mC: 5-Methylcytosines

DNMT: DNA Methyltransferases

ATP: Adenosine Triphosphate

H2A(X): H2A Histone Family Member X

siRNAs: Small Interfering RNAs

miRNAs: MicroRNAs

mRNA: Messanger RNA

3'-UTR: Three Prime Untranslated Region

IncRNA: Long Non-Coding RNAs

HPG: Hypothalamic Pituitary Gonadal

GnRH: Gonadotropin Releasing Hormone

LH: Luteinizing Hormone

FSH: Follicular Stimulating Hormone

POF: Premature Ovarian Failure

POI: Premature Ovarian Insufficiency

IFN: Interferon

E3: Estriol

CBL: Casitas B-lineage Lymphoma

UBE21: Ubiquitin-Conjugating Enzyme 21

PML: Progressive Multifocal Leukoencephalopathy

HIV: Human Immunodeficiency Virus

MID1: Midline1

ADHD: Attention-Deficit Hyperactivity Disorder

RBCC: Ring-B-box-Coiled-Coil

E2: Estradiol

ER-alpha: Estrogen Receptor-Alpha

EL: Erythrocyte Lysis

PCR: Polymerase Chain Reaction

RT-PCR: Real Time-Polymerase Chain Reaction

BW: Wash Buffer

BD: Desulfonation Buffer

MS-HRM: Methylation Sensitive – High Resolution Melting

dNTP: Deoxyribose Nucleotide Triphosphate

Std: Standard Deviation

dsDNA: Double Stranded DNA

ssDNA: Single Stranded DNA

CHAPTER I
Introduction

Epigenetics refers to inheritable changes in gene expression that do not result in alterations in DNA (deoxyribonucleic acid) sequence (Zovkic, 2021). Epigenetic changes can be reversed and don't change the DNA sequence. Epigenetic modifications, such as chemical alteration to DNA and histone proteins are regulate and control gene expression (Aboud et al., 2021). DNA methylation, RNA modifications, non-coding RNA, and histone modifications are the main types of epigenetics alterations.

Epigenetic modifications are critical for cellular identity and function (Lei et al., 2020). Epigenetic modifications can be influenced by various factors, including physiologic, pathologic stimuli and environmental factors such as stress, physical activities, age, eating habits, tobacco usage, and alcohol consumption (Pagiatakis et al., 2021). Studies have demonstrated that epigenetic processes play a significant role in developing various pathological mechanisms such as aging, diabetes mellitus, coronary artery disease, and hypertension (Lei et al., 2020).

Hormones are substances inside every multicellular organism which acts as a chemical messenger and affect many different processes in human. In females, hormones play an essential role throughout a female's life, starting from puberty, going through the chance to experience being a mother, and the possibility to play cardioprotective role. Hormonal disturbance leads to emotional, physical, and biological changes, some of these changes can be temporary, and others may last for an extended period of time. Regardless of their cultural background or health state (Hajj et al., 2020). Menopause is believed to be the point in time after twelve continuous months of amenorrhea; this long-term absence of menses is caused by a lack of ovarian follicular activity (Greendale et al., 1999).

In the years preceding up to menopause, the most important clinical findings that have been recorded about the effect of menopause are sleeplessness, changes in monthly periods, hot flashes, sadness, and weariness, which all are examples of the physiological and psychological difficulties that women go through during the period before the menopause (Hajj et al., 2020). This period is time is called the menopausal transition period and also known medically as perimenopause; this transition usually occurs between the ages of mid-forties to mid-fifties (Hajj et al., 2020).

Researchers discovered that the onset of menopause is influenced by epigenetic age. It was found that earlier menopause is associated with higher epigenetic age, owing to higher levels of DNA methylation than expected (Levine et al., 2016).

TRIM-3 gene is a protein-coding gene that is located in chromosome 11p15.4, the protein coded by this gene is called Tripartite motif-containing protein 3 hence the name (Ozato et al., 2008). In recent years, tripartite motif (TRIM) proteins have been extensively researched as essential regulators of a wide range of biological processes, in different signaling pathways (Yang et al 2020).

TRIM-3 is considered a tumor suppressor gene; for example, in colorectal cancer progression, it acts as a tumor suppressor. *TRIM3* is required for the activation of estrogen signaling in whole genomic scale (Zhuang et al., 2022)

The aim of this study is:

(a) to determine the methylation level of the *TRIM-3* gene

(b) to investigate the effect of the methylation pattern of this gene on menopause

The research question is as follows, in perspective of the study's goal of determining the association between methylation of the *TRIM-3* gene and menopause:

(a) is there any relationship between the methylation status of the *TRIM-3* gene and Post-menopausal term?

In literature, the possible effects of the methylation pattern of the *TRIM-3* gene during menopause; have not been shown yet. This study will be the first to show the relationship between the methylation status of the *TRIM-3* gene and menopause.

CHAPTER II
General information

2.1 Epigenetic

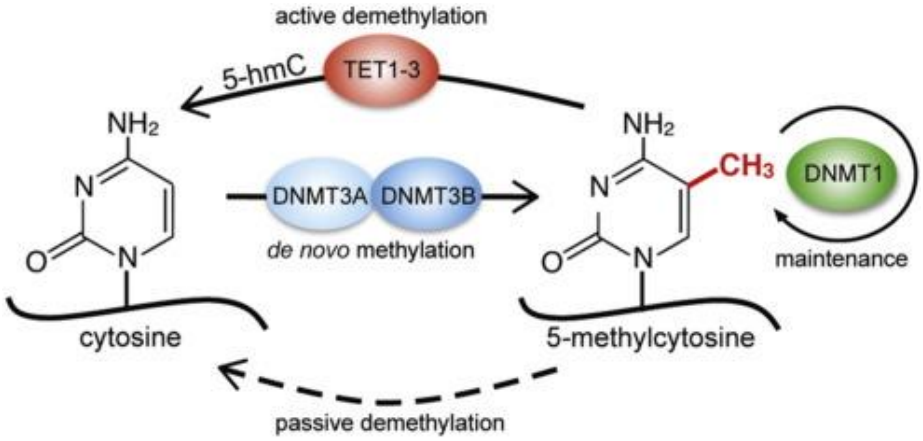
Epigenetics refers to heritable or acquired alterations in the expression of gene level that do not result in any changes in DNA sequence (Strachan and Read, 2010). Epigenetic alterations are changes to DNA that control whether genes are turned on or off. These changes are applied to DNA and have no effect on the sequence of the DNA (Esteller, 2008). Different epigenetic patterns exist in individuals, tissues within persons, and even cells within tissues. From the implantation of the embryo to the death of the individual, epigenetic processes play a critical part in the establishment of every biological condition in our body system (Moosavi and Ardekani, 2016). The main epigenetic modifications are chromatin remodeling, histone modification, DNA methylation, non-coding RNA (ncRNA)-associated gene silencing, and RNA modifications (Lei et al., 2020).

2.1.1 DNA methylation

The process of covalently attaching methyl groups to DNA is known as methylation. (each methyl group consist of three hydrogen atoms and one carbon atom) to DNA building blocks (Esteller, 2008). DNA methyltransferases (DNMTs) enzymes convert cytosines to 5- methylcytosines (5mC) by attaching to the C5 carbon of cytosine phosphate guanine dinucleotides (CpGs). In humans, three DNMT enzymes are found: DNMT1, DNMT3A, and DNMT3B (Loaeza et al., 2020). DNMT3A and DNMT3B maintain the de novo methylation while DNMT1 keeps the methylation of DNA (Figure 1) (Gardini, 2020). DNA methylation is considered the most common epigenetic mechanism (Weinhold, 2006).

Figure 1

DNA Methylation (Ambrosi et al., 2017)



2.1.2 Chromatin remodeling

Chromatin remodeling, also known as the regulated alteration of chromatin structure, it can be carried out in two different ways: either by the covalent modifications of histones or by the function of ATP-dependent remodeling complexes (Jeff et al., 2000). There are several different processes that can be utilized to modify chromatin. Some of these processes work broadly while others work locally on a single nucleosome. It is critical to establish a direct link between in vivo remodeling activities and in vitro remodeling of complex molecular capabilities (Jeff et al., 2000). Chromatin remodeling factors lead to the gene being silenced by the structure of chromatin is immensely packed through organizing the nucleosomes on the DNA; therefore, access to the transcription proteins is prevented (Pagiatakis et al.,2021).

2.1.3 Non-coding RNA (ncRNA)

Non-coding RNAs (ncRNAs) are regulatory molecules involved in gene silencing. They include miRNA (microRNAs), siRNA (small interfering RNAs), PIWI-interacting RNAs, and a variety of long ncRNAs (Taft et al.,2010). These RNAs now clearly serve as transcriptional and post-transcriptional regulators, as well as guides for chromatin-modifying complexes (Taft et al., 2010). 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 significant 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 gene expression is inhibited by the joining of miRNAs to the untranslated region of (3'- UTR) messenger RNAs (mRNAs), resulting in deterioration of target messenger RNA and suppression of subsequent protein translation processes (Pagiatakis et al., 2021).

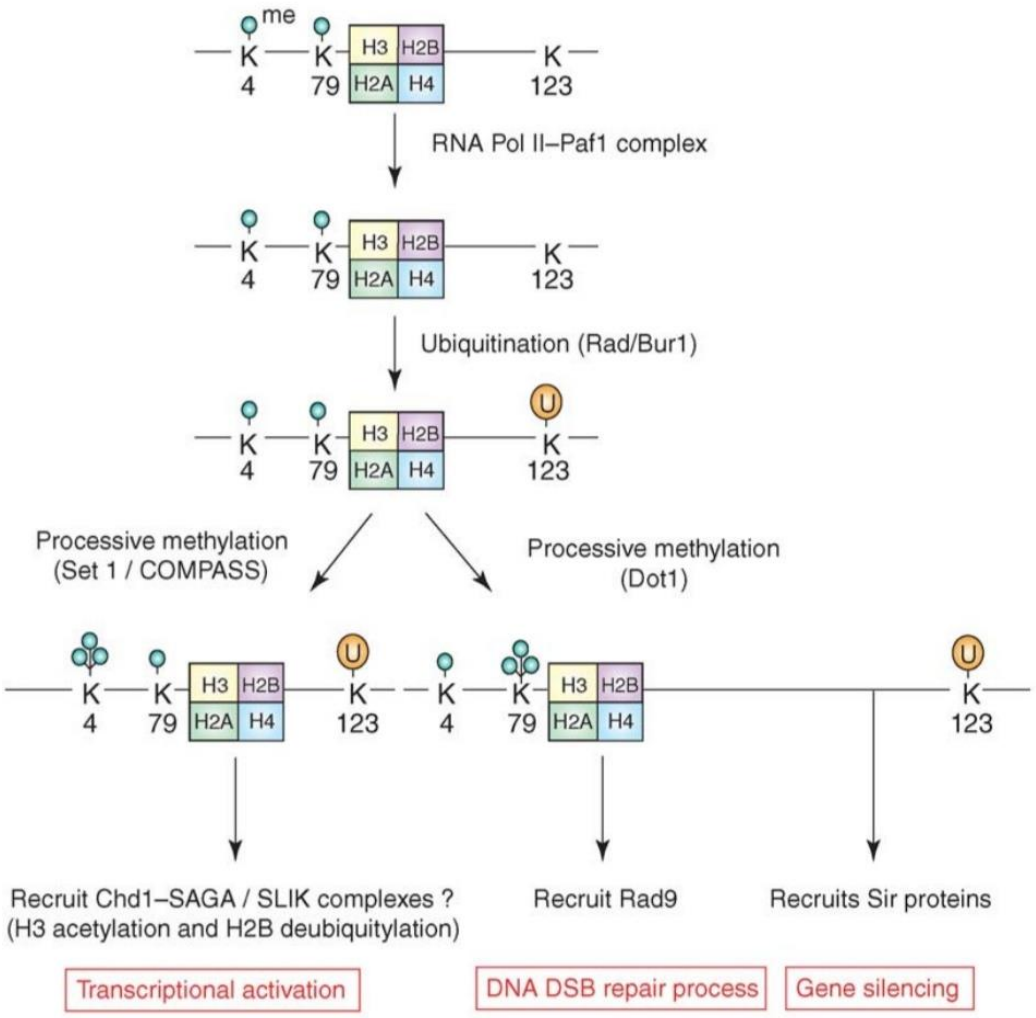
2.1.4 Histone modification

Histones are cellular structural proteins present in the nucleus. The way DNA wraps around histones determine the structure of chromosomes (Esteller, 2008). Histones can be altered by removing or adding several chemical groups such as acetyl and methyl groups (each consisting of one oxygen atom, two carbon, and three hydrogens) (Esteller, 2008).

The post-translational modification of histone proteins is; phosphorylation, acetylation, ubiquitination, and methylation (Figure 2) (Aboud et al., 2021) (Weinhold, 2006). These modifications play a key role in DNA packaging (Berger et al., 2002). Histone acetylation consists of a lysine residue, which is charged as positive, which leads to weakness in the interplay of DNA and histone (Gujral, 2020). Therefore, the structure of chromatin is opened, and transcription is activated. By histone methylation, one, two, or three methyl groups are added to lysine while one or two 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 (Whetstine, 2010). 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 ubiquitination, and it plays a role in both gene silencing and transcription. (Li et al., 2020).

Figure 2

Histone Modifications (Nightingale et al., 2006).



Errors in the epigenetic process can lead to alterations in gene activity or expression status (Esteller, 2008). Epigenetic aberrations have been associated with diseases like metabolic disorders, cancers, degenerative problems (Esteller, 2008), obesity (Kalkan and Becer,2019) and post-menopausal vasomotor symptom (Kalkan et al., 2020).

2.2 Menopause

The hypothalamic-pituitary-gonadal (HPG) axis controls the female reproductive system (Shao et al., 2021). The hypothalamus secretes gonadotropin-releasing hormone (GnRH), which regulates the ovulation cycle. The pituitary is stimulated by GnRH to produce and secrete follicular stimulating hormone (FSH) and luteinizing hormone (LH) (Goldstein and Smith, 2016). The loss of ovarian follicular function,

which results in permanent cessation of menstruation, is termed menopause. It is also thought to be the point in time after twelve continuous months of amenorrhea (Greendale et al., 1999).

Estrogen and progesterone play a critical role in the female reproductive system (Nelson et al., 2001). Besides affecting the reproductive system, estrogen affects the heart, blood vessels, bones, breast, brain, and other organs (Satoskar et al., 2017). Progesterone is crucial for the menstrual cycle and also the maintenance of the early stages of pregnancy (Conneely et al.,2002). Generally, any disturbances in hormonal levels have consequences (Bishop. ,2017). Alteration in estrogen and progesterone levels are associated with irregular menses and/or amenorrhea. The effects of low levels of estrogen and progesterone have been observed in the perimenopausal phase (Greendale et al., 1999). Perimenopause, often known as the menopausal transition, occurs when the body begins the process of shifting to menopause (Burkman, 2002). Alteration in hormone levels during this transition, lead menstrual cycle to become inconsistent or irregular (Burkman, 2002). Clinically, the symptoms of this hypo-production of hormones during the menopausal period of time are mood swings, bones that are weak or fragile, finding trouble concentrating, night sweats (Bishop, 2017), and hot flashes (Kalkan et al., 2020), missing menstrual periods (Bishop, 2017) osseous loss, corruption of lean body mass, and raised fat mass are also figured out (Prakash et al., 2021). In addition to physical changes, social, emotional, and familiar variables may have a role in the establishment of symptoms during menopause (Huerta et al.,1995).

2.2.1. Early menopause

Menopause that occurs between the ages of 40 and 45 is known as "early menopause". Several genetic and environmental factors interact to influence the onset of early menopause, making it an extremely complex and variable condition (Fu et al., 2019). Women who reach menopause at a younger age than the average are estimated to account for 5% of the population. Premature menopause, also known as premature ovarian insufficiency (POI) or premature ovarian failure (POF), affects approximately 1% of women before the age of 40 (Qin et al., 2015). Although, there are a variety of causes for spontaneous early menopause or premature menopause, including autoimmune diseases, infections, enzyme deficiencies, inflammatory conditions, or metabolic abnormalities (Shuster et al.,2010). Early menopause has

also been demonstrated to be influenced by genetic diseases or mutations (Fu et al., 2019). Autoimmune diseases, Turner syndrome, Fragile X syndrome, chemotherapy, and radiation have all been linked to early menopause (Bachelot et al., 2009) (Luisi et al., 2015). Non-genetic variables, on the other hand, only account for a minor percentage of early menopause. Menopause onset age is significantly heritable, with heritability scores ranging from 44% to 66 %, according to family and twin studies (Murabito et al., 2005) (Van et al., 2004).

2.3 *TRIM* genes

The tripartite motif (*TRIM*) proteins are a medium-sized post-translational modifier subfamily (Reymond et al., 2001). *TRIMs* are classified into thirteen subtypes based on their amino-terminal tripartite domain arrangement - RING–Bbox1/2–coiled-coil (RBCC) - but their carboxy-terminal domains are different (Torok et al., 2001). Despite domain arrangement, *TRIM* proteins play a role in a variety of biological processes. These roles include DNA repair, immunological and cell stress response modulation, proliferation, differentiation, transcription, viral restriction, and apoptosis (Ozato et al., 2008). The inclusion of a RING domain promotes the biological flexibility of TRIM proteins by facilitating protein conjugation with ubiquitin, the small ubiquitin-like modifier SUMO, or the ubiquitin-like molecule IFN-stimulated protein of 15 kDa (ISG15) (Ozato et al., 2008). Furthermore, *TRIM* proteins commonly form large protein complexes that reside in different cytoplasmic nuclear sub-compartments via their coiled-coil domains (Reymond et al., 2001). *TRIM* proteins play a vital function in a wide variety of other neurological diseases, cancer, and play a key role in human defensive capabilities against pathogens (Meroni et al., 2005). *TRIM* family members are found in varying numbers in different species. Humans and mice have the largest families. However, due to the different domain makeup of the *TRIM* family members, the actual number of TRIM family members in each species is unknown. *TRIM* genes evolved independently, although having a common ancestor, as indicated by their extensive distribution everywhere across the genome and attainment of species-specific activities (Ozato et al., 2008).

Different *TRIMs* can work together to modulate the immune response, and also have many activities (Yang et al., 2020). The evolution of both innate and adaptive immunity is closely linked to the expansion of *TRIM* genes. Thus, it seems apparent

that *TRIM* family proteins evolve as regulators to maintain optimum immune responses to remove pathogen infections without causing autoimmunity, considering the numerous activities of *TRIM* proteins in both innate and adaptive immunity (Yang et al., 2020).

2.3.1 *TRIMs* and cell cycle

Cell cycle progression and mitosis are controlled by *TRIM* proteins at various points throughout the process. Delaying the expression of *TRIMs* during the cell cycle triggers cell proliferation and causes cells more resistant to death signals. *TRIM* proteins are most important during mitosis (Venuto et al., 2019). The *TRIMs* superfamily has been implicated in the regulation of key components of the mitotic spindle machinery, such as kinetochores, centrosomes, and mid-bodies, during mitosis. These components are necessary for optimal chromosomal orientation and segregation (Venuto et al., 2019)

2.3.2 The ring domain

N-terminal regions of practically all *TRIM* proteins contain the zinc-binding motif, the RING domain, which can be found within 10–20 amino acids of the first methionine (Torok et al., 2001) (Reymond et al., 2001). The general understanding of RING domain activity stems primarily from investigations of the RING domain-containing protein CBL (Casitas B-lineage lymphoma), which demonstrated that RING domains mediate ubiquitination events (Joazeiro et al., 1999) (Waterman et al., 1999). Recently, the crystal and solution structures of the RING domains of members of the *TRIM* family were identified (Stevens et al., 2019).

Numerous members of the *TRIM* family, including *TRIM5, TRIM8*, *TRIM11, TRIM21* (also known as RO52), *TRIM22*, and *TRIM25*, have been shown to have E3 ubiquitin-ligase activity **(**Sabile et al.,2006). Innate immune receptor stimulation triggers the E3 ubiquitin ligase activity of several *TRIM* proteins, which has now been shown to be critical for the anti-HIV actions of these proteins and for regulating their effects on signaling cascades. When conjugated with ISG15, *TRIM25* can additionally change itself and other proteins in a ring domain unrelated manner (Zou et al., 2006) (Zou et al. 2007). Another intriguing finding from this study is that *TRIM63* and PML both have the ability to connect with UBE2I (ubiquitin-

conjugating enzyme E2I), a SUMO-conjugating enzyme, which shows that they are engaged in sumoylation (Meroni et al., 2005).

2.3.3 The B-box domains

The B-box domains are likewise zinc-binding motifs, with different consensus sequences for B-box 1 and B-box 2 among *TRIM* superfamily members. Both of these components can be found in *TRIM* proteins, however, B-box 1 have never been seen without B-box 2. The fact that several human TRIM B-box 1 and B-box 2 domains have ternary structures that are similar to RING domains demonstrates that all three domains originated from a same ancestor domain (Ozato et al., 2008).

The development of a genetic disease with a mendelian inheritance pattern, in addition to innate resistance to HIV, have both been linked to B-box domains. B-box 2 has been shown to change the way the viral capsid is recognized by the host (Li et al., 2007) (Brass et al.,2008). X-linked Opitz G/BBB syndrome individuals have also been found to have mutations in both B-box domains of *TRIM18* (known as Midline1; MID1) (Ferrentino et al., 2007). In patients with Opitz G/BBB syndrome, the most prevalent changes are located in the C-terminal fibronectin type 3 and PRYSPRY domains (Schweiger and Schneider, 2003).

2.3.4 The coiled-coil domains

Coiled-coil domains are a common motif in many protein families. *TRIM* family members and other proteins interact via this domain in both homomeric and heteromeric ways, particularly self-association. According to sequence and structural investigations, these domains have a variety of topologies, ranging from three contiguous but non-continuous coiled subdomains to a single continuous coil (Ozato et al., 2008). A crucial function in *TRIM5α*'s ability to limit viral infectivity has been identified in its coiled-coil domain (Javanbakht et al., 2006).

2.3.5 The PRY and SPRY domains

The most common C-terminal sequences identified in *TRIM* family members are the 140-amino-acid SPRY domains and 61-amino-acid PRY. These domains can be identified in 11 different protein families of humans; however, they are most commonly seen in members of the *TRIM* family (Woo et al., 2006). The SPRY domain is found by itself in 39 members of the human *TRIM* family, and the SPRY

domain is joined to the PRY domain to create the PRYSPRY domain. The evolutionary history of the SPRY domain is shared by animals, plants, and fungi. Humans, chickens, frogs and mice are the only vertebrates with the PRYSPRY domain (Rhodes et al., 2005).

2.3.6 Classification of *TRIM* genes

In regard to the RBCC motif36, the sequences that make up the C-terminal region and their composition was used to classify human *TRIM* family members (Short and Cox, 2006). C-terminal sections can contain any of ten different motifs, either alone or in combination, giving rise to nine families (Short and Cox, 2006). In this classification method, the relationship between domains and cellular localization, expression, and function is emphasized (Short and Cox, 2006).

The *TRIM* genes encode a large number of mRNAs and proteins that are distributed among different tissues (Reymond et al., 2001). As a result of variable splicing of multiple *TRIM* genes, different subcellular localization and protein-protein interactions may occur (Reymond et al., 2001). Moreover, post-translational modifications, such as ubiquitination, phosphorylation, and sumoylation, can have a significant impact on protein expression levels for particular isoforms (Caglioni et al., 2006).

2.3.7 *TRIM* genes and diseases

Abnormalities in *TRIM* gene are involved in developmental disorders, chromosomal abnormalities, cardiovascular and metabolic disorders, and most importantly, associated with neuropsychiatric disorders such as Alzheimer's disease, attention deficit hyperactivity disorder (ADHD), multiple sclerosis, and schizophrenia (Watanabe and Hatakeyama, 2017). Variations in *TRIM* gene have been linked to a wide range of disorders, according to increasing evidence. It has not yet been understood how the mutations create abnormalities, even though it has been revealed they are crucial for the etiology of each disease. Therefore, understanding the molecular mechanisms behind the observed events is critical. Synonymous variations and changes in intergenic areas have long been known to affect phenotype via affecting splicing, mRNA export, transcription and translation efficiency. Mutations in the *TRIM* gene have the potential to alter gene expression levels. The *TRIM* proteins can be altered physiologically in numerous ways when amino acid

sequences are changed by non-synonymous mutations (Watanabe and Hatakeyama, 2017). The non-synonymous mutations in *TRIM* proteins have the potential to cause diseases or influence their symptoms by altering polyubiquitin chains on their targets.

2.4 *TRIM-3* gene

TRIM-3 gene is a protein-coding gene that is located in chromosome 11p15.4. The protein generated by this gene belongs to tripartite motif (*TRIM*) family of RING finger proteins, also known as the 'RING-B-box-coiled-coil' (RBCC) subgroup. A coiled-coil region is one of the components of the TRIM motif, along with three zinc-binding domains, a RING, a B-box type 1, and a B-box type 2. This protein is thought to be involved in cargo transport mediated by myosin V. There have been reports of transcript variations that have been alternatively spliced yet still encode the same isoform. It acts as a tumor suppressor gene that appears to be associated with many cancers, such as breast cancer, gastric cancer, and liver cancer (Wang, 2020). In terms of domain structure and genetic organization, the *TRIM* family is divided into two subgroups (Sardiello et al., 2008). Members of Group 1, which can be found in both vertebrates and invertebrates, are distinguished by their diverse C-terminal domains. Invertebrates lack Group 2, but they do have a C-terminal SPRY domain (Ponting et al., 1997). *TRIM3* gene is a member of the first group. *TRIM3* gene has a wide distribution as it is expressed in many different tissues, such as the duodenum, kidneys, ovary, testis, and 22 more tissues.

2.4.1 *TRIM3* and menopause

Menopause is described as the cessation of menstruation, which corresponds to the termination of ovulation due to ovarian follicle loss (Gold, 2011). The normal menopausal average age is 51, but it varies from one female to another, but the main cause of this variation remains unclear. Environmental variables are thought to play a significant role in menopausal age. Tobacco usage, dietary habits, and body fat composition have all been identified as modifiable risk factors for menopause (Cramer and Xu, 1996) (Gold, 2011). While genetic variables do have an effect on the age of menopause, family studies have revealed that inheritable disorders affecting the timing of menopause also vary (Gold, 2011). The estrogen hormone binds to a receptor known as the estrogen receptor alpha (ER), which is a member of the nuclear receptor superfamily. The ER alpha protein is 595 amino acids in length

and is composed of three functional domains: a ligand-binding domain, a DNA-binding domain, and a transcription activation domain (Renaud et al., 2005). When 17-estradiol (E2) activates ER-alpha, the ER-alpha protein may translocate into the nucleus and bind to the promoter regions of ER-alpha target genes, resulting in an increase in the production of downstream target genes (Zhuang et al., 2022). A study was done on breast cancer patients, and it showed that the transcription factor *TRIM3* is necessary for the establishment of ER-alpha-positive breast cancer. It also showed that estrogen signaling is coordinated by *TRIM3* (Zhuang et al., 2022). Since the *TRIM3* gene can affect estrogen signaling, we can suggest that maybe it has an effect associated with menopause.

CHAPTER III

Methodology

3.1 Study case

A total of 60 post menopause and 54 pre menopause women were enrolled in this study. Cengiz Topel Government Hospital gathered blood specimens of participants from the Department of Obstetrics and Gynecology.

Participants' smoking, alcohol, and physical activity habits were carefully assessed, as well as their exposure to exogenic hormones such as birth control pills and hormone replacement therapy throughout the postmenopausal stage.

The inclusion criteria: All women in the postmenopausal stage should have been in the stage for at least one year. Women who are experiencing menopause unnaturally, women who are taking antianxiety, antidepressants, or exogenic hormones, and women who have a significant illness or mental retardation are excluded from the study.

The Near East University Scientific Research Ethics Committee approved the study after informed consent was given by all the participants (Project No: YDU/2021/93-1830).

3.2 Materials

The list of equipment’s has been shown in the table (Table 1).

Table 1:

The List Of Equipments.

Micropipettes and Micropipette Tips (2-20-100-1000 µl)	**Micro-Centrifuge**	**Vortex Mixers**
Eppendorf Tubes (1.5 ml)	**Vortex Mixers**	
Water bath	**Deep-Freeze**	

Table 1 (continued) :

PCR Tubes (0.2 mL)	**Thermal Cycler**
Real-Time PCR System (Rotor-Gene)	**NanoDrop Spectrophotometer**

3.3 Methods

3.3.1 DNA extraction

The protocol for the AllPrep DNA/RNA/Protein isolation kit (Qiagen GmbH, Hilden, Germany) was followed for the extraction of DNA from the blood specimens of the study participants. The protocol used for the DNA isolation is presented in below.

1. Erythrocyte lysis (EL) buffer was incubated with blood samples at 4°C for 15 minutes. Centrifugation at 1000 x g, 4 0C, for 15 minutes followed the incubation step to remove any remaining contaminants.
2. The pellet was added with five milliliters of EL and centrifuged at 1000 x g at four degree Celsius for ten minutes for discarding the supernatant.
3. Pellet (lysis cells) were dried after the centrifugation process, and supernatant was thrown away.
4. The cells were disrupted by the addition of 350-600 microliter buffer RLT, then vortexed.
5. After the lysate was pipetted onto spinning columns that were located in the collecting tube with a diameter of two millimeters, the tube was centrifuged at the maximum speed of 14,000 revolutions per minutes.
6. 500 µl AW1 buffer (wash buffer) was put into the collection tube. The tubes were centrifuged at 8000 x g (10 000 rpm) for 15 seconds. After the centrifugation period is done, collection tubes should be checked, and all of the filtrates 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 were carried out.

7. Afterward, 500 µl AW2 was put in each tube, lids were closed and centrifuged at 20000 x g (14 000 rpm), which is full speed as 2 minutes.

8. The DNA spin columns were transferred to 1.5 microliter collection tubes. 100 µl EB buffer to each spin column and lids should be closed. Then, the incubation step of samples were carried out at room temperature for two minutes. Following incubation, samples were centrifuged at 8000 x g (10,000 rpm) for one minute to elute the DNA. After the DNA extraction procedure was completed, A NanoDrop ND-1000 Spectrophotometer was used to evaluate the concentration and purity of the isolated DNA, which was used specifically for this purpose (Thermo Fisher Scientific).

3.3.2 Bisulfite Modification

The sodium bisulfite conversion is considered to be the best method for determining the methylation conditions of a DNA sequence. Unmethylated cytosines are converted to uracil upon application of the sodium bisulfite conversion method to target DNA, but methylated cytosines remain unchanged. After the sodium bisulfite treatment, the conversion of cytosine to uracil is accomplished by removing the amino groups (deamination) from cytosine, which results in cytosine turning into uracil sulfonate, which is followed by the conversion of uracil sulfonate to uracil by desulfonation. As a result, two distinct DNA sequences are revealed: one that has been methylated and one that has unmethylated (Shiraishi and Hayatsu, 2004).

Consequently, DNA is incubated in a low pH environment with a high concentration of sodium bisulfate and high temperature as well. The most common result of these challenging conditions is DNA fragmentation followed by complete loss during the purification stage. Removal of bisulfite salts and other chemicals engaged in the conversion process is done in the purification step. Protect buffer in this kit has a pH-indicator dye that is used to verify that the proper pH value is being maintained during the reaction. This ensures that DNA fragmentation is prevented.

The Qiagen's EpiTect Bisulfite Kit (Qiagen) were used to apply the bisulfite modification technique to DNA from low concentration solutions containing unmethylated cytosine. The following steps are involved:

1. DNA was dissolved to be utilized in the bisulfite conversion process.

The appropriate number of aliquots of Bisulfite Mix were thawed by adding to each aliquot 800 µl of water free of RNase . Bisulfite Mix was vortexed until totally dissolved.

2.According to the table below (Table 2), bisulfite reactions were performed in 200 µl PCR tubes. 40 microliters of RNase-free water and DNA solution should be the total volume.

Table 2

Bisulfite Reaction Components (Qiagen, 2014).

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

3. The tubes were sealed, and the bisulfite reactions were well mixed at room temperature until the DNA Protect Buffer turned green to blue. Turning buffer from green to blue is the indication that mixing is adequate and pH is correct for being converted of bisulfite reaction.

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

Table 3

Thermal Cycler Conditions (Qiagen, 2014)

Denaturation	Incubation	Denaturation	Incubation	Denaturation	Incubation	Hold
95°c	60°c	95°c	60°c	95°c	60°c	20 °c
5min	25min	5min	85min	5min	175min	∞

5. For the step of incubation, the bisulfite reaction included PCR tubes located in the thermal cycler.

Cleaning the bisulfite modified DNA

6. When bisulfite conversion has been finished, the brief centrifugation process of bisulfite reactions included PCR tubes and the bisulfite reactions were transferred to clean 1.5 microliter micro centrifuge tubes.

7. Each sample should be added to 560 µl of newly produced buffer BL along with 10 µg/ml carrier RNA. Vortexing was used to mix the solution, then centrifuged shortly.

8. Mixtures were transfused to EpiTect spin columns. The centrifugation of spin columns were carried out at full speed as 1 minute afterward. It was carried out to discard flow through and return spin columns to collection tubes.

9. 500 µl of Buffer BW (wash buffer) was added to each spin column and centrifuged for one minute at maximum speed. Then, the procedure of discarding flows through and returning spin columns to collecting tubes was accomplished.

10. Each spin column was filled with 500 µl of desulfonation buffer (BD) and incubated at 15-25 0C (room temperature) for 15 minutes. After the incubation step has been completed; flow through was discarded and the spin columns were returned to the collection tubes after one minute of centrifugation at high speed.

11. Each spin column received 500 µl Buffer BW, which was then centrifuged for one minute at maximum speed. Spin columns were returned to collection tubes after the flow through was discarded.

12. Centrifugation at maximum speed for one minute was applied to fresh 2 ml collecting tubes to eliminate the potential of liquid residue.

13. New 1.5 ml micro centrifuge tubes were filled with spin columns. 20µl of Buffer EB were added to each tube. And centrifugation at about 15,000 x g (12,000 rpm) for one minute to elute DNA.

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

The NanoDrop Spectrometer was applied to control the concentration and purity of the bisulfite-treated specimens.

3.3.3. Methylation Sensitive High-Resolution Melting Analysis

Rotor-Gene Q (Qiagen GmbH) was used for Methylation-Sensitive High-Resolution Melting (MS-HRM) analysis to detect the DNA methylation status of the TRIM-3 gene. Primers were constructed in accordance with the EpiTect HRM PCR Handbook (Qiagen GmbH). The HRM analysis was used to analyze DNA samples that had undergone PCR amplification in order to determine their melting behavior. The HotStarTaq Plus DNA Polymerase, EpiTect HRM PCR Buffer, RNase free water, EvaGreen, and dNTPs are all included in the EpiTect HRM PCR Master Mix. The EpiTect HRM PCR Master Mix, control DNAs, RNase free water, primer solutions, and template DNAs were dissolved. The reaction mix was prepared according to the table (Table 4).

Table 4

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

Component	Volume/10µl reaction	Final concentration
2X EpiTect Master Mix	5µl	1x
10 µM (each) primer mix	0.75µl	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	10µl	

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

Cycling Conditions for HRM Analysis (Qiagen, 2009)

Initial PCR Activation Step	5min	95°c

Table 5 (continued)

3-step cycling		
Denaturation	10s	95°c
Annealing	30s	55°c
Elongation	10s	72°c
Number of cycles 40-45		
Denaturation	30s	95°c
Pre-hold	30s	50°c
HRM Analysis for Rotor-Gene Q	2s	65°c - 95°c 0.1°c increment

5. HRM analysis was performed after the PCR tubes were placed in the real-time cycler and the PCR operation was started.

3.4. Statistical Analysis

For statistical analysis, the chi-square test was performed. For calculations, the SPSS 15.0 program (SPSS, Chicago, IL, USA) was used, with a statistical significance of $P < 0.05$.

CHAPTER IV

Result

A total of 114 women are involved in this study, 60 of them were post-menopausal women, and 54 are in a premenopausal period (controls).

The World Health Organization's standard of at least one year of amenorrhea was used to establish menopause. Participants who use steroid hormones or biological therapy were excluded from the study.

The average age of pre-menopausal individuals was 33.4 ± 6.8 (mean ± Std. Deviation), whereas the average age of menopausal participants was 56.6± 4.8years (mean ± Std. Deviation).

4.1. Methylation Pattern of *TRIM3* Gene

Universal unmethylated and methylated control DNA (EpiTect Control DNA Set) were used as a control (Figure 3).

TRIM-3 gene methylated in 26 out of 54 patients with pre menopause (48.1%) and *TRIM-3* gene methylated in 34 out of 60 patients with menopause (56.7%). No statistically significant difference found between methylation status and post menopausal term identified ($p > 0.05$) (Table 6) (Figure 4).

TRIM-3 gene unmethylated in 28 out of 54 patients with pre menopause (51.9%) and *TRIM-3* gene unmethylated in 26 out of 60 patients with menopause (43.3%). There was no correlation between TRIM3 gene methylation and menopause ($p > 0.05$) (Table 6) Figure 5).

Table 6

Methylation Status of TRIM3 Gene in Pre-Menopause and Post-Menopause Subjects

Table 6 (conitued)

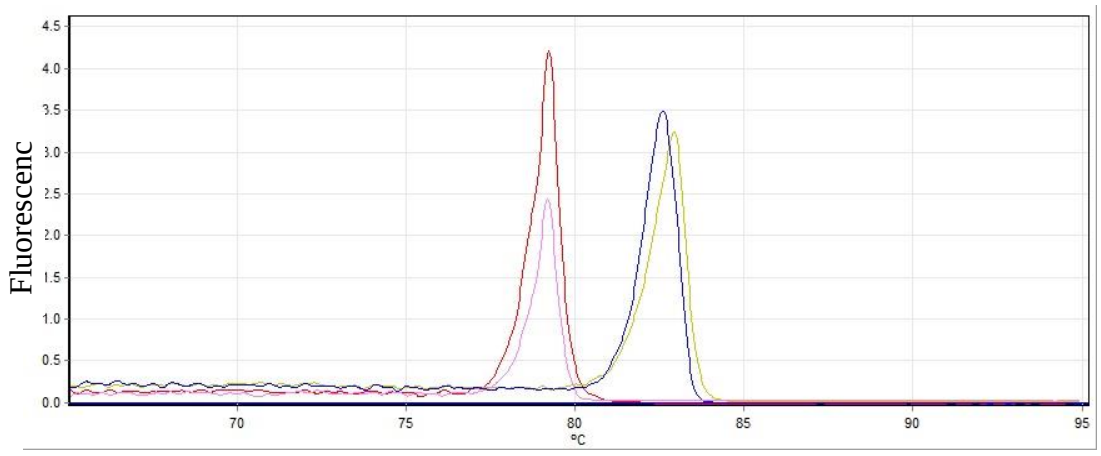
TRIM		Status			
		Post-menopause	**Pre-menopause**	**Total**	**P value**
Unmethylated	observed	26	28	54	P > 0.05
	%within column	43.3%	51.9%	47.4%	
Methylated	observed	34	26	60	
	%within column	56.7%	48.1%	52.6%	
Total	observed	60	54	114	
	%within coulmn	100.0%	100.0%	100.0%	

Figure 3

TRIM3 Gene Methylated Control and TRIM3 Gene Unmethylated Control.

The *TRIM3* gene's global methylated control was illustrated in yellow, whereas the *TRIM3* gene's universal unmethylated control was shown in purple.

Unmethylated case was illustrated in red, while the methylated case was displayed in blue. In X axis temperature (^{0}C) is shown, while Fluorescence (dF/dT) is shown in Y axis.



Temperature

Figure 4

Unmethylated TRIM3 Patient

This figure shows an unmethylated case that displayed in blue, and the unmethylated control in purple. In X axis temperature (^{0}C) is shown, while Fluorescence (dF/dT) is shown in Y axis.

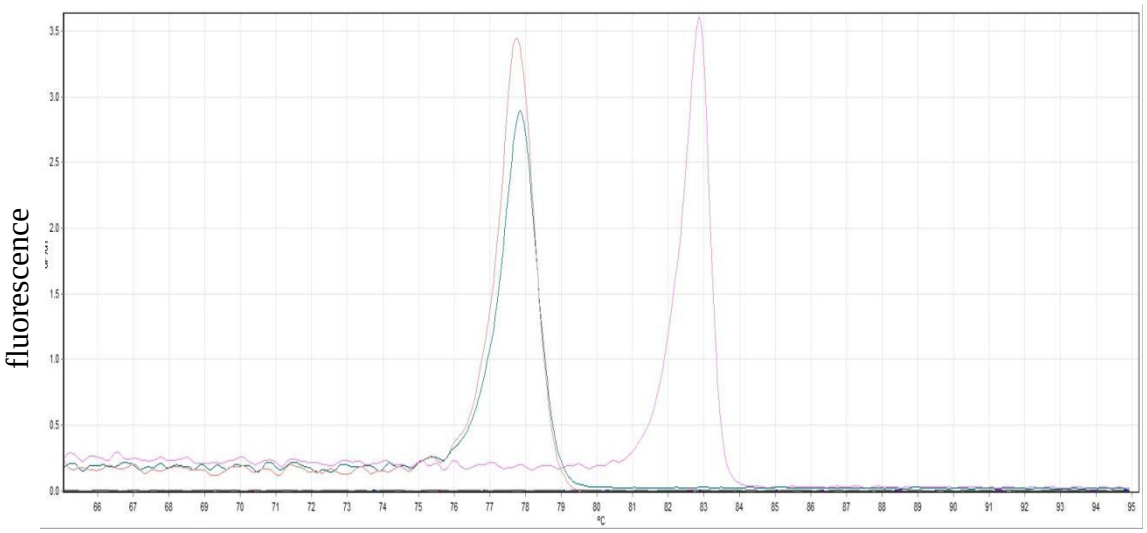
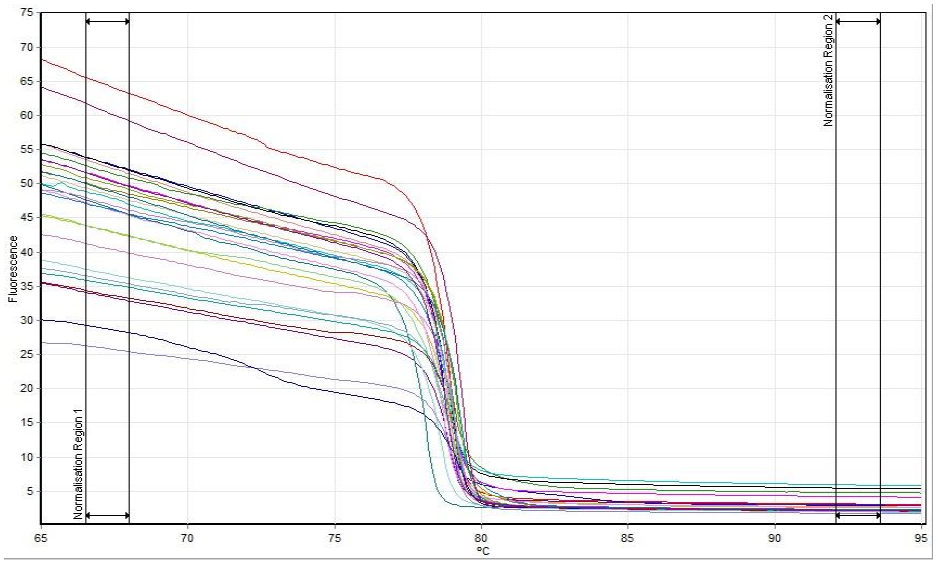


Figure 5

Analysis of HRM Data.

Normalization data derived from the raw data plots are shown in Figure 5.



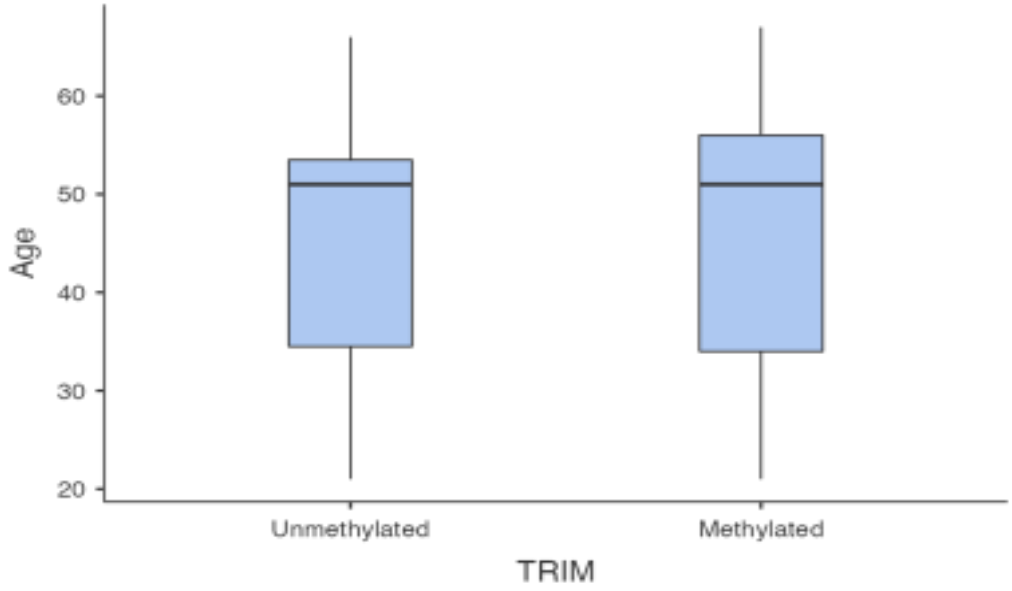
4.1.1. Interaction Between Age and *TRIM3* Methylation

The mean age of total 60 unmethylated samples were 44.2 and a standard deviation of 13.6 years. While 42 methylated samples mean age was 46.7 and a standard deviation was 12.2 (Graphic 1).

No significant association found between the methylation pattern of the *TRIM3* gene and age ($p>0.05$). (Table 7).

Graphic 1

Plot Graphic of Age and TRIM3 Methylation



CHAPTER V

Discussion

DNA methylation is the most well-known epigenetic method for regulating gene activity (Moore et al., 2012). DNA methylation has been demonstrated in number of studies to have a role in imprinting disorders and cancer (Jin and Liu, 2018). Recent research indicates that DNA methylation also have a role in metabolic diseases, autoimmune disorders, obesity, psychological diseases, and aging (Kalkan and Becer, 2019).

Menopause is considered as one of the normal aging phases in females which associated with different environmental factors (Ouzounian, and Christin-Maitre, 2005). Production of estrogen and progesterone hormones by the body is likely to vary during the perimenopausal transition period (Ouzounian, and Christin-Maitre, 2005).

Several genetic variations have been found to be linked with age at a normal menopause, the results of the study as a whole have been quite unsatisfactory (Voorhui *et al.,* 2010). In light of an earlier discovery that a variant in the *AMHR2* gene affects the age at which a woman enters menopause. Voorhuis and colleagues observed that there is evidence that genes involved in primary follicle recruitment influence the timing of menopause. This finding was supported by the observation that *BMP15* was associated with the age at which women entered menopause (Voorhuis *et al.,* 2011). It is becoming increasingly possible to identify the genetic and epidemiological factors that influence the timing of menopause. As a result, the extent to which these factors can account for the risk of early menopause now be evaluated in a more methodical manner in both individuals and populations. Genetic factors and modifiable epidemiological factors, such as smoking, are probably also responsible for the gradual decline in oocyte quality that occurs up to ten years or more before menstruation stops (Forman et al., 2011). Pelosi and his colleagues reached to conclusion that suggested using measures such as AMH levels to promote reproductive life optimization (Pelosi et al., 2015).

A growing body of evidence suggests that *TRIM* gene mutations are associated with wide range of disorders. Although it is obvious that the mutations have a role in the etiology of each disease, the molecular processes by which the alterations induce

abnormalities have yet to be discovered (Watanabe and Hatakeyama, 2017). A study was done on breast cancer patients which conclude that by maintaining the ER alpha protein, *TRIM3* can stimulate breast cancer cell proliferation and migration. Also disrupting *TRIM3* protein expression or activity could be a viable method for treating ER alpha-positive breast cancer (Zhuang *et al.,* 2022). Moreover, by reducing the effects of cancer-progressive genes, *TRIM3* may play a preventive function in gastric cancer. Farhadi and and his fellow researchers indicates that in individuals with gastric cancer, a low level of *TRIM3* mRNA expression is associated with a poor prognosis (Farhadi et al., 2022).

In terms of immunity, Yang and his colleagues came to the conclusion that given the diverse roles of *TRIM* proteins in adaptive and innate immunity, it appears that *TRIM* family proteins evolve as regulators to guarantee appropriate immune responses to remove pathogen infections without triggering autoimmunity (Yang et al., 2020).

There are a few studies about menopause related genes and the genetic factors are not very well studied. This is study might be one of a few studies that investigate the relation between menopause and specific gene.

In this study, the relationship between the mean age of participants and their *TRIM3* gene methylation status was investigated by using universal controls depending on reading melting temperature (TM) of each case sample.

The mean age of patients with unmethylated *TRIM3* gene was 44.0 (mean ± Std. Deviation, 44.0 ± 14.7), and the mean age of patients with methylated *TRIM3* gene was 47.9 (mean ± Std. Deviation, 47.9± 13.0). There was no statistical significance detected (P>0.05. The methylation status of the *TRIM3* gene was determined in both premenopausal and postmenopausal patients. The *TRIM3* promoter was methylated in 48.1% of premenopausal women and 56.7% of postmenopausal women, according to our findings. In 51.9 % of the premenopausal women the *TRIM3* gene was unmethylated and 43.3 % of the postmenopausal patients were unmethylated.

According to the findings of the statistical study, there was not found to be any significant linkage between the age of the participants and their methylation status of the *TRIM3* gene ($p > 0.05$).

CHAPTER VI

Conclusion and recommendation

Epigenetic modifications have a significant impact on women's lives. Several papers demonstrate the involvement of epigenetics in menopause. This study was performed to investigate the relation of *TRIM3* methylation pattern and menopause.

The association between the methylation pattern of *TRIM3* gene and menopausal conditions couldn’t be demonstrated, also, there was no association between the methylation pattern of the *TRIM3* gene and age.

TRIM3 helps enhance estrogen signaling and regulates the growth of breast cancer cells. Estrogen hormone level’s fluctuation is linked with symptoms like: hot flashes, anxiousness, and moodiness which are also experienced during the menopause. This is the first study to look into the relationship between the *TRIM3* gene's methylation status and menopause. This study will help future studies by being as a starting point for further research. In addition, examining the relation between other *TRIM* gene family members and menopause are recommended.

CHAPTER VII

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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
Yakın Doğu Üniversitesi
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