T.R.N.C

NEAR EAST UNIVERSITY

GRADUATE SCHOOL OF HEALTH SCIENCES

THE INVESTIGATION OF DNA METHYLATION OF ESTROGEN RELATED RECEPTOR ALPHA (*ESRRA*) AND RECEPTOR ACTIVATOR OF NUCLEAR FACTOR KAPPA B LIGAND (*RANKL*) GENES IN MENOPAUSE WOMAN

MOHAMMED AHMMAD ALTARDA

MEDICAL BIOLOGY AND GENETICS

MASTER OF SCIENCE THESIS

NICOSIA

2018

T.R.N.C

NEAR EAST UNIVERSITY

GRADUATE SCHOOL OF HEALTH SCIENCES

THE INVESTIGATION OF DNA METHYLATION OF ESTROGEN RELATED RECEPTOR ALPHA (*ESRRA*) AND RECEPTOR ACTIVATOR OF NUCLEAR FACTOR KAPPA B LIGAND (*RANKL*) GENES IN MENOPAUSE WOMAN

Mohammed Ahmad Altarda

MEDICAL BIOLOGY AND GENETICS

MASTER OF SCIENCE THESIS

SUPERVISOR:

Associate Professor RASiME KALKAN

CO- SUPERVISOR:

Associate Professor PINAR TULAY

NICOSIA

2018

The Directorate of Graduate Scholl of Health Sciences,

This study has been accepted by the Thesis Committee in Medical Genetics and Biology Program as a Master of Science Thesis.

Thesis Committee:

Chair:	Professor Doctor NEDIME SERAKINCI Near East University
Supervisor:	Associate Professor RASiME KALKAN Near East University
Member:	Assistant Professor TİJEN ATAÇAĞ University of Kyrenia

According to the relevant articles of the Near East University Postgraduate Study-Education and Examination Regulations, this thesis has been approved by the above mentioned members of the thesis committee and the decision of the Board of Directors of the Institute.

Professor Hüsnü Can BAŞER
Director of the Graduate School of Health Sciences

DECLARATION

I hereby declare that all information in this document has been obtained and presented in accordance with academic rules and ethical conduct, and no part of this thesis was previously presented for another degree or diploma in any university elsewhere. I also declare that, as required by these rules and conduct, I have fully cited and referenced all material and results that are not original to this work.

Name, Last name:

Signature:

Date:

ACKNOWLEDGEMENTS

Firstly, I would like to offer my unlimited thanks and great gratitude to my project guide Associate Professor RASiME KALKAN.
For her help, endless support, understanding spirit and constant encouragement. She has stood beside me throughout all steps of research study.
I could not have made this project without her precious help.

Secondly, I would like to show my appreciation to Associate Professor PINAR TULAY who has been providing such nice advice and support through the duration of my thesis.

Worthy of note also is my mother who defied financial odds to support my education up to the secondary school level, from where a string of scholarships brought me this far.

Last but not the least, to all my family members, friends and others who have in one way or another shared their support, either morally, emotionally and physically.

Thank you all,

Mohammed Altarda

ABSTRACT

Altarda M. The Investigation of DNA Methylation of Estrogen Related Receptor Alpha (*ESRRA*) and Receptor Activator of Nuclear Factor Kappa B Ligand (*RANKL*) Genes in Menopause Woman. Near East University, Graduate School of Health Sciences, M.Sc. Thesis in Medical Biology and Genetics Program, Nicosia, 2018.

Menopause occurs when the follicle pool in the ovaries has become exhausted and is insufficient to maintain menstrual cycles. Genetic and environmental factors are the major determinants of the timing of ending and starting these periods. Increased number of studies tried to identify genetic background of reproductive ageing and disorders of reproduction. Candidate-gene and genome-wide approach, including both a linkage mapping and association design tried to highlight related genes in humans. Estrogen is an important hormone during the menopause. During the menopause follicle-stimulating hormone and luteinizing hormone levels increase and estrogen levels decrease. The polymorphisms of *Estrogen* gene were associated with osteogenesis and menopause. The *RANKL*/*RANK* pathway is important for both formation and function of the osteoclast.

In this study, the aim was to detect methylation status of *RANKL* gene and *ESRRA* gene in post-menopausal woman. DNA was purified from blood taken from 35 post-menopausal patients and 30 pre-menopause participants. Methylation status of *RANKL* and *ESRRA* promotor regions was analyzed by Methylation Specific High Resolution Melting (MS-HRM) technique. There was no statistically significant association between post-menopausal and methylation of *RANKL* promotor ($P > 0.05$). Only a relationship between *ESRRA* promotor methylation and post-menopausal patients ($P < 0.05$) has been identified. Furthermore, significant association between methylation status of *RANKL* and *ESRRA* genes was associated. The results of this study showed that the *ESRRA* unmethylation increased the methlation level of *RANKL*, which means repressed *RANKL* gene expression and function.

Keywords: Menopause, RANKL, ESRRA, MS-HRM, Methylation

ÖZET

Altarda M. *Estrogen Related Receptor Alpha* (*ESRRA*) ve *Receptor Activator of Nuclear Factor Kappa B Ligand* (*RANKL*) Genlerinin DNA Metilasyonlarının Menopoz dönemindeki kadınlarda araştırılması.

Yakın Doğu Üniversitesi, Sağlık Bilimleri Enstitüsü, Tibbi Biyoloji ve Genetik Programı, Yüksek Lisans Tezi, Lefkoşa 2018.

Menopoz, yumurtalıklarda folikül rezervinin azalması ve menstüral döngüler için yetersiz sayıya ulaşması durumudur. Genetik ve çevresel faktörler menopoz zamanı periyotların başlama zamanı ile ilgili esas belirleyicilerdir. Reproduktif yaşlanma ve üreme hastalıkları ile ilgili olarak son dönemde oldukça fazla çalışma yapılmaktadır. Aday gen ve genom-wide asosiasyon ve linkage haritalama çalışmaları ile insanlarda ilişkili genlerin tanımlanılması çalışılmaktadır. Menopoz döneminde östrojen oldukça önemli bir hormondur. Menopoz da FSH ve LH hormone seviyesi artmakta ve östrojen seviyesi ise azalmaktadır. Östrojen gen polimorfizmlerinin osteogenez ve menopozda önemli rol oynadıkları bilinmektedir. *RANKL*/*RANK* yolağı ise özellikle osteoklast fonksiyonunda ve oluşumunda önemli rol oynamaktadır.

Bu çalışmada, *RANKL* ve *ESRRA* genlerinin metilasyon durumlarının post-menopoz kadınlarda araştırılması amaçlanmıştır. DNA 35 post-menopoz ve 30 pre-menopozlu bireyden alınan kandan izole edilmiştir. Metilasyon Spesifik Yüksek Rezolüsyonlu Erime Eğrisi Analizi ile (MS-HRM) *RANKL* ve *ESRRA* promotor metilasyon durumları analiz edilmiştir. Post-menopoz ve *RANKL* promotor metilastoyonu arasında istatistiksel olarak önemli bir ilişki saptanmamıştır ($P>0.05$). Sadece *ESRRA* promotor metilasyonu ve post-menopoz arasında önemli bi istatistiksel ilişki saptanmıştır ($P<0.05$). Ayrıca *RANKL* ve *ESRRA* genlerinin metilasyonları arasında istatistiksel olarak önemli bir ilişki saptanmıştır. Ayrıca *ESRRA* geminin unmetilasyonunun *RANKL* metilasyon seviyesini artırdığı, yani RANKL gen ekspresyonunu ve fonksiyonunu baskıladığı saptanmıştır.

Anahtar Kelimeler: Menopoz, RANKL, ESRRA, MS-HRM, Metilasyon

TABLE OF CONTENT

Page No

DECLARATION iii

ACKNOWLEDGEMENTS v

ABSTRACT vi

ÖZET vii

TABLE OF CONTENT viii

LIST OF TABLES x

LIST OF FIGURES xi

1. INTRODUCTION 1

1.1 Menopause 1

1.2 Genetics of Menopause 2

1.3 Estrogen 4

1.3.1 Relation of Estrogen with Diseases 5

1.4 RANKL / RANK Gene 5

1.4.1 RANKL and Related Diseases 7

1.5 Epigenetics 8

1.5.1 DNA Methylation 8

1.6 High Resolution Melting Analysis 9

1.7 Methylation Specific High Resolution Melting Analysis (MS-HRM) 10

1.8 Bisulfite Modification 11

2. MATERIALS AND METHODS 12

2.1 Study Case 12

2.2 Materials 12

2.2.1 Used Equipment 12

2.2.2 Kits 13

2.3 Methods 13

2.3.1 DNA extraction 13

2.3.2 Bisulfite Modification 14
2.3.3 MS-HRM 15
2.4 Statistical analyses 17
3. RESULTS 18
2.5 Methylation of RANKL Gene 18
2.6 Methylation of ESRRA Gene 22
3.1 Relationship between promotor methylation of ESRRA, RANKL and menopause 25
4. DISCUSSION 27
CONCLUSION 30
5. REFERENCES 31

LIST OF TABLES

Page No

Table 1: The common symptoms of PMS. 2

Table 2: Bisulfite reaction components. 14

Table 3: Thermal cycler conditions for bisulfite conversion. 14

Table 4: Reaction composition using EpiTect HRM PCR Master Mix, 2x. 16

Table 5: Optimized cycling protocol for HRM analysis on the Rotor-Gene Q. 16

Table 6: Characteristics of study population. 18

Table 7: Methylation frequency of RANKL promotor in 65 samples. 19

Table 8: According to study group promotor methylation status of RANKL. 19

Table 9: Methylation frequency of ESRRA in 65 samples. 22

Table 10: According to study group promotor methylation status of ESRRA. 22

Table 11: RANKL and ESRRA Interaction. 26

LIST OF FIGURES

Page No

Figure 1.1: Location of ERα and ERβ genes on the chromosome 6 and chr14. 4

Figure 1.2:The RANKL/RANK extracellular domains, ... 7

Figure 1.3: DNA cytosine methylation 9

Figure 1.4: A) The binding of transcriptional factors at gene promoter results. 9

Figure 1.5: Paradigm illustrating the steps to detect DNA methylation by HRM 11

Figure 3.1: Universal methylated control and universal unmethylated control 20

Figure 3.2: Unmethylated RANKL patient. RANKL methylated control. 20

Figure 3.3: Unmethylated RANKL patient. RANKL methylated control. 21

Figure 3.4: Unmethylated RANKL patient. RANKL unmethylated control 21

Figure 3.5: General overview of the methylated and unmethylated patients in ESRRA. ... 23

Figure 3.6: Universal methylated control and universal unmethylated control. 23

Figure 3.7: Unmethylated ESRRA patient.. 24

Figure 3.8: Unmethylated ESRRA patient.. 24

Figure 3.9: Methylated and unmethylated ESRRA patients.. 25

LIST OF ABBREVIATIONS

ANM	Age At Natural Menopause
BMU	Basic Multicellular Unit
BMD	Bone Mineral Density
ERα	Estrogen Receptor Alpha
ESRRA	Estrogen Related Receptor Alpha (gene)
ERs	Estrogen Receptors
FMP	Final Menstrual Period
FSH	Follicle-Stimulating Hormone
HPLC	High-Performance Liquid Chromatography
HRM	High Resolution Melting Analysis
HRT	Hormone replacement therapy
LH	Luteinizing Hormone
MS-HRM	Methylation Specific High Resolution Melting
MSP	Methylation Specific-PCR
OBs	Osteoblasts
OCs	Osteoclasts
OPG	Osteoprotegerin
POI	Premature Ovarian Insufficiency
RA	Rheumatoid Arthritis
BD	Desulfonation Buffer
RANKL	Receptor Activator Of Nuclear Factor Kappa-B Ligand
SNP	Single-Nucleotide Polymorphism
TLC	Thin-Layer Chromatography
TNF	Tumor Necrosis Factor

1. INTRODUCTION

1.1 Menopause

Menopause defined in Greek as “ending of month”; “meno” means month and “pause" means ending. The hormone levels are altered then menstrual cycle has ceased and this ends the reproductive competence (Currie & Martin, 2006). Many symptoms have been noted in menopause due to irregular secretion of hormones such as hot flashes, sweating and sleep disorders (Table 1). The symptoms show differences between the early and late stage of menopause (Stachoń, 2013; Joffe et al 2010).

Depending on the severity of symptoms and changing of the hormone levels, two periods have been classified; called pre-menopause and post-menopause. Pre-menopause refers to the stage two or three years before final menstrual period (FMP), sometime defined as the whole reproductive duration until FMP (World Health Organization (WHO), 1996).

Peri-menopause or “menopausal transition” identified as the initial stage of loss of menstrual cycle where the final years of reproductive life starts to biological fluctuation and endocrinological altering (Santoro, 2016; The North American Menopause Society, 2000). Additionally, the peri-menopausal period takes two to eight years according to World Health Organization and starts around 45.5-47.5 years of age. During the normal menstrual cycle, estrogen inhibits the stimulation of follicle-stimulating hormone (FSH). The lack of feedback inhibition (Su & Freeman, 2009) during the menopause causes a decreased level of estrogen level and increased level of the FSH and luteinizing hormone (LH). Alterations in hormone level has an effect on the length and regularity of the period (Trickey, 2003). Post-menopausal woman has severe symptoms than peri-menopausal woman (Monteleone et al., 2018). During the post-menopause period; serum FSH level increased ten times and LH has increased three times (Peacock & Ketvertis, 2018) and serum estradiol level decreased to less than 80 pmol/l (Lobo et al., 2000). Additionally, ovarian activity decreases and testosterone level drops by 20% (Couzinet et al., 2001). Estrogen has also been shown to be important in bone loss and osteoporosis in post-menopausal women (Khosla et al., 2012). Signaling pathways are important in the regulation of osteoclasts, osteoblasts and control bone turnover which leads to bone loss after menopause and during the normal aging.

Table 1: The common symptoms of PMS (from (Trickey, 2003)).

PHYSICAL	EMOTIONAL AND MENTAL
Abdominal Distension	Nervous Tension
Headaches	Mood Swings
Joint Pains and/or Backache	Depression
Fluid Retention	Forgetfulness
Cyclic Weight Gain	Excess Sleepiness
Premenstrual Acne	Anxiety
Fatigue and Weakness	

1.2 Genetics of Menopause

Hormone levels, nutrition, medical care, genetic features and environmental factors all have influence on the severity of menopause and the time of onset. Nowadays, studies focused on the identification of the relationship between environmental factors and genetic background of the neutral menopause. Many studies aimed to identify the responsible genes and the interactions among genetics, biological and environmental factors and menopause. Genome-wide linkage analysis, candidate gene association studies and genome-wide association studies (GWAS) tried to highlight the associated genotype of age natural menopause (ANM) (He & Murabito, 2014). Previously published studies reported that there is a significant association with X chromosome, chromosome 9q21.3 and menopause in Dutch population. Asselt and colleagues (2004) showed that Xp21.3 locus is associated with premature ovarian insufficiency (POI) (van Asselt et al., 2004).

Several studies showed the association between single nucleotide polymorphism (SNP) and menopause (Voorhuis et al 2010). Genome-wide association studies scanned more than 500,000 SNP of the whole human genome (Voorhuis et al., 2010). Mitchell and colleagues (2006) showed the importance of Cytochrome P450 Family 19 Subfamily a Member 1(CYP19A1) during the estrogen synthesis and demonstrated that the CYP19A1 polymorphism is associated with late stage of menopause transition and age of menarche (Mitchell et al., 2008). Zhang and colleagues (2006) demonstrated that rs854163 SNP in Decarboxylase gene (HDC) was associated with age of menopause (Zhang et al., 2006).

He and colleagues (2009) and Stolk and colleagues (2009) discovered novel SNPs that are related to the age at menarche and age at natural menopause (He et al., 2009; Stolk et al., 2009). Researchers identified early menopause, before 40 years old, in Fragile X syndrome (Laml et al., 2002) and high level FSH was detected (Murray et al 2000). Qin and coleagues (2015) showed the ineractions between genetic abnormalities and early menopause (ER) or POI (Qin et al., 2015). Over the past decades, numerous genes have been listed as POI candidate genes, in which these genes are crucial in cell apoptosis (FMR1), migration and differentiation of primordial germ cell (NANO53) and transcription factors for oocyte (NOBOX, WT1) (Mercadal et al., 2014; Qin et al., 2015; Wang et al., 2016). Recent studies also highlighted genetic variants which were associated with earlier age at menopause (Sarnowski et al 2018).

Estrogen have two isoforms, ERα and ERβ which has generated by alternative splicing of mRNA (Heldring et al., 2007). The importance of ERα in bone metabolism has been demonstrated in many studies. Smith and collegues, showed that elavated bone turn over and osteopenia was due to a point mutation in the ERα gene (Smith et al., 1994). ERα mRNA expression in primary hOB (human osteoblasts)cells was higher than human osteosercoma cell (Chen et al., 2004). Also, the expression of ERα detected in the myometrium and viginal wall of post and premenopusal woman (Chen et al., 1999; Sakaguchi et al., 2003).

Until now, polymorphisms of estrogen- related genes and their role in timing of menopause has been tested by different researchers. ERα gene polymorphism include (TA) repeat, *Pvull* and *Xbal* are associated with bone mass in pre- and peri-menopuseal woman (Albagha et al., 2009; Sowers et al., 2009; Willing et al., 2009). Malacara and collegues showed that some post-menopuse symptoms including vaginal dryness and hot flashes that are associated with ERα polymorphism (Malacara et al., 2004). Furthermore, ERα expression levels have been associated with various cancers. ERα expression was significantly higher in post-menopusal thyriod papillary cancer cells than control subjects. Rubio et al. concluded that elevated ERα gene expression levels can elevate the risk of thyroid papillary cancer which occurs after menopuse (Rubio et al., 2018). The methylation of ERα gene was higher in Chinese woman with breast cancer than normal samples (Zhao et al., 2008).

Furthermore, Ramos and colleagues reported that DNA methylation increases the risk of cardiovascular abnormalities (CDV) in post-menopausal woman, in such decreased level of global methylation was associated with cardiovascular risk in postmenopausal women (Ramos et al., 2016).

The reproductive ageing has also been suggested to change the epigenetic profiles of different genes. Levine and colleagues used different tissue types to be able to demonstrate epigenetic aging and found strong interaction between age at menopause and epigenetic age acceleration in blood samples (Levine et al., 2016).

1.3 Estrogen

Estrogen is an important hormone for the sexual development and it is also important for menstrual cycle control during reproduction age. Estrogen used their nuclear estrogen receptors (ERs) to be able to apply their biological functions (Mangelsdorf et al., 1995). ERs are composed of five domains: amino-terminal domain (A/B- domain) which are defined by AF1 and AF2, whereas AF1 responsible for active transcription of gene, while AF2 is specific ligand (Heldring et al., 2007), a DNA-binding domain (C- domain), a hinge region (D- domain), a ligand-binding domain (E-domain) and the carboxyl-terminal domain (F-domain) which is specific to ERs not for other members of the steroid/ nuclear receptor superfamily. Estrogen have two isoforms, ERα and ERβ which is generated by alternative splicing of mRNA (Heldring et al., 2007).

ERα consists of 595 amino acid which encoded by *ESRA* gene or ESR1 and located at the long arm of chromosome 6, while estrogen receptor beta is encoded by *ESRB* gene and located at 14q23.2- q23.3 position (NCBI, 2018).

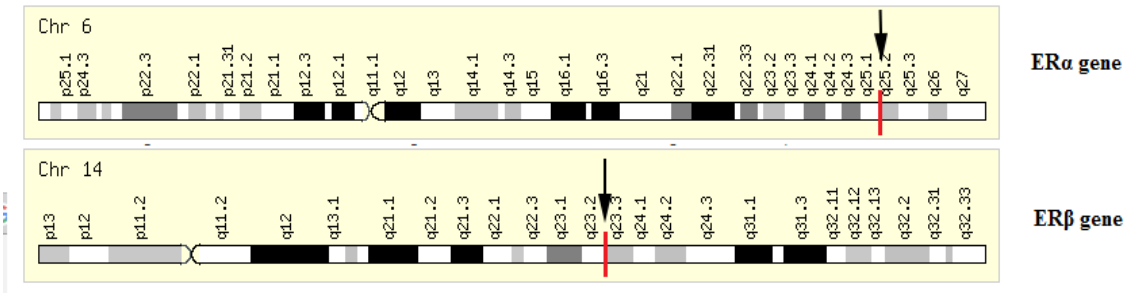

Figure 1.1: Location of ERα and ERβ genes on the chromosome 6 and chr14 (adapted from genecard.com).

Generally, ERs are expressed on different types of tissues including uterus, liver, cervix and pituitary gland, while high level expression of ERs and specially ERβ has been observed in ovarian (granulosa cells) tissue. The function of estrogen is not limited to the regulation of menstrual cycle, various new reports showed the importance in both male and female

reproductive system, cardiovascular system, neuroendocrine system, skeletal system, and adipose tissues. In reproductive system, ERs knockout mice "ERKO" showed that low number of normal follicles and oocytes are obtained (Drummond & Fuller, 2010). Also, undeveloped mammary glands have been observed due to loss of estrogen signalling further stopping the ovulation (Hewitt & Korach, 2003). Additionally, ERs influence the male reproduction system through prostate development and prostatic homeostasis (McPherson et al 2008), in such loss of ERs reduce the fertility on men by decreasing the sperm concentration and quality (Lee et al., 2009).

1.3.1 Relation of Estrogen with Diseases

In cardiovascular system, many studies demonstrated that low levels of ERs caused decreased LDL level and reduce the level of cholesterol (Knowlton & Lee, 2012).

Estrogen related receptor alpha (*ESRRA*) was localized on chromosome 11q13.1 and encoded by this gene is a nuclear receptor that is closely related to the estrogen receptor. This protein acts as a site-specific transcription regulator and has been also shown to interact with estrogen and the transcription factor TFIIB by direct protein-protein contact. The binding and regulatory activities of this protein have been demonstrated in the regulation of a variety of genes including lactoferrin, osteopontin, medium-chain acyl coenzyme A dehydrogenase (MCAD) and thyroid hormone receptor genes (Casaburi et al., 2015).

Estrogen has an important role on bone by either cellular mechanism or molecular mechanism. The deficiency of estrogen caused features on bone turnover and this deficiency leads to increasing number of BMUs (Basic Multicellular Unit) during the bone remodelling (Weitzmann & Pacifici, 2006). Estrogen deficiency in post-menopausal women plays an effectual role on osteloclast (OC) formation. Deficiency of estrogen increased the production of RANKL and TNF by osteoblasts. Excessive RANK ligand causes increased number of osteoclasts, increased bone remodelling activity and greater bone loss (Nanes, 2003). Furthermore, estrogen extend the life span of osteoblasts by active the Src/Shc/ERK pathway (Lee et al., 2006).

1.4 RANKL / RANK Gene

RANKL is one of the members of the tumor necrosis factor (TNF) family (Boyce & Xing, 2007). *RANKL* considered as polypeptide protein with TNF-related apoptosis including

ligand (TRAIL), CD40 and Fas ligand (Muñoz et al., 2005). *RANKL* or *TNFSF11* gene is located at chromosome number 13 long arm whereas two form of *RANKL* has been produced from proteolysis or alternative splicing (Ikeda et al., 2001). *RANKL* is expressed in many types of tissues including lymph node, lung, spleen, osteocytes, T-cells and bone at different levels (Kearns et al., 2007).

RANK is a of 625 amino acids polypeptide that has 4.5% signal peptide, 29% - 30% N-terminal extracellular domain, 62% cytoplasmic C-terminal domain and 4% short transmembrane domain (Wright et al., 2009).

RANK or Tumor necrosis factor receptor superfamily member 11A is a type I trans-membrane protein. This protein has been detected in many type of cells including osteoclasts, dendritic cells, fibroblasts, and T- and B-cell lineages (Boyce & Xing, 2007).

RANK has a high affinity to bind to RANK ligand and OPG (Osteoprotegerin), and its activation of RANK stimulates osteoclastogenesis (Figure 1.2) (Boyce & Xing, 2008).

RANKL has been identified as a helper to stimulate epithelial cells to produce the milk during lactation period in mammary glands, while knockout and RANK overexpressing mice show lactation failure by suppression of prolactin pathway. Cordero and colleagues conclude that the pharmacological blockage of RANK signaling at mid-gestation in wild type mice leads to precocious and exacerbated lacto-genesis (Cordero et al., 2016). Recently, has been discover another important role of *RANKL* which is a thermoregulator, after central *RANKL* injections in both rats and mice, severe fever has been noted, data shows that the changing of body temperatures was only in the females, an author suggested that *RANKL/ RANK* might explains hot flashes, sudden bursts of high body temperature in the older woman (Hanada et al., 2009).

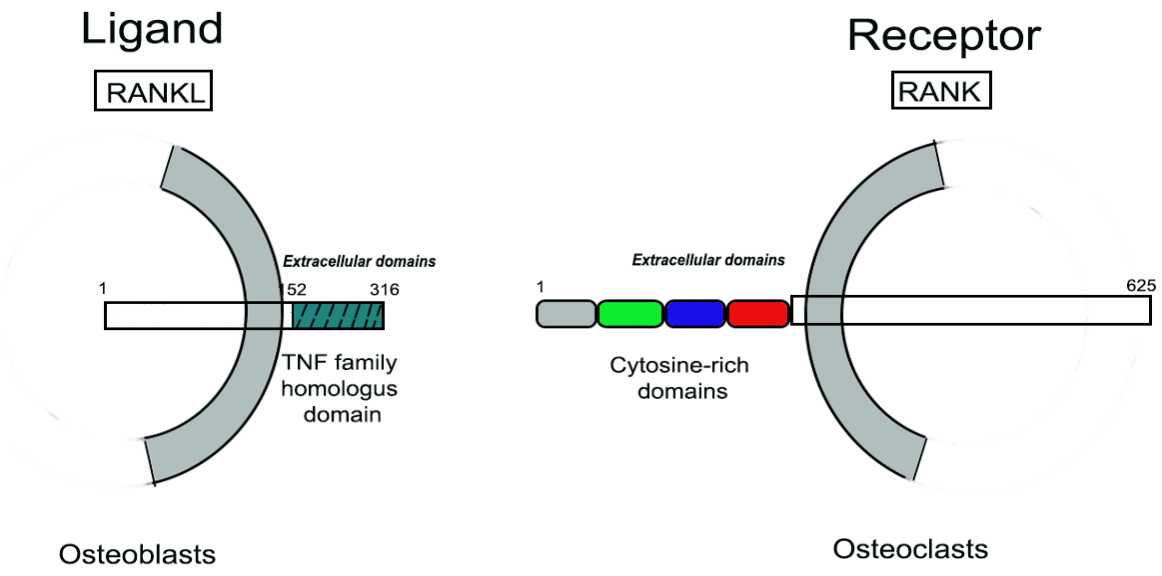

Figure 1.2: The RANKL/RANK extracellular domains, RANKL protein is consist of 316 amino acid residues, TNF homologues domain placed between ASP152-ASP316 (Adapted from Takahashi et al., 2008).

1.4.1 RANKL and Related Diseases

The importance of *RANKL* in the skeletal system has been detected by several studies. Firstly, in 1988 it has been shown that *RANKL* plays a significant role in osteoclast generation process. *RANKL* expressed as a membrane-associated protein at osteoblasts and binds to RANK in osteoclasts through cell-cell interaction and stimulate the differentiation of osteoclasts (Takahashi et al., 1988). *RANKL* not limited to induce the differentiation of osteoclasts but also plays a key role in osteoclast function and survival (Pérez-Sayáns, 2010). *RANKL* pathway regulated by estrogen and OPG, where estrogen limits the amount of RANK ligand expression by osteoblasts and OPG blocks the binding of RANK ligand to RANK (Shevde et al., 2000). Rare autosomal disorder which is called familial expansile osteolysis shows skeletal changes including damage of dentition and heighten bone remodeling in focal areas lead to painful, defecting to pathologic fracture, newly has discovered that there are two insertion mutation in first exon of *RANKL* (Ralston, 2008). Huges and colleagues reported heterozygous insertion mutations in exon 1 of *RANKL* in familial expansile osteolysis and demonstrated that both mutations caused an increase in

RANK-mediated nuclear factor-kappaB (NF-kappaB) signaling *in vitro*. (Hughes et al., 2000).

As a result of the age-related changes in osteoclast and osteoblast function; bone density and structure deterioration over time can cause a higher fracture risk in human. Distal forearm fractures increased rapidly in menopause women. Signaling pathways are important for the regulation of osteoclasts and osteoblasts bone turnover after menopause or during normal aging (Iñiguez-Ariza & Clarke, 2015). *RANKL* gene also plays a major role in menopausal-related disorders. Stynkarsdottir and colleagues showed that SNPs in *RANKL* promoter and 5'UTR region associated with BMD (Styrkarsdottir et al., 2008). Furthermore, high *RANKL* rate has been detected in osteoporosis and low BMD patients, author suggested that transcription activity of those SNPs at *RANKL* gene which causes abnormalities in bone metabolism (Mencej et.al., 2008). Eghbali and colleagues showed that upregulation of *RANKL* has an effect on osteoclast formation and activity, this effective leads to elevating the bone resorption in early post-menopausal woman (Eghbali-Fatourechi et al., 2003).

1.5 Epigenetics

In 1942, Conrad Waddigton introduced a new term called epigenetic to explain "the interaction between genes and the environment that leads to appear a new phenotype without changing in nucleotide sequence. Multiple interactions have been demonstrated such as imprinting gene silencing, X chromosome inactivation, paramutation, RNA interference, chromatin modification and DNA methylation (Yen et al., 2007; Lande et al 2007; Kouzarides, 2007; Tollefsbol, 2017). These are important factors which control the function and the expression of genes (Armstrong, 2014). Histone (chromatin) modification and DNA methylation are the most common epigenetic mechanisms that strongly have an impact on gene expression and bring many diseases into being. However, the numerous studies on epigenetics mechanisms offer a new way for management of the disorders.

1.5.1 DNA Methylation

DNA methylation is an epigenetic modification happening on the pyrimidine ring at fifth carbon of cytosine, by using specific enzyme called methyltransferases (DNMTs) which brings methyl group from S-adenyl methionine (SAM) to setup 5mC (5- methylctosine) (Figure 1.7).

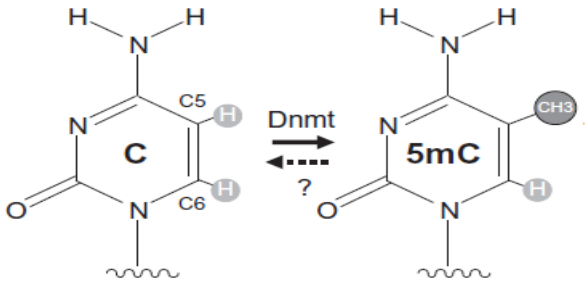

Figure 1.3: DNA cytosine methylation (from Tollefsbol, (2017))

DNA methylation is important for the gene regulation, activation, expression and cell differentiation without changing the nucleotide sequence (Tollefsbol, 2017; Miranda, & Jones, 2007; Derek et al., 2010). CpG islands which localized near to the promotor regions of genes can be methylated or unmethylated and both have an effect on gene expression CpG (Figure 1.8).

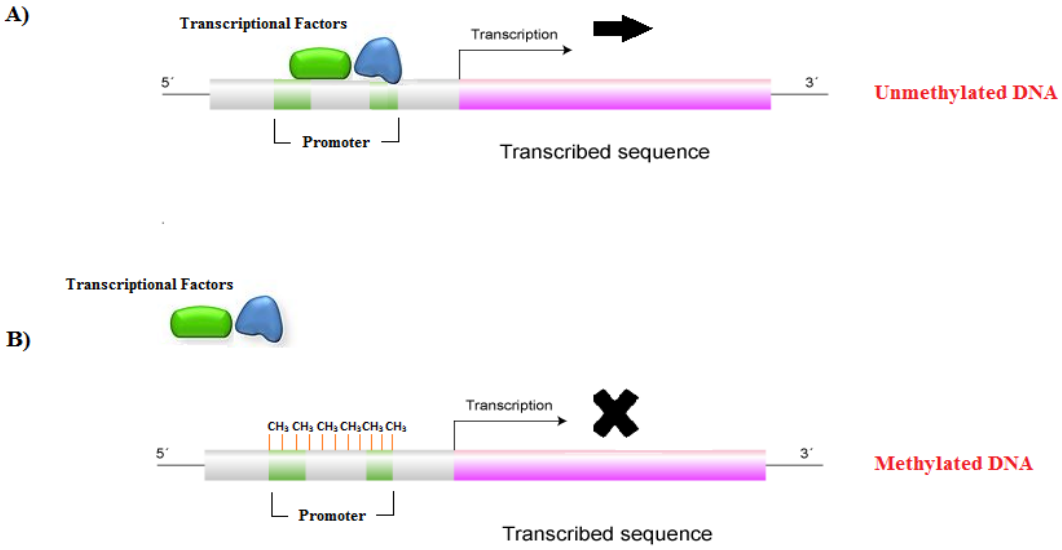

Figure 1.4: A) The binding of transcriptional factors at gene promoter results in transcription initiation. B) Methylated promoter does not allow transcriptional factors from attached to produce silence in the gene. Adapted from (Nikolova & Hariri, 2015).

1.6 High Resolution Melting Analysis

High Resolution Melting (HRM) analysis is one of the new approaches which has been performed in 2003 for the first time by Gundry and colleagues for detection of multiple type

of genetic mutations and DNA sequence variants such as SNPs and DNA methylation (Gundry et al., 2003). This technique depends on melting temperature differences between nucleotides during the denaturation of DNA. HRM procedure started by the amplification of DNA and labeling of the interested region of the DNA is important because HRM is fluorescent based technique (Biosystems, 2010). HRM technique used fluorescent dyes which includes, LC Green, SYTO Dye, EvaGreen® or Chromofy. These dyes are characterized by a high amount of fluorescent bright that are emitted when linked to double strand DNA (dsDNA) leading to exposure related to the PCR product to a range of temperature denaturing the DNA. HRM detects temperature changes and gives melting curve where the highest point of fluoresce will be at the beginning. Emitting rate of fluoresce dye depends on many factors including length of the product, GC content and sequence of interest DNA target (Reed & Wittwer, 2004).

1.7 Methylation Specific High Resolution Melting Analysis (MS-HRM)

The methods used for the identification of the methyl group started even before discovering the influence of methylation on gene expression in 1975 (Holliday & Pugh, 1975). These methods are still progressing and improving the importance of DNA methylation in molecular mechanism.

The first techniques for methylation analysis were HPLC (High-performance liquid chromatography) or TLC (Thin-layer chromatography) made with splitting the unmethylated deoxynucleosides from methylated one (Vischer & Chargaff, 1948). After that, biological pathways had appeared. In 1985, Adouard and colleagues introduced an immunological DNA methylation assay. This method used the polyclonal antibodies that can observe the main differences in methylation (Adouard et al., 1985). Restriction enzymes which are defined as specific enzymes that are able to cut the DNA at specific recognition sites, both *Hpa II* and *Msp I* can be used to identify DNA methylation (Cedar et al., 1979). Over the years, sensitive, accurate, and effective methods have been developed. Bisulfite sequencing designed by Frommer used for identification of the DNA methylation pattern (Frommer et al., 1992, Li & Tollefsbol, 2011). In this regard, bisulfite modified DNA used during the PCR amplification with specific primers (Methylation specific PCR) and this followed by sequencing (Figure 1.9) (Shen & Waterland, 2007).

However, those methods are not sensitive enough to detect the global variety in DNA methylation. The use of HRM increased recently and this technology shows the differences of melting temperature between methylated and unmethylated cytosines (Switzeny et al., 2016).

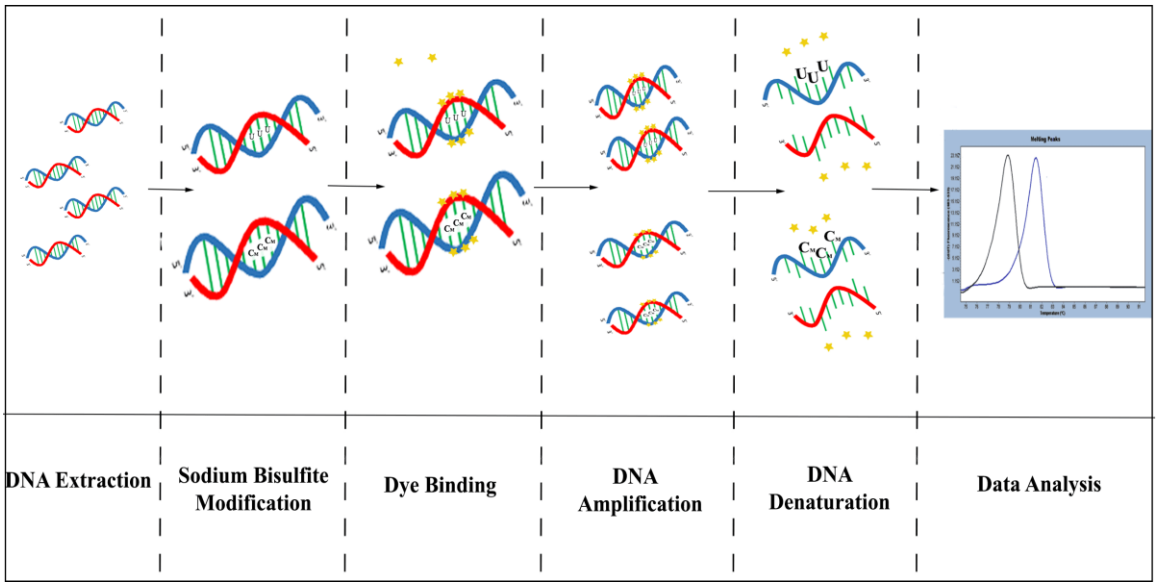

Figure 1.5: Paradigm illustrating the steps to detect DNA methylation by HRM (Adapted From https://www.qiagen.com).

1.8 Bisulfite Modification

Bisulfite conversion or modification was discovered in 1992 by Frommer and colleagues (Frommer et al., 1992). This method was based on conversion of the cytosine to uracil after treatment with sodium bisulfite and removing the amino group in cytosine and makes uracil sulfonate. Then this process is followed by desulfonation which produces uracil (Grunau & Clark, 2001). Frommer and colleagues have proposed that sulfonation reaction of cytosine becomes slower if there is a methyl group located on the fifth position of pyrimidine ring (Frommer et al., 1992).

The aim of this study is; investigates the methylation status of both estrogen receptor alpha gene (*ESRRA*) and *RANKL* gene in pre and post-menopausal women by using MS-HRM.

2. MATERIALS AND METHODS

2.1 Study Case

We studied the *ESRRA* and *RANKL* gene methylation in a series of 30 pre-menopausal and 35 post-menopausal women. Blood samples were collected by the Department of Obstetrics and Gynecology from Cengiz Topel Government Hospital.

Bone mineral density (BMD) information and exposure to exogenous hormones (oral contraception or post-menopausal hormone replacement therapy, smoking habits, alcohol and physical activity was carefully reviewed.

Inclusion and exclusion criteria's are as follow;

Inclusion criteria: All post-menopausal ladies (menopause for > 1 year).

Exclusion criteria: Women with un-natural menopause, women who took medications such as anxiolytics, anti-depressants, exogenous hormone, women who have serious disease or mental retardation.

Informed consent was obtained from each patient and the study was approved by the Ethical Committee of the Near East University (Project No: SAG-2016-2-012). The clinical and biochemistry data were carefully reviewed and compared.

2.2 Materials

2.2.1 Used Equipment

Micropipettes and tips (2–20–100–1000 µL) (Gilson)
Eppendorf Tubes (1.5 mL)
PCR Tubes (0.2 mL) (Perkin Elmer)
Water Bath
Micro-centrifuge (Eppendorf)
Vortex Mixers (Heidolph)
Deep-freeze (Arçelik)
Spectrophotometer (Nano-Drop)
Thermal cycler (Eppendorf Ag, 533151744)
Real-time PCR System (Rotor gene)

2.2.2 Kits

The AllPrep DNA/RNA/Protein Mini Kit - QIAGEN

The EpiTect Bisulfite Kit - QIAGEN

EpiTect HRM PCR Kit - QIAGEN

2.3 Methods

2.3.1 DNA extraction

The following isolation procedure is used.

1- Incubated blood samples into the erythrocyte lysis (EL) buffer at 4° C for 15 min. than centrifuge at 1000 x *g*, for 15 min at 4°C and supernatant was discarded.
2- 5ml EL buffer was added to the pellet and centrifuge at 1000 x *g* for 10 min at 4°C and supernatant was discarded.
3- Pellet (lysis cells) were dried after being disposed of the supernatant
4- 350 µl – 600 µl of buffer RLT- ß-mercaproethanol were added with vortex.
5- Samples loaded to the QIAshredder spin column and centrifuged at 14000 rpm for 3 min.
6- 500 µl AW1 buffer added and then centrifuged for 15 s at 10000 rpm.
7- 500 µl of AW2 buffer added and centrifuged for 2 min at full speed.
8- 100 µl of preheated Elution Buffer (EB) was added to the tube and incubated at room temperature for 2 min and centrifuged at 10000 rpm for 1 min.

DNA was extracted from the fresh blood tissues by AllPrep DNA/RNA/Protein Mini Kit and its quantity was measured using a NanoDrop ND-1000 Spectrophotometer (Thermo Fisher Scientific).

2.3.2 Bisulfite Modification

Unmethylated cytosine residues were converted to the uracil by bisulfite treatment of DNA by using the EpiTect Bisulfite Kit (QIAGEN) according to the Unmethylated Cytosine's in DNA from Low-Concentration Solutions protocol.

1- 800 µl of RNase-free water was added to dissolve bisulfite mix
2- Add each component in the order listed (Table 2).

Table 2: Bisulfite reaction components.

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

3- Performed the bisulfite DNA conversion using a thermal cycler. Program the thermal cycler according to following table.

Table 3: Thermal cycler conditions for bisulfite conversion.

	Temperature (°C)	Time (min)
Denaturation	95°C	5 min.
Incubation	60°C	25 min.
Denaturation	95°C	5 min.
Incubation	60°C	85 min.
Denaturation	95°C	5 min.
Incubation	60°C	175 min.
Hold	20°C	∞

4- After bisulfite modification samples were transferred to the 1.5 ml micro-centrifuge tubes and then added 560 µl freshly prepared Buffer BL containing 10 µg/ml carrier RNA. Mix the solutions by vortexing and then centrifuge briefly. Transfer the mixture into the EpiTect spin columns than centrifuge the spin columns at maximum speed for 1 min.
5- Discarded the flow-through, and place the spin columns back into the collection tubes and added 500 µl Buffer BW to each spin column, and centrifuge at maximum speed for 1 min.
6- Discarded the flow-through, and place the spin columns back into the collection tubes and added 500 µl Buffer BD to each spin column, and incubate for 15 min at room temperature (15–25°C) and then centrifuge the spin column for 1 min at maximum speed.
7- Discarded the flow-through, and place the spin columns back into the collection tubes and added 500 µl Buffer BW to each spin column and centrifuge at maximum speed for 1 min.
8- Discarded the flow-through, and place the spin columns back into the collection tubes and placed the spin columns into new 2 ml collection tubes, and centrifuge the spin columns at maximum speed for 1 min to remove any residual liquid.
9- Added 20 µl Buffer EB onto the center of each membrane. Elute the purified DNA by centrifugation for 1 min at approximately 15,000 x *g* (12,000 rpm.)

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

After DNA isolation and bisulfite modification, DNA concentration was measured by using Nanodrop and the samples which have 10 ng and upper in DNA concentration was included in this study.

2.3.3 MS-HRM

We used QIAGEN Rotor Gene Q for MS-HRM to detect the methylation status of our samples. Primers were designed according to the EpiTect® HRM™ PCR Handbook. *RANKL* and *ESRRA* genes were scored according to their cut-off value was set above the mean methylation levels +2Å~ standard deviations of the four unmethylated controls. We used comparable amounts of template genomic DNA for all samples resulting in CT values

below 30 and differing by no more than 3 CT values. Reaction composition using EpiTect HRM PCR Master Mix, 2x was shown in Table 4.

Table 4: Reaction composition using EpiTect HRM PCR Master Mix, 2x.

Component	Volume per 10 µl reaction	Final concentration
2X EpiTect HRM PCR 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	

Program the real-time cycler according to the following table 5.

Table 5: Optimized cycling protocol for HRM analysis on the Rotor-Gene Q.

Initial PCR Activation Step	5 min	95 °C
3-step cycling		
Denaturation	10 s	95 °C
Annealing	30 s	55 °C
Extension	10 s	72 °C
Number Of Cycles **40-45**		
Denaturation	30 s	95 °C
Pre-hold	30s	50 °C
HRM Analysis For Rotor-Gene Q	2s	65–95° C 0.1 °C increments

2.4 Statistical analyses

The statistical analyses and their associations with patient characteristics were performed by chi-square test and two tailed Fisher's exact test. Calculations were performed using SPSS 15.0 software (SPSS, Chicago, IL, USA), with a statistical significance of $P < 0.05$.

3. RESULTS

This study was carried out on 30 pre-menopausal (control) and 35 post-menopausal women. Menopause was defined according to the recent World Health Organization criteria (at least 1 year of amenorrhea, retrospectively) and serum estradiol level was less than 30 pg/mL. None of the participants received any steroid or biological therapies either. There were no significant differences between the pre-menopausal and post-menopausal groups in BMD, smoking habits, alcohol and physical activity.

Lumbar and femoral BMD were determined by dual-energy X-ray absorptiometry (DEXA). No differences were found in the femoral neck, trochanter, Ward's Triangle, L2, L3, L4, L2-L4, or between the femoral neck and total hip BMD after further adjustment for potential confounding factors ($P > 0.05$) (age and BMI).

The mean age of 30 pre-menopausal patients was 33.5 years (mean ± Std. Deviation, 33.5 ± 6.9) and 56.7 (mean ± Std. Deviation, 56.7± 4.9) was the post-menopausal patients (Shown in Table 6).

Table 6: Characteristics of study population.

Menopause Status	Mean	Std. Deviation	Median	Minimum	Maximum	N
Menopause	56.7714	4.91747	56.0000	51.00	71.00	35
Control	33.5000	6.92198	34.0000	21.00	52.00	30
Total						65

After DNA isolation, bisulfite modified DNA was used for methylation analysis. Methylation status of related genes has been determined by MS-HRM method with CT value equals 0.001 (Figure 3.1). We used comparable amounts of template genomic DNA for all samples resulting in CT values below 30 and differing by no more than 3 CT values.

2.5 Methylation of RANKL Gene

Universal methylated and unmethylated control DNA (EpiTect Control DNA Set, Cat No./ID: 59568) used as a reference to determine the methylation situation for our samples in each reaction (Figure 3.1).

In total; *RANKL* promotor was methylated in 26 samples and 39 samples were unmethylated (Table 7).

In the study group, *RANKL* promotor was methylated in 16 (45.7%) post-menopausal women (Figure 3.4.) and 19 (54.3%) of the pre-menopausal women is unmethylated (Table 10) (Figure 3.2, Figure 3.3) (Table 8).

In the control group, 10 (33.3%) of the samples were methylated and 20 (66.7%) of the samples were unmethylated (Table 8).

There is no statistically significant association between menopause and methylation of *RANKL* promotor (P > 0.05) (Table 8). There was no significant differences between the methylation of *RANKL* and BMD, smoking habits, alcohol and physical activity.

Table 7: Methylation frequency of RANKL promotor in 65 samples.

RANKL					
		Frequency	Percent	Valid Percent	Cumulative Percent
Valid	M	26	40.0	40.0	40.0
	U	39	60.0	60.0	100.0
	Total	65	100.0	100.0	

Table 8: According to study group promotor methylation status of RANKL.

			RANKL		**Total**	**p Value**
			M	U		
Menopause Status	Menopause	Count	16	19	35	p> 0.05
		% within Menopause Status	45.7%	54.3%	100.0%	
	Control	Count	10	20	30	
		% within Menopause Status	33.3%	66.7%	100.0%	
Total		Count	26	39	65	
		% within Menopause Status	40.0%	60.0%	100.0%	

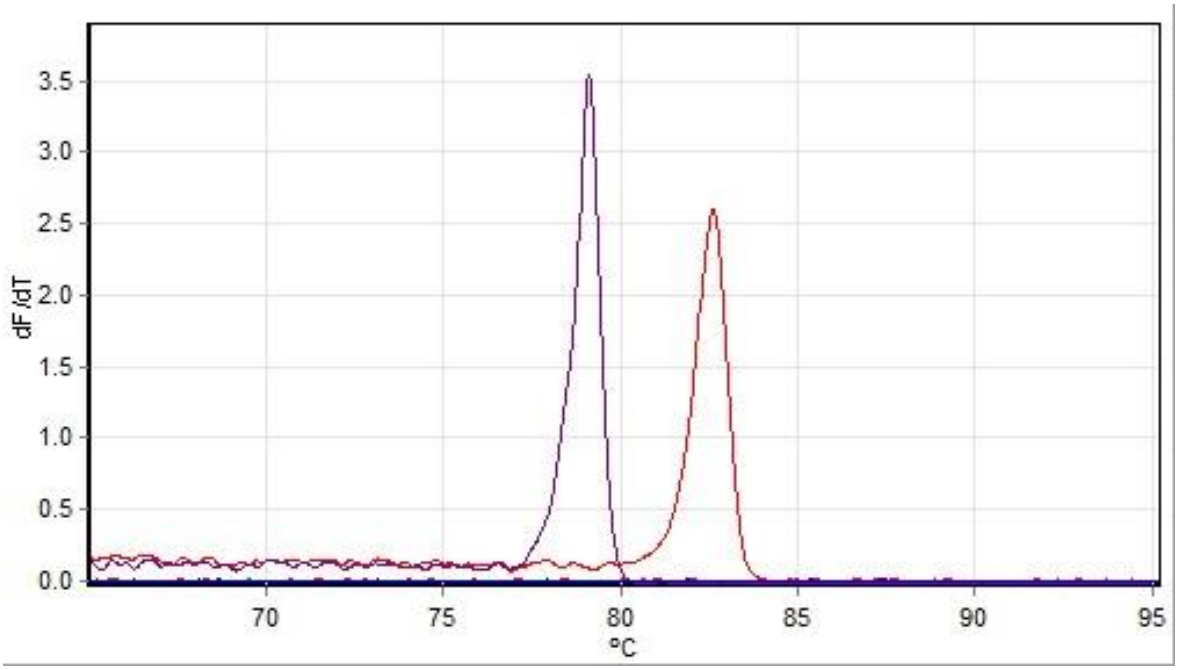

Figure 3.1: Universal methylated control and universal unmethylated control for RANKL gene. Purple peak was associated with universal unmethylated control for RANKL gene and red peak was associated with universal methylated control for RANKL gene.

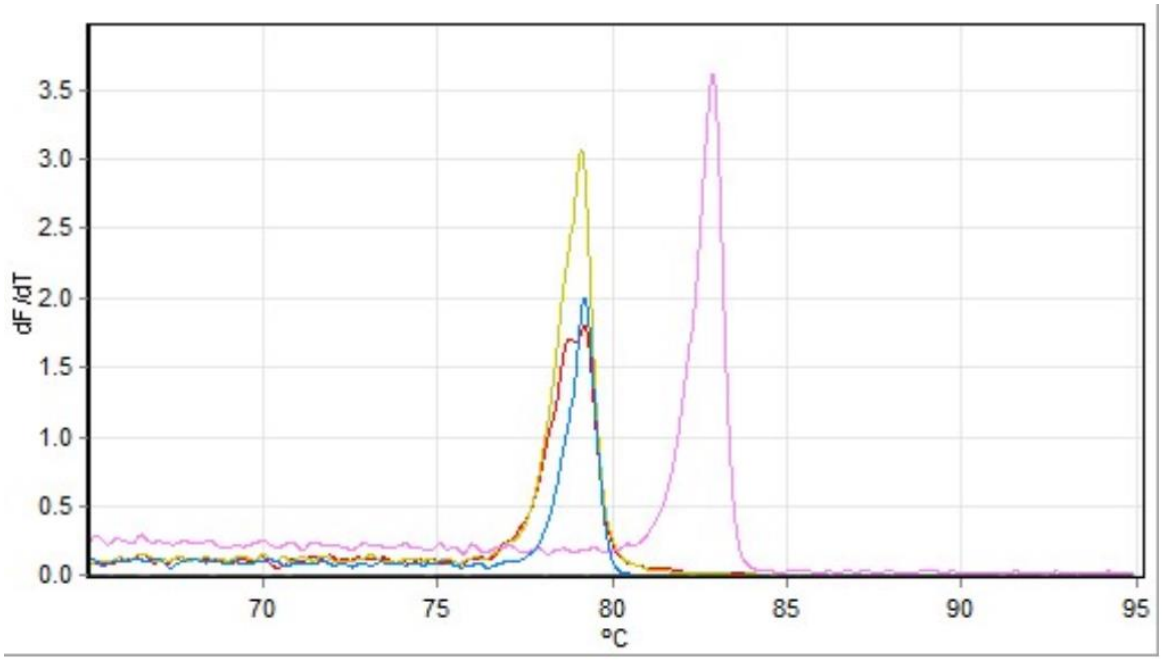

Figure 3.2: Unmethylated RANKL patient. RANKL methylated control was pink, unmethylated control was yellow. Patient number 62 and 63 were unmethylated.

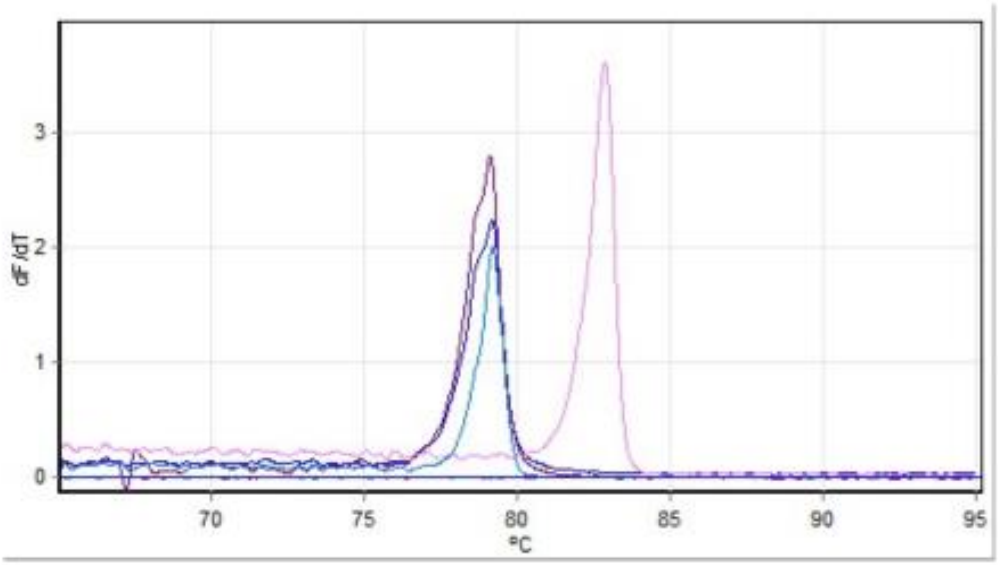

Figure 3.3: Unmethylated RANKL patient. RANKL methylated control was pink, unmethylated control was blue. Patient number 64 and 65 were unmethylated.

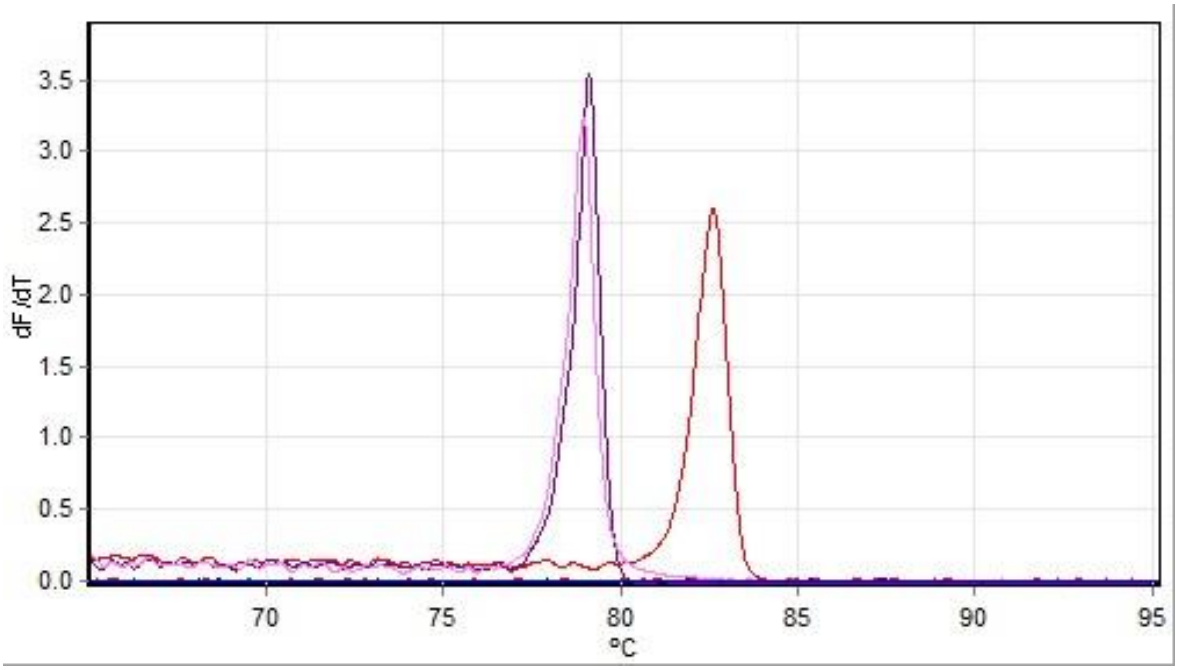

Figure 3.4: Unmethylated RANKL patient. RANKL unmethylated control was purple, methylated control was red. Patient number 49 was unmethylated.

2.6 Methylation of ESRRA Gene

In total; ESRRA was methylated in 18 (27%) samples and unmethylated in 47 (72%) samples (Table 9).

In the study group, ESRRA gene was methylated in 6 of 35 (17.1%) post-menopausal samples and unmethylated in 29 of 35 (82.9%) post-menopausal samples (Table 10).

In the control group, *ESRRA* gene was methylated in 12 of 30 samples (40%) and unmethylated in 18 of 30 (60%) samples (Table 10).

There is statistically significant association between menopause and methylation of *ESRRA* promotor ($p < 0.05$) (Table 10).

Table 9: Methylation frequency of ESRRA in 65 samples.

ESRRA					
		Frequency	Percent	Valid Percent	Cumulative Percent
Valid	M	18	27.7	27.7	27.7
	U	47	72.3	72.3	100.0
	Total	65	100.0	100.0	

Table 10: According to study group promotor methylation status of ESRRA.

			ESRRA		**Total**	**p Value**
			M	U		
Menopause Status	Menopause	Count	6	29	35	p< 0.05
		% within Menopause	17.1%	82.9%	100.0%	
	Control	Count	12	18	30	
		% within Menopause	40.0%	60.0%	100.0%	
Total		Count	18	47	65	
		% within Menopause	27.7%	72.3%	100.0%	

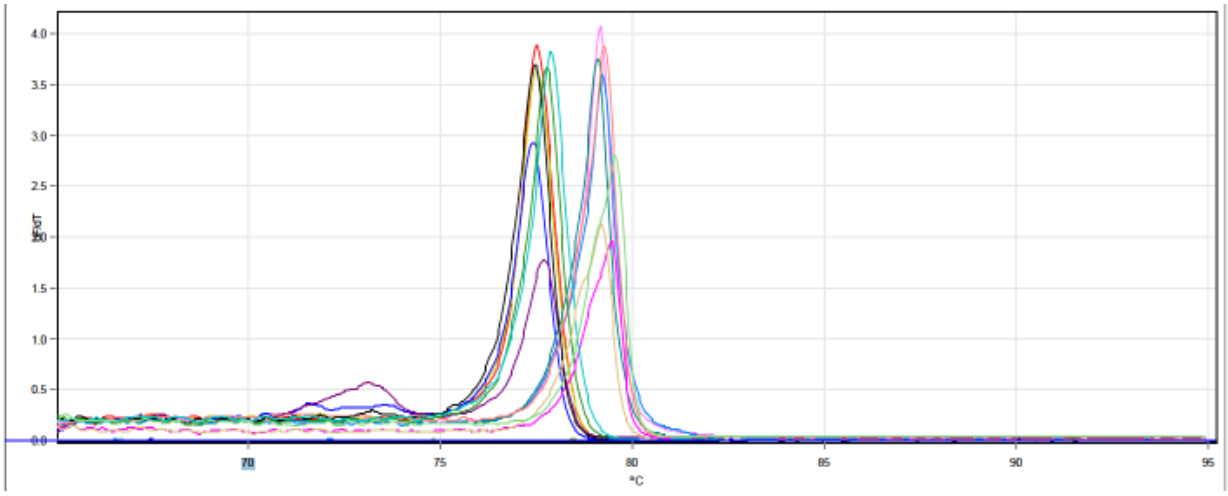

Figure 3.5: General overview of the methylated and unmethylated patients in ESRRA gene.

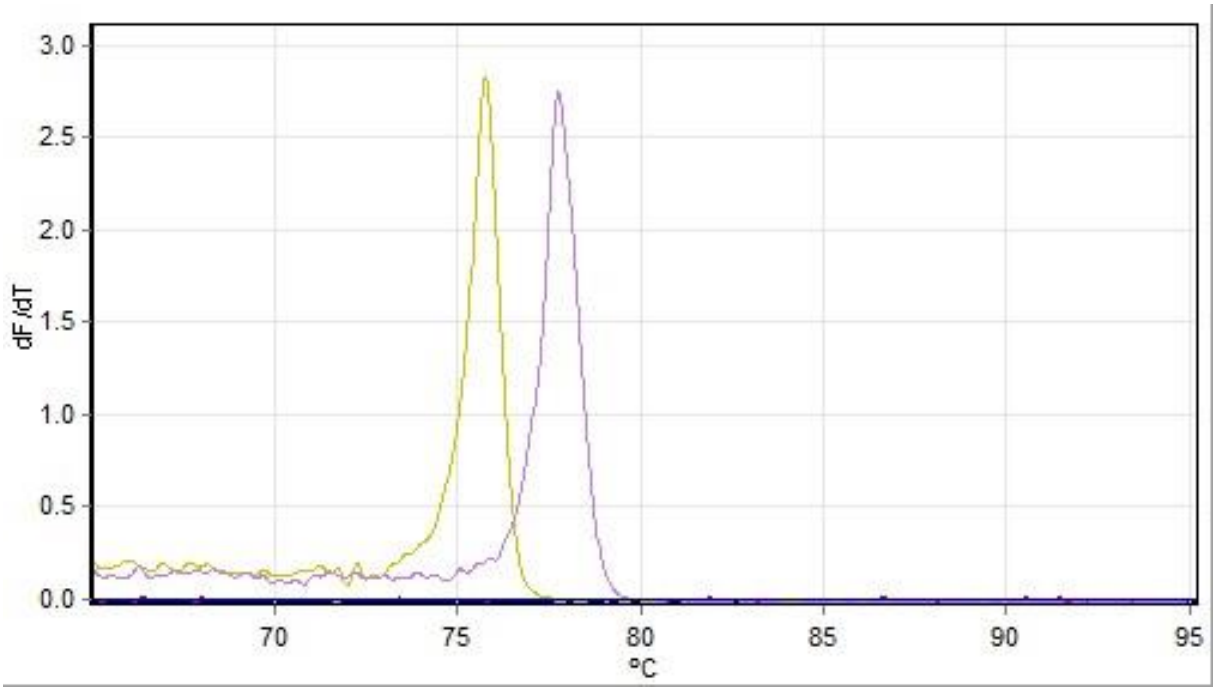

Figure 3.6: Universal methylated control and universal unmethylated control for ESRRA gene. Yellow peak was associated with universal unmethylated control for ESRRA gene and purple peak was associated with universal methylated control for ESRRA gene.

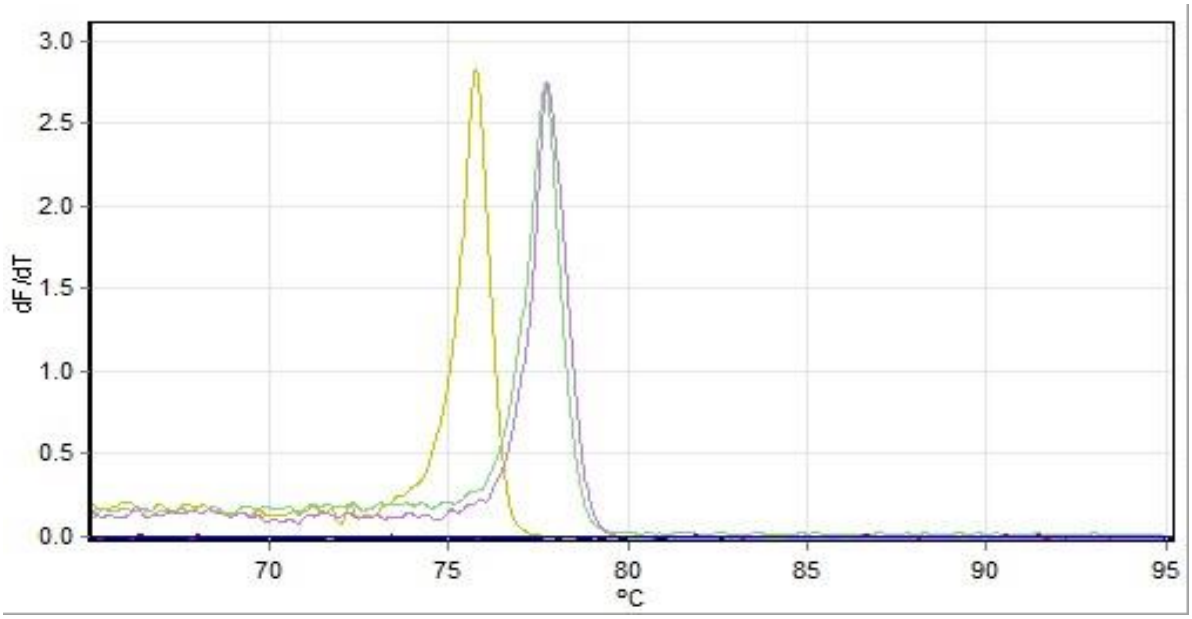

Figure 3.7: Unmethylated ESRRA patient. Yellow peak was associated with universal unmethylated control for ESRRA gene and purple peak was associated with universal methylated control for ESRRA gene and green peak associated with methylated patient number 54.

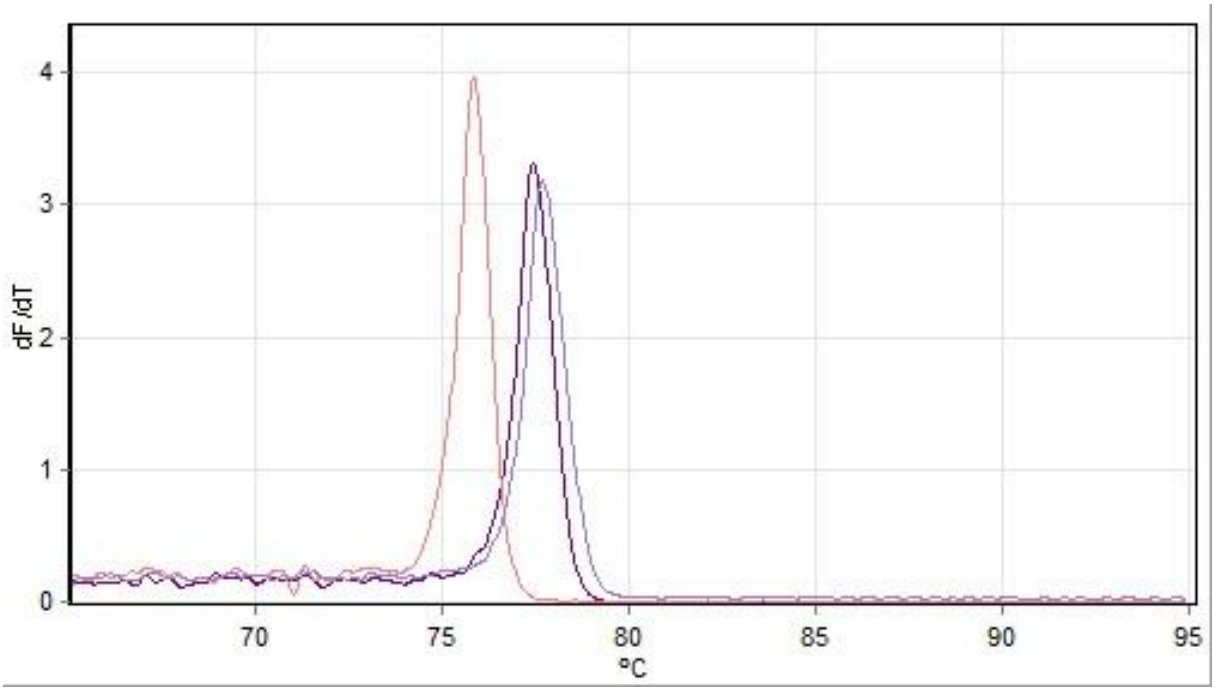

Figure 3.8: Unmethylated ESRRA patient. Pink peak was associated with universal unmethylated control for ESRRA gene and blue peak was associated with methylated control for ESRRA gene and purple peak associated with methylated patient number 38.

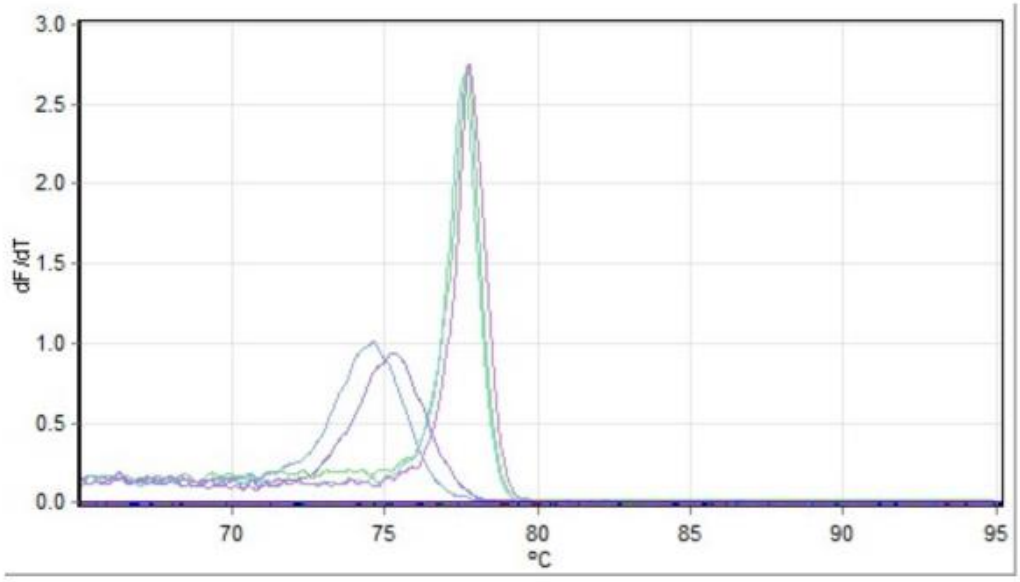

Figure 3.9: Methylated and unmethylated *ESRRA* patients. *ESRRA* unmethylated control was purple, methylated control was green. Patient number 3 is methylated and patient number 54, 55 were unmethylated.

3.1 Relationship between promotor methylation of ESRRA, RANKL and menopause

The relationship between *ESRRA* and *RANKL* was analyzed. Both of the genes were methylated in 11 samples, *ESRRA* was unmethylated and *RANKL* was methylated in 15 samples.

In 7 samples, ESRRA was methylated but *RANKL* was unmethylated and in 32 samples both of the genes were unmethylated.

There was statistically significant association between *RANKL* and ESRRA methylation status ($p < 0.05$) (Table 11).

Table 11: RANKL and ESRRA Interaction.

			ESRA		Total	p Value
			M	U		
RANKL	M	Count	11	15	26	p<0.05
		% within RANKL	42.3%	57.7%	100.0%	
	U	Count	7	32	39	
		% within RANKL	17.9%	82.1%	100.0%	
Total		Count	18	47	65	
		% within RANKL	27.7%	72.3%	100.0%	

4. DISCUSSION

Methylation has an impact on gene expression (Tost, 2009). Numerous studies showed the role of DNA methylation in cancer and imprinting disorders. Fragile X, ICF (Immune deficiency, centromeric instability, and facial abnormalities) are examples of neurodevelopmental disorders where aberrant methylation pattern has been observed (Robertson, 2005). Furthermore, loss of DNA methylation (hypo-methylation) and hyper-methylation has been detected in different type of tumors (Laird, 2005).

Thaler and colleagues concluded that hypermethylation at Lysyl Oxidase (LOX) promoter has an effect on bone quality (Thaler et al., 2011). Lysyl oxidase (lox) are expressed in osteoblasts and considered as collagen, elastin cross liking enzyme which is necessary in bone formation and strength (Grau-Bové et al 2015). Also, the relationship of the DNA methylation and ALPL gene was related mineralization and demineralization of bone cells (Delgado-Calle et al., 2011).

On other side, Reppe and colleagues screened 100 BMD-related genes and 480000 CpG site and found differences on DNA methylation between osteoporotic woman and healthy controls (Reppe et al., 2015).

RANKL provides a ligand/receptor/receptor antagonist system for controlling bone homeostasis and related biological processes (Bucay et al., 1998). *RANKL* stimulates bone resorption by activating RANK signaling (Trouvin & Goëb, 2010; Eghbali-Fatourechi et al., 2003; Dougall et al., 1999; Bucay et al., 1998) and considered as a candidate gene for regulating susceptibility to osteoporosis (Ralston & de Crombrugghe, 2006). The *RANKL* gene encodes a protein of 316 amino acids with a molecular mass of 38 kDa. *RANKL* produced by osteoblastic lineage cell and activated T cells, promotes osteoclast formation, fusion, differentiation, activation, and survival. Also leads to enhanced bone resorption and bone loss (Lacey et al., 1998; Kong et al., 1999). In fact, the *RANKL* gene has been associated with osteoporosis susceptibility in candidate gene association studies and then confirmed by genome-wide association studies (GWAS) as a susceptibility locus regulating bone mineral density (BMD) (Styrkarsdottir et al., 2008). Single nucleotide polymorphisms (SNPs) of the *RANKL* promoter showed association with BMD (Hofbauer, 1999; Hsu et al., 2006; Mencej et al., 2008). Mencej and colleagues detected promoter polymorphisms of *RANKL* gene (−290C>T, −643C>T and −693G>C) which was related with elevated transcriptional

activity of gene and correlated with increased *RANKL* protein rate. This caused imbalances in bone metabolism and was related with low BMD (Mencej et al., 2008).

Shang and colleagues analyzed SNPs of RANK and *RANKL* in a Chinese female population and showed that *RANKL* polymorphisms related with BMD in the femoral neck in peri- and postmenopausal Chinese women (Shang et al, 2013)

Researchers tried to identify the importance of genetic variations in peri- and postmenopausal women and BMD. The importance of SNPs in BMD has been shown by different researchers. (Shang et al., 2013; Tu et al., 2015; Wang et al., 2016; Zheng et al., 2015)

Delgado-Calle and colleagues showed that increased expression of *RANKL* during rhe osteoporotic fractures but they did not show statistically significant association between methylation and osteoporotic fractures (Delgado-Calle et al., 2012).

Hanada and colleagues demonstrated that central *RANKL* injections in mice and rats triggered severe fever and they mapped RANK during the fever response on astrocytes but when they applied high doses of *RANKL* in intraperitoneal this did not result in any changes in body temperature nor in activity. They highlighted the *RANKL* was important fever inducer in central nervous system (Hanada et al 2009). In this work, we detected *RANKL* promotor methylation in 16 (45.7%) post-menopausal women and 10 control group (33.3%). The *RANKL* gene was unmethylated in 19 post-menopausal women (33.3%) and 20 (66.7%) of the control samples. There is no statistically significant association observed between menopause and methylation of *RANKL* promotor.

Estrogen gene contains two receptors (Estrogen receptor-α and Estrogen receptor-β) and has important physiological functions in bone and in other tissue (Chen et al., 2004) . ERα consists of 595 amino acid residues with a molecular mass of 66 kDa and encoded by Estrogen Receptor Alpha gene (ESRA) (Bord et al., 2001). Although, ERα is well reported in the female reproductive system.

Penolazzi and collegues showed that 4 CpG within ~ 700 bp promoter F region (which's control the expression of ERα) were methylated, also this region localized near to the transcription factor activator protein (AP1) and RUNX2 binding site and important for proliferation and differentiation of the osteoblasts (Penolazzi et al., 2004) . Until now, based on our literature research, there is no research study was applied with *ESRRA* gene methylation in pre and post-menopause women. In this study, we identified methylation

status of Estrogen Receptor Alpha gene (*ESRRA*) in post-menopause woman. We detected *ESRRA* promotor methylation in 6 post-menopause (%17.1) and 12 (%40) of the control samples. In 29 of the post-menopause (%82.9) and 18 of the control samples (%60) were unmethylated. We observed statistically significant association between pre-menopause and unmethylation of *ESRRA* promotor. So, this shows that this gene is unmethylated during the pre-menopause and it is methylated during the post-menopause. *ESRRA* is related to the estrogen receptor and interacts with estrogen and the transcription factor. So this also confirms the basic literature information which was the expression of estrogen was decreased during the post-menopause.

Both *ESRRA* and *RANKL* were detected to be methylated in 42.3% of patients and both of the genes were unmethylated in 82.1% of patients. When the *RANKL* gene was methylated the *ESRRA* gene was unmethylated in %57.7 samples and contrary when the *ESRRA* gene was methylated, *RANKL* gene was unmethylated in %17 of the patients. *ESRRA* can caused increased methylation level in *RANKL* gene. This implies that when the *ESRRA* gene is unmethylated, the methylation level of *RANKL* gene was increased. The significant interaction was detected between methylation status of *RANKL* and *ESRRA* genes ($P<0.05$).

5. CONCLUSION

In conclusion, *ESRRA* and *RANKL* are interacting with each other regardless the menopausal status of the women. When the *ESRRA* is unmethylated, the methylation profile of the *RANKL* was increased. This suggests that *ESRRA* can suppress the *RANKL* gene expression. Researchers highlighted that *RANKL* used COX2-PGE(2)/EP3R pathway for thermoregulation and induction of fever. Therefore, the increased methylation of *RANKL* may directly affect the expression on gene and this causes abnormal fluctuation of temperature on post-menopause patients. At the same time, researchers highlighted that the deficiency of RANKL/RANK expression is related with osteoporosis and irregular resorption of bone. In accordance with literature, we can conclude DNA methylation is another mechanism which decreased gene expression and increased irregular resorption of bone but this hypothesis also needs confirmation on bone cells. On the other hand, we have found significant differences in the gene methylation profiles of the post-menopausal and pre-menopausal women. Our findings may provide further insight into the process of postmenopausal changes and gene methylation status. In conclusion, this is the first study which shows the *RANKL* and *ESRRA* methylation in post-menopausal women. Also, this is the first study which shows the *ESRRA* induced *RANKL* methylation.

6. REFERENCES

Adouard, V., Dante, R., Niveleau, A., Delain, E., Revet, B., and Ehrlich, M. (1985) the accessibility of 5-methyl-cytosine to specific antibodies in double-stranded DNA of Xanthomonas phage XP12. *Eur.J. Biochem*. 152, 115–121.

Albagha, O. M. E., McGuigan, F. E. A., Reid, D. M., & Ralston, S. H. (2009). Estrogen Receptor α Gene Polymorphisms and Bone Mineral Density: Haplotype Analysis in Women from the United Kingdom. *Journal of Bone and Mineral Research*, 16(1), 128–134. https://doi.org/10.1359/jbmr.2001.16.1.128.

Armstrong, L. (2014). Epigenetics. New York, NY: *Garland Science*.

Biosystems, A. (2010). Methylation Analysis Using Methylation-Sensitive HRM and DNA Sequencing. *Life Technologies Corporation*, 1–6. Retrieved from papers3://publication/uuid/ACD61081-E5EE-41A5-AE5B-CD074E40A860

Bord, S., Horner, A., Beavan, S., & Compston, J. (2001). Estrogen Receptors α and β Are Differentially Expressed in Developing Human Bone1. *The Journal of Clinical Endocrinology & Metabolism*, 86(5), 2309–2314. Retrieved from http://dx.doi.org/10.1210/jcem.86.5.7513

Boyce, B. F., & Xing, L. (2007). Biology of RANK, RANKL, and osteoprotegerin. *Arthritis Research and Therapy*, 9(SUPPL.1). https://doi.org/10.1186/ar2165

Boyce, B. F., & Xing, L. (2008). Functions of RANKL/RANK/OPG in bone modeling and remodeling. *Archives of Biochemistry and Biophysics*, 473(2), 139–146. https://doi.org/10.1016/j.abb.2008.03.018

Bucay, N., Sarosi, I., Dunstan, C., Morony, S., Tarpley, J., Capparelli, C., … Scott Simonet, W. (1998). Osteoprotegerin-deficient mice develop early onset osteoporosis and arterial calcification. *Genes & development* (Vol. 12). https://doi.org/10.1101/gad.12.9.1260

Casaburi, I., Avena, P., De Luca, A., Chimento, A., Sirianni, R., Malivindi, R., … Pezzi, V. (2015). Estrogen related receptor α (ERRα) a promising target for the therapy of adrenocortical carcinoma (ACC). Oncotarget, 6(28), 25135–25148. Retrieved from http://www.ncbi.nlm.nih.gov/pmc/articles/PMC4694820/

Cedar, H., Solage, A., Glaser, G., and Razin, A.(1979).Direct detection of methylated cytosine in DNA by use of the restriction enzyme MspI. *Nucleic Acids Res*. 6, 2125–2132.

Chen, F.-P., Hsu, T., Hu, C.-H., Wang, W.-D., Wang, K.-C., & Teng, L.-F. (2004). Expression of estrogen receptors alpha and beta in human osteoblasts: identification of exon-2 deletion variant of estrogen receptor beta in postmenopausal women. *Chang Gung Medical Journal*, 27(2), 107–115.

Chen, G.-D., Oliver, R. H., Leung, B. S., Lin, L.-Y., & Yeh, J. (1999). Estrogen receptor α and β expression in the vaginal walls and uterosacral ligaments of premenopausal and postmenopausal women. *Fertility and Sterility*, 71(6), 1099–1102. https://doi.org/https://doi.org/10.1016/S0015-0282(99)00113-2

Cordero, A., Pellegrini, P., Sanz-Moreno, A., Trinidad, E. M., et al. (2016) Rankl Impairs Lactogenic Differentiation Through Inhibition of the Prolactin/Stat5 Pathway at Midgestation. *Stem Cells*, 34(4), 1027-1039.

Couzinet, B., Meduri, G., Lecce, M.G., et al. (2001) the postmenopausal ovary is not a major androgen- producing gland. *J Clin Endocrinol Metab*, 86, 5060–5066.

Currie, H., and Martin, K. (2006) Menopause: Answers At Your Fingertips. *Class Publishing.*

Delgado-Calle, J., Sañudo, C., Fernández, A. F., García-Renedo, R., Fraga, M. F., & Riancho, J. A. (2012). Role of DNA methylation in the regulation of the RANKL-OPG system in human bone. *Epigenetics*, 7(1), 83–91. https://doi.org/10.4161/epi.7.1.18753

Delgado-Calle, J., Sañudo, C., Sánchez-Verde, L., García-Renedo, R. J., Arozamena, J., & Riancho, J. A. (2011). Epigenetic regulation of alkaline phosphatase in human cells of the osteoblastic lineage. *Bone*, 49(4), 830–838. https://doi.org/10.1016/j.bone.2011.06.006

Derek K Lim, A. H., Maher, E. R., K Lim DCH MRCPCH, D. H., & Maher FRCP FMedSci Professor, E. R. (2010). SAC review DNA methylation: a form of epigenetic control of gene expression. *SAC Review*, 1212, 37–4237. https://doi.org/10.1576/toag.12.1.037.27556

Dougall, W. C., Glaccum, M., Charrier, K., Rohrbach, K., Brasel, K., De Smedt, T., … Schuh, J. (1999). RANK is essential for osteoclast and lymph node development. *Genes & Development*, 13(18), 2412–2424. Retrieved from http://www.ncbi.nlm.nih.gov/pmc/articles/PMC317030/

Drummond, A.E., Fuller, P.J. (2010) the importance of ERbeta signalling in the ovary. *J Endocrinol*, 205(1), 15–23.

Eghbali-Fatourechi, G., Khosla, S., Sanyal, A., Boyle, W. J., Lacey, D. L., & Riggs, B. L. (2003). Role of RANK ligand in mediating increased bone resorption in early postmenopausal women. *Journal of Clinical Investigation*, 111(8), 1221–1230. https://doi.org/10.1172/JCI200317215.

ESR1 estrogen receptor 1 [Homo sapiens (human)] - Gene - NCBI. (n.d.). Retrieved from https://www.ncbi.nlm.nih.gov/gene/2099.

ESR2 estrogen receptor 2 [Homo sapiens (human)] - Gene - NCBI. (n.d.). Retrieved from https://www.ncbi.nlm.nih.gov/gene/2100.

Frommer, M., McDonald, L.E., Millar, D.S., Collis, C.M., Watt, F., Grigg, G.W., Molloy, P.L., Paul, C.L. (1992) A genomic sequencing protocol that yields a positive display of 5-methylcytosine residues in individual DNA strands. *Proc Natl Acad Sci*. 89, (5), 1827-31.

Grau-Bové, X., Ruiz-Trillo, I., & Rodriguez-Pascual, F. (2015). Origin and evolution of lysyl oxidases. Scientific Reports, 5, 10568. https://doi.org/10.1038/srep10568

Grunau, C., Clark,S.J. (2001) Bisulfite genomic sequencing: systematic investigation of critical experimental parameters. *Rosenthal A Nucleic Acids Res*, 29, (13):E65-5.

Gundry, C.N., Vandersteen, J.G., Reed, G.H., Pryor, R.J., Chen, J., Wittwer, C.T. (2003) Amplicon melting analysis with labeled primers: a closed-tube method for differentiating homozygotes and heterozygotes. *Clin Chem*, 49, 396-406.

Hanada, R., Leibbrandt, A., Hanada, T., Kitaoka, S., Furuyashiki, T., Fujihara, H., … Penninger, J. M. (2009). Central control of fever and female body temperature by RANKL/RANK. Nature, 462(7272), 505–509. https://doi.org/10.1038/nature08596

He, C., Murabito, J.M. (2014) Genome-wide association studies of age at menarcheand age at natural menopause, Mol. Cell. *Endocrinol*. 382, 767–779.

Heldring, N., Pike, A., Andersson, S., Matthews, J., Cheng, G., Hartman, J., . . . Gustafsson, J. (2007). Estrogen Receptors: How Do They Signal and What Are Their Targets. *Physiological Reviews*, 87(3), 905-931.

Hewitt, S.C., Korach, K.S. (2003) Oestrogen receptor knockout mice: roles for oestrogen receptors alpha and beta in reproductive tissues. *Reproduction*, 125(2), 143–149.

Hofbauer, L. C. (1999). Osteoprotegerin ligand and osteoprotegerin: Novel implications for osteoclast biology and bone metabolism. *European Journal of Endocrinology*, 141(3), 195–210. https://doi.org/10.1530/eje.0.1410195

Holliday, R., Pugh, J.E. (1975) DNA modification mechanisms and gene activity during development. *Science*, 187, 226–232.

Hsu, Y.-H., Niu, T., Terwedow, H. A., Xu, X., Feng, Y., Li, Z., … Xu, X. (2006). Variation in genes involved in the RANKL/RANK/OPG bone remodeling pathway are associated with bone mineral density at different skeletal sites in men. *Human Genetics*, 118(5), 568–577. https://doi.org/10.1007/s00439-005-0062-4

Hughes, A. E., Ralston, S. H., Marken, J., Bell, C., MacPherson, H., Wallace, R. G. H., … Anderson, D. M. (2000). Mutations in TNFRSF11A, affecting the signal peptide of RANK, cause familial expansile osteolysis. *Nature Genetics*, 24, 45. Retrieved from http://dx.doi.org/10.1038/71667

Ikeda, T., Kasai, M., Utsuyama, M., Hirokawa, K. (2001) Determination of three isoforms of the receptor activator of nuclear factor-[kappa]B ligand and their differential expression in bone and thymus. *Endocrinology*, 142, 1419–1426. [PubMed: 11250921].

Iñiguez-Ariza, N. M., & Clarke, B. L. (2015). Bone biology, signaling pathways, and therapeutic targets for osteoporosis. *Maturitas*, 82(2), 245–255. https://doi.org/https://doi.org/10.1016/j.maturitas.2015.07.003

Joffe, H., Massler, A., Sharkey, K.M. (2010) Evaluation and Management of Sleep Disturbance during the Menopause Transition. *Semin Reprod Med*, 28, 404-421.

Kearns, A.E., Khosla, S., Kostenuik, P. (2007) RANKL and OPG Regulation of Bone Remodeling in Health and Disease. *Endocr Rev*.

Knowlton, A.A., Lee, A.R. (2012) Estrogen and the cardiovascular system. *Pharmacol Ther*, 135(1), 54–70.

Kong, Y.-Y., Feige, U., Sarosi, I., Bolon, B., Tafuri, A., Morony, S., … Penninger, J. M. (1999). Activated T cells regulate bone loss and joint destruction in adjuvant arthritis through osteoprotegerin ligand. *Nature*, 402, 304. Retrieved from http://dx.doi.org/10.1038/46303

Kousteni, S., Chen, J. R., Bellido, T., Han, L., Ali, A., OBrien, C., … C Manolagas, S. (2002). Reversal of Bone Loss in Mice by Nongenotropic Signaling of Sex Steroids. *Science (New York, N.Y.)* (Vol. 298). https://doi.org/10.1126/science.1074935

Lacey, D. L., Timms, E., Tan, H.-L., Kelley, M. J., Dunstan, C. R., Burgess, T., … Boyle, W. J. (1998). Osteoprotegerin Ligand Is a Cytokine that Regulates Osteoclast Differentiation and Activation. *Cell*, 93(2), 165–176. https://doi.org/https://doi.org/10.1016/S0092-8674(00)81569-X

Laird, P. W. (2005). Cancer epigenetics. *Human Molecular Genetics*, 14(suppl_1), R65–R76. Retrieved from http://dx.doi.org/10.1093/hmg/ddi113

Laml, T., Preyer, O., Umek, W., Hengstschläger, M., & Hanzal, E. (2002). Genetic Disorders in Premature Ovarian Failure. *Human Reproduction Update*, 8(5), 483. Retrieved From Http://Search.Ebscohost.Com/Login.Aspx?Direct=True&Db=Edb&An=44427235&Site=Eds-Live

Lande-Diner, L., Zhang, J., Ben-Porath, I., Amariglio, N., Keshet, I., Hecht, M., et al. (2007) Role of DNA methylation in stable gene repression. *J Biol Chem*, 282, 12194–200.

Lee, K.H., Park, J.H., Bunick, D., Lubahn, D.B., Bahr, J.M. (2009) Morphological comparison of the testis and efferent ductules between wild-type and estrogen receptor alpha knockout mice during postnatal development. *J Anat*, 214(6), 916–925.

Lee, S.K. et al. (2006) T lymphocyte-deficient mice lose trabecular bone mass with ovariectomy. *J. Bone Miner*. Res. 21, 1704–1712.

Levine, M. E., Lu, A. T., Chen, B. H., Hernandez, D. G., Singleton, A. B., Ferrucci, L., … Horvath, S. (2016). Menopause Accelerates Biological Aging. *Proceedings of the National Academy of Sciences*, 113(33), 9327–9332. Https://Doi.Org/10.1073/Pnas.1604558113

Li, Y., & Tollefsbol, T. O. (2011). DNA methylation detection: Bisulfite genomic sequencing analysis. *Methods in Molecular Biology (Clifton, N.J.)*, 791, 11–21. https://doi.org/10.1007/978-1-61779-316-5_2

Lobo, R. A., Kelsey, J. L., & Marcus, R. s. (2000). Menopause: Biology and pathobiology. *Academic Press*. Retrieved from https://www.sciencedirect.com/book/9780124537903/menopause#book-description

Malacara, J. M., Pérez-Luque, E. L., Martínez-Garza, S., & Sánchez-Marín, F. J. (2004). The relationship of estrogen receptor-α polymorphism with symptoms and other characteristics in post-menopausal women. *Maturitas*, 49(2), 163–169. https://doi.org/https://doi.org/10.1016/j.maturitas.2004.01.002

Mangelsdorf, D.J., Thummel, C., Beato, M., Herrlich, P., Schutz, G., Umesono, K., et al. (1995) The nuclear receptor superfamily: The second decade. *Cell*, 83, pp. 835-839.

McPherson, S.J., Ellem, S.J., Risbridger, G.P. (2008) Estrogen-regulated development and differentiation of the prostate. *Differentiation*, 76(6), 660–670.

Mencej, S., Albagha, O., Prezelj, J., Kocjan, T., & Marc, J. (2008). TNFSF11 gene promoter polymorphisms modulate promoter activity and influence bone mineral density in

postmenopausal women with osteoporosis. *Journal of Molecular Endocrinology*, 273–279. https://doi.org/10.1677/JME-08-0003.

Mercadal, B. A., Imbert, R., Demeestere, I., Gervy, C., Leener, A. D., Englert, Y., . . . Delbaere, A. (2015). AMH mutations with reduced in vitro bioactivity are related to premature ovarian insufficiency. *Human Reproduction*, 30(5), 1196-1202. doi:10.1093/humrep/dev042.

Miranda, T.B., Jones, P.A. (2007) DNA methylation: the nuts and bolts of repression. *J Cell Physiol*, 213, 384–90.

Mitchell, E.S., Farin, F.M., Stapleton, P.L., Tsai, J.M., Tao, E.Y., Smith-DiJulio, K., and Woods, N.F. (2008) Association of estrogen-related polymorphisms with age at menarche, age at final menstrual period, and stages of the menopausal transition. *Menopause*, 15, 105–111.

Monteleone, P., Mascagni, G., Giannini, A., Genazzani, A. R., & Simoncini, T. (2018). Symptoms of menopause — global prevalence, physiology and implications. *Nature Reviews Endocrinology*, 14, 199. Retrieved from http://dx.doi.org/10.1038/nrendo.2017.180

Muñoz, J.B., Nuñez, G., Ebratt, R., Álvarez, J.J. (2005) Estudio clínico controlado aleatorizado para evaluación del Emdogain como terapia adjunta en el tratamiento de recesiones gingivales/ Randomized controlled clinical trial for evaluation of Emdogain in adjunctive therapy in gingival recession treatment. *Univ Odontol*, 25, 4-11.

Murray, A., Ennis, S., & Morton, N. (2000). No Evidence For Parent Of Origin Influencing Premature Ovarian Failure In Fragile X Premutation Carriers. *American Journal of Human Genetics*, 67(1), 253–254. Retrieved From Http://Www.Ncbi.Nlm.Nih.Gov/Pmc/Articles/Pmc1287086/

Nanes M.S. (2003) Tumor necrosis factor-alpha: molecular and cellular mechanisms in skeletal pathology. *Gene*, 321, 1–15.

Nikolova, Y. S., & Hariri, A. R. (2015). Can we observe epigenetic effects on human brain function? Trends in Cognitive Sciences, 19(7), 366–373. https://doi.org/10.1016/J.TICS.2015.05.003

Peacock, K., & Ketvertis, K. M. (2018). Menopause. Treasure Island (FL).

Penolazzi, L., Lambertini, E., Giordano, S., Sollazzo, V., Traina, G., del Senno, L., & Piva, R. (2004). Methylation analysis of the promoter F of estrogen receptor alpha gene: effects on the level of transcription on human osteoblastic cells. *The Journal of Steroid*

Biochemistry and Molecular Biology, 91(1–2), 1—9. https://doi.org/10.1016/j.jsbmb.2004.02.005

Pérez-Sayáns, M., Somoza-Martín, J.M., Barros-Angueira, F., Rey, J.M., García-García, A. (2010) RANK/RANKL/OPG role in distraction osteogenesis. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod*. 109(5):679-86.

Qin, Y., Jiao, X., Simpson, J. L., & Chen, Z.-J. (2015). Genetics of primary ovarian insufficiency: new developments and opportunities. Human Reproduction Update, 21(6), 787–808. Retrieved from http://dx.doi.org/10.1093/humupd/dmv036

Ralston, S. H. (2008). Juvenile Paget's disease, familial expansile osteolysis and other genetic osteolytic disorders. *Best Practice and Research: Clinical Rheumatology*, 22(1), 101–111. https://doi.org/10.1016/j.berh.2007.11.005

Ralston, S., & de Crombrugghe, B. (2006). Genetic regulation of bone mass and susceptibility to osteoporosis. *Genes & development* (Vol. 20). https://doi.org/10.1101/gad.1449506

Ramos, R. B., Fabris, V., Lecke, S. B., Maturana, M. A., & Spritzer, P. M. (2016). Association between Global Leukocyte DNA Methylation and Cardiovascular Risk in Postmenopausal Women. *BMC Medical Genetics*, 17(1), 1–6. Https://Doi.Org/10.1186/S12881-016-0335-X

Reed, G. H., & Wittwer, C. T. (2004). Sensitivity and specificity of single-nucleotide polymorphism scanning by high-resolution melting analysis. *Clinical Chemistry*, 50(10), 1748–1754. https://doi.org/10.1373/clinchem.2003.029751

Reppe, S., Datta, H., & Gautvik, K. M. (2015). The Influence of DNA Methylation on Bone Cells, 384–392.

Robertson, K. (2005). DNA methylation and human disease. *Nature reviews. Genetics* (Vol. 6). https://doi.org/10.1038/nrg1655

Rubio, G. A., Catanuto, P., Glassberg, M. K., Lew, J. I., & Elliot, S. J. (2018). Estrogen receptor subtype expression and regulation is altered in papillary thyroid cancer after menopause. *Surgery*, 163(1), 143–149. https://doi.org/https://doi.org/10.1016/j.surg.2017.04.031

Sakaguchi, H., Fujimoto, J., Aoki, I., & Tamaya, T. (2003). Expression of estrogen receptor α and β in myometrium of premenopausal and postmenopausal women. *Steroids*, 68(1), 11–19. https://doi.org/https://doi.org/10.1016/S0039-128X(02)00111-3

Santoro, N. (2016). Perimenopause: From Research to Practice. *Journal of Women's Health*, 25(4), 332–339. http://doi.org/10.1089/jwh.2015.5556.

Sarnowski C, Kavousi M, Isaacs S, Demerath EW, Broer L, Muka T, Franco OH, Ikram MA, Uitterlinden A, Franceschini N, Lunetta KL, Murabito JM. Genetic variants associated with earlier age at menopause increase the risk of cardiovas cularevents in women. Menopause. 2018 Apr;25(4):451-457.

Shang, M., Lin, L., & Cui, H. (2013). Association of genetic polymorphisms of RANK, RANKL and OPG with bone mineral density in Chinese peri- and postmenopausal women. *Clinical biochemistry* (Vol. 46). https://doi.org/10.1016/j.clinbiochem.2013.03.011.

Shen, L., & Waterland, R. A. (2007). Methods of DNA methylation analysis. *Current Opinion in Clinical Nutrition & Metabolic Care*, 10(5), 576–581. https://doi.org/10.1097/MCO.0b013e3282bf6f43\r00075197-200709000-00003.

Shevde, N. K., Bendixen, A. C., Dienger, K. M., & Pike, J. W. (2000). Estrogens suppress RANK ligand-induced osteoclast differentiation via a stromal cell independent mechanism involving c-Jun repression. *Proceedings of the National Academy of Sciences*, 97(14), 7829–7834. https://doi.org/10.1073/pnas.130200197

Smith, E. P., Boyd, J., Frank, G. R., Takahashi, H., Cohen, R. M., Specker, B., … Korach, K. S. (1994). Estrogen Resistance Caused by a Mutation in the Estrogen-Receptor Gene in a Man. *New England Journal of Medicine*, 331(16), 1056–1061. https://doi.org/10.1056/NEJM199410203311604

Sowers, M., Willing, M., Burns, T., Deschenes, S., Hollis, B., Crutchfield, M., & Jannausch, M. (2009). Genetic Markers, Bone Mineral Density, and Serum Osteocalcin Levels. *Journal of Bone and Mineral Research*, 14(8), 1411–1419. https://doi.org/10.1359/jbmr.1999.14.8.1411

Stolk, L., Zhai, G., Van Meurs, J. B. J., Verbiest, M. M. P. J., Visser, J. A., Estrada, K., … Uitterlinden, A. G. (2009). Loci At Chromosomes 13, 19 And 20 Influence Age At Natural Menopause. *Nature Genetics*, Vo 41, (6), 645. Retrieved From Http://Search.Ebscohost.Com/Login.Aspx?Direct=True&Db=Edsgao&An=Edsgcl.201086683&Site=Eds-Live

Styrkarsdottir, U., Halldórsson, B., Gretarsdottir, S., Guðbjartsson, D., Walters, G., Ingvarsson, Þ., … Stefansson, K. (2008). Multiple Genetic Loci for Bone Mineral

Density and Fractures. *The New England journal of medicine* (Vol. 358). https://doi.org/10.1056/NEJMoa0801197

Su, H. I., & Freeman, E. W. (2009). Hormone changes associated with the menopausal transition. *Minerva Ginecologica*, 61(6), 483–489.

Switzeny, O. J., Christmann, M., Renovanz, M., Giese, A., Sommer, C., & Kaina, B. (2016). MGMT promoter methylation determined by HRM in comparison to MSP and pyrosequencing for predicting high-grade glioma response. *Clinical Epigenetics*, 8, 49.

Takahashi, N., Akatsu, T., Udagawa, N., Sasaki, T., Yamaguchi, A., Moseley, J. M., … Suda, T. (1988). OSTEOBLASTIC CELLS ARE INVOLVED IN OSTEOCLAST FORMATION. *Endocrinology*, 123(5), 2600–2602. Retrieved from http://dx.doi.org/10.1210/endo-123-5-2600

Thaler, R., Agsten, M., Spitzer, S., Paschalis, E. P., Karlic, H., Klaushofer, K., & Varga, F. (2011). Homocysteine Suppresses the Expression of the Collagen Cross-linker Lysyl Oxidase Involving IL-6, Fli1, and Epigenetic DNA Methylation. *The Journal of Biological Chemistry*, 286(7), 5578–5588. https://doi.org/10.1074/jbc.M110.166181

The North American Menopause Society. (2000) Clinical challenges of perimenopause: consensus opinion of the North American Menopause Society. *Menopause*, 7, 5-13.

Tollefsbol, T. O. (2017). Handbook of epigenetics. *Elsevier*.

Tost, J. (2009). DNA Methylation: Methods and Protocols (Vol. 507). https://doi.org/10.1007/978-1-59745-522-0

Trickey, R. (2003). Women, Hormones and the Menstrual Cycle, 1074. https://doi.org/10.1007/s13398-014-0173-7.2.

Trouvin, A.-P., & Goëb, V. (2010). Receptor activator of nuclear factor-κB ligand and osteoprotegerin: maintaining the balance to prevent bone loss. *Clinical Interventions in Aging*, 5, 345–354. https://doi.org/10.2147/CIA.S10153

Tu, P., Duan, P., Zhang, R.-S., Xu, D.-B., Wang, Y., Wu, H.-P., … Si, L. (2015). Polymorphisms in genes in the RANKL/RANK/OPG pathway are associated with bone mineral density at different skeletal sites in post-menopausal women. *Osteoporosis International*, 26(1), 179–185. https://doi.org/10.1007/s00198-014-2854-7

van Asselt, K. M., Kok, H. S., Putter, H., Wijmenga, C., Peeters, P. H. M., van der Schouw, Y. T., … Pearson, P. L. (2004). Linkage analysis of extremely discordant and concordant sibling pairs identifies quantitative trait loci influencing variation in human menopausal

age. *American Journal of Human Genetics*, 74(3), 444–453. https://doi.org/10.1086/382136.

Vischer, E., and Chargaff, E. (1948) the composition of the pentose nucleic acids of yeast and pancreas. *J. Biol.Chem*. 176, 715–734.

Voorhuis, M., Onland-Moret, N. C., Van Der Schouw, Y. T., Fauser, B. C. J. M., & Broekmans, F. J. (2010). Human Studies on Genetics of the Age at Natural Menopause: *A Systematic Review*. Human Reproduction Update, 16(4), 364–377. Https://Doi.Org/10.1093/Humupd/Dmp055

Wang, J., Wang, Y., Zhao, Y., Li, Y., Sun, M., Na, R., … Yang, X. (2016). Polymorphisms of genes in the OPG/RANKL/RANK pathway in the Mongols of Inner Mongolia China: Relationship to other populations (Vol. 9).

Weitzmann, M. N., & Pacifici, R. (2006). Estrogen deficiency and bone loss: an inflammatory tale. *Journal of Clinical Investigation*, 116(5), 1186–1194. https://doi.org/10.1172/JCI28550

Willing, M., Sowers, M., Aron, D., Clark, M. K., Burns, T., Bunten, C., … Jannausch, M. (2009). Bone Mineral Density and Its Change in White Women: Estrogen and Vitamin D Receptor Genotypes and Their Interaction. *Journal of Bone and Mineral Research*, 13(4), 695–705. https://doi.org/10.1359/jbmr.1998.13.4.695

World Health Organization (WHO). (1996) Research on the menopause in the 1990s: report of a WHO scientific group. *Geneva: WHO Technical Report Series number*, 866 ,1-107.

Wright, H. L., McCarthy, H. S., Middleton, J., & Marshall, M. J. (2009). RANK, RANKL and osteoprotegerin in bone biology and disease. *Current Reviews in Musculoskeletal Medicine*, 2(1), 56–64. https://doi.org/10.1007/s12178-009-9046-7

Yen, Z.C., Meyer, I.M., Karalic, S., Brown, C.J. (2007) A cross-species comparison of X chromosome inactivation in Eutheria. *Genomics*, 90, 453–63.

Zhang, F., Xiong, D.-H., Wang, W., Shen, H., Xiao, P., Yang, F., … Deng, H.-W. (2006). Hdc Gene Polymorphisms Are Associated With Age at Natural Menopause in Caucasian Women. *Biochemical and Biophysical Research Communications*, 348, 1378–1382. Retrieved From Http://10.0.3.248/J.Bbrc.2006.08.008

Zhao, L., Wang, L., Jin, F., Ma, W., Ren, J., Wen, X., … Wei, M. (2008). Silencing of estrogen receptor α (ERα) gene by promoter hypermethylation is a frequent event in Chinese women with sporadic breast cancer. *Breast Cancer Research and Treatment*, 117(2), 253. https://doi.org/10.1007/s10549-008-0192-1

Zheng, H., Wang, C., Wei, H., Fu, W.-Z., & Zhang, Z.-L. (2015). OPG, RANKL, and RANK gene polymorphisms and the bone mineral density response to alendronate therapy in postmenopausal Chinese women with osteoporosis or osteopenia. *Pharmacogenetics and Genomics* (Vol. 26). https://doi.org/10.1097/FPC.0000000000000181x.