



**Arab American University**

**Faculty of graduate studies**

**Title of thesis: Association of selected polymorphisms  
in the VDR gene and BMD among Palestinian Type 1  
diabetes Mellitus patients**

By

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This thesis was submitted in partial fulfillment of the  
requirements for the master's degree in  
Molecular Genetics and Genetics Toxicology  
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## Thesis approval

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**By**

**Yara Hazim Tawfek Yassin**

**This thesis was defended successfully on 11.06.2022 and approved by:**

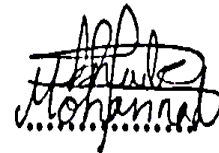
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## Declaration

I certify that this thesis is submitted for the degree of master in Molecular genetics and genetics toxicology the result of my own research. This thesis has not been submitted for a higher degree in any other university or institution .

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A handwritten signature in black ink, appearing to read 'Yara Yassin', is written over a light grey rectangular background.

Date: September 20<sup>th</sup>, 2022

## **Dedication**

I dedicate this work to my beloved family  
Mom, dad and my brothers  
Thank you for believing in me.

## **Acknowledgments**

I would like to express my appreciation to all the participants who made this research successfully completed. The subjects including, patients and controls for donating their time and effort. I would like to extend my deep appreciations to my supportive supervisor Prof. Hisham Darwish who offered invaluable supervision and guidance, and the staff of Arab American University academic Research lab Miss. Roua Thawabtah and Mr. Hussam Sallam who accompanied me during the practical work from the first to the last moment. Many thanks to my brother's wife Miss Hadel Naamneh for her assistance in the editing and assisting in the final organizing of the thesis. Lastly, I would like to express my deep love and gratitude to my beloved family for their endless support and love.

## Abstract

**Objectives:** The aims of this investigation was to study the association between the in VDR gene polymorphisms including rs10735810 (exon 2) and rs1544410 (intron 8), BMD and osteoporosis in type 1 diabetes mellitus and its significance in the pathophysiology of type 1 diabetes.

**Materials and methods:** A 88 healthy control and 86 diabetic patients aged 18-30 years where recruited in this study. All subjects were exposed for bone mineral density (BMD) examination using DEXA scan in three main locations ( femur neck, total hip and lumber spine L1- L4). Genomic DNA was prepared from whole blood samples obtained from all subject. Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) was ultimately used to determine the distribution of the various genotypes and haplotypes in rs10735810 (exon 2) and rs1544410 (intron 8). Comprehensive statistical analysis to correlate between the indicated haplotypes and genotypes in the VDR gene with type 1 diabetes and BMD in the various locations and osteoporosis was performed using SPSS program.

**Results:** Our data confirmed a strong association between type 1 diabetes, BMD, and total osteoporosis. The data evidently showed that (rs10735810 (exon 2) but not rs1544410 (intron 8) was associated with lower BMD and osteoporosis status at the indicated sites. The data also showed no association between rs10735810 (exon 2) and rs1544410 (intron 8) haplotypes and genotypes with diabetes. However, it showed significant association of rs10735810 (exon 2) C allele as a risk of osteoporosis and the T as protective allele with negative between the various alleles in rs1544410 (intron 8) and osteoporosis. Furthermore, the data showed rs10735810 (exon 2) but not rs1544410 (intron 8) alleles represent causative factor of low BMD and osteoporosis but is not linked to in diabetes type 1 patients, The cause of osteoporosis in diabetic

type 1 patients might be due to other genetic factors and/or to environmental factors including vitamin D status and other nutritional factors.

**Conclusions:**

- 1- Strong association between type 1 diabetes, BMD and total osteoporosis is confirmed.
- 2- Evidently, variants in rs10735810 (exon 2) but not rs1544410 (intron 8) was associated with lower BMD and osteoporosis status at the indicated sites.
- 3- No association between rs10735810 (exon 2) and rs1544410 (intron 8) haplotypes and genotypes with diabetes was detected.
- 4- Significant association between rs10735810 (exon 2) C allele as a risk of osteoporosis and the T as protective allele with negative between the various alleles in rs1544410 (intron 8) and osteoporosis was evident.
- 5- The data showed rs10735810 (exon 2) but not rs1544410 (intron 8) alleles is associated with low BMD and osteoporosis but not linked to diabetes type 1.
- 6- The cause of osteoporosis in diabetic type 1 patients might be due to other genetic factors and/or to environmental factors including vitamin D status and other nutritional factors.

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## Abbreviations

PI3K: Phosphoinositide 3-kinase  
IRS-1: Insulin receptor substrate 1  
GLUT-4: GLUCOSE TRANSPORTER 4  
MODY: Maturity onset of diabetes of the young  
ICA: Islet cells autoantibodies  
IAA: Insulin autoantibodies  
IA2: Protein tyrosine phosphate  
GAD: Glutamic acid decarboxylase  
ZNT8A: Zinc transportive protein  
HLA: Human leukocyte antigen  
AIRE: Autoimmune regulator  
INS: Insulin gene  
PTPN22: Protein tyrosine phosphatase non-receptor type 22  
CTLA4: Cytotoxic T-lymphocyte antigen  
BMI: Body mass index  
VDR: Vitamin D receptor  
ER: Estrogen receptor  
CT: Calcitonin  
RANKL: Receptor activator of nuclear factor kappa B ligand  
OPJ: Osteoprotegerin  
BMD: Bone mineral density  
DEXA: Dual-Energy X-ray absorptiometry  
WHO: World health organization  
RANK: Receptor activator of nuclear factor k  
FOK-1: Flavobacterium okeanoikoites 1  
BSM-1: Submaxillary mucin 1  
T1DM: Type 1 diabetes mellitus  
EDTA: Ethylenediamine tetraacetic acid  
DNA: Deoxyribonucleic Acid  
PCR: Polymerase chain reaction  
RFLP: Restriction Fragments Length Polymorphism  
TAE: Tris Acetate EDTA  
SPSS: Statistical package for the social sciences  
SNP'S: Single nucleotide polymorphism  
ANOVA: Analysis of variance

## Chapter 1

### Introduction

#### 1.1 Diabetes mellitus

Defect in insulin release and action is a major cause of a metabolic disorder with chronic hyperglycemia known as diabetes mellitus. Insulin is produced in the pancreas by beta cells (Voet D 2011). Insufficient levels of the insulin hormone does not affect the glucose level only, it is also associated with metabolic abnormalities in lipids and proteins (Kharroubi AT 2015). The difference in symptoms between type 1 and type 2 diabetes is the symptoms onset, in other words patients with type 2 diabetes show late onset of the symptoms, while type 1 patients suffer from significant symptoms shows in early ages characterized by polyuria, polydipsia, polyphagia, weight loss and blurred vision caused by complete insulin loss (Voet D 2011) figure (1.1).

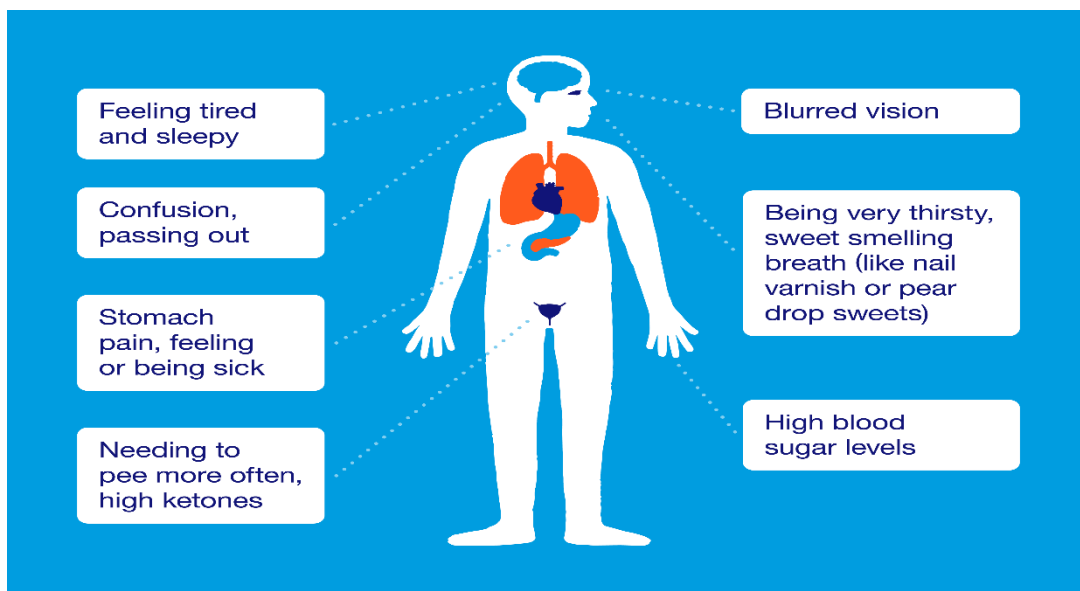


Figure 1.1: Diabetes mellitus symptoms

Untreated diabetes may cause coma or even death caused by ketoacidosis due to lack of insulin that in turn helps the sugar to enter the cells. lack of insulin prompts the release of a series of hormones responsible for using fat as a fuel to the cells instead of sugar. Ketones are one of the products of fat breakage mechanism and excess ketones build up in the body and cause a number of complications in the body (Craig ME 2009, Galtier F 2010) as summarized in figure 1.2.

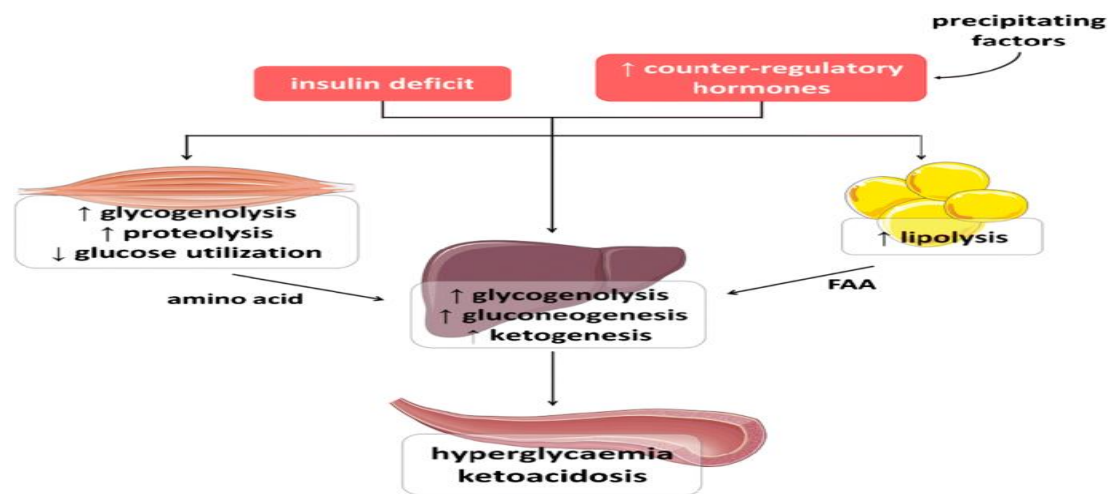


Figure 1.2: Changes in the body parameters caused by insulin defect.

The consequences of insulin defect are not far from insulin resistance consequences, which take place mainly at the insulin receptor level or signal transduction (Timothy G 2004 ).

The cell recognizes insulin by the alpha subunit of its receptor in the plasma membrane which has a tyrosine kinase activity, Insulin-insulin receptor binding stimulate the activation of downstream mediators including PI3K and IRS-1, which in the end allow the diffusion of glucose through GLUT-4 in the plasma membrane. Absence of tyrosine phosphorylation in the insulin receptor or its own substrate, unfunctional GLUT4 trafficking of the major enzymes in the pathway, mutations in The cell recognizes insulin by the alpha subunit of its receptor in the plasma membrane which has a tyrosine kinase activity, Insulin-insulin receptor binding stimulate the activation of downstream mediators including PI3K and IRS-1, which in the end allow the diffusion of glucose through GLUT-4 in the plasma membrane.

Absence of tyrosine phosphorylation in the insulin receptor or its own substrate, unfunctional GLUT4 trafficking of the major enzymes in the pathway, mutations in different genes, are the main causes of the insulin resistance (Timothy G 2004, Voet D 2011) as shown in Figure 1.3.

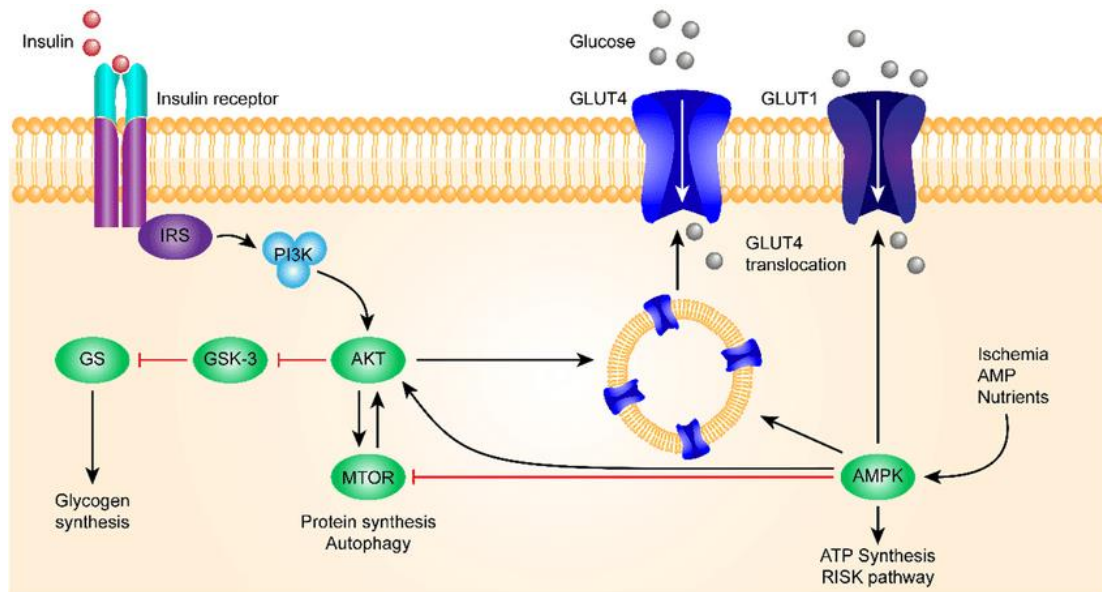


Figure 1.3: Insulin pathway.

## 1.2 Diabetes classification

Although all Diabetes types shares the same final result which is accumulation of glucose in the blood stream, except that they are divided into four groups according to the causes, age of onset etc: type 1 diabetes, type 2 diabetes, gestational diabetes, other types (MedicineNet 2019).

**1. Type1 diabetes:** Type 1 diabetes has an early onset compared to other types. It occurs when some or all of the beta cells in the pancreas are destroyed due to autoimmune disorder that targets the pancreas, which lead to abnormal high glucose level in the blood stream as a result of insulin absence which is the main factor to

assist glucose entrance and metabolism in various cells (MedicineNet. 2019) as demonstrated in figure 1.4.

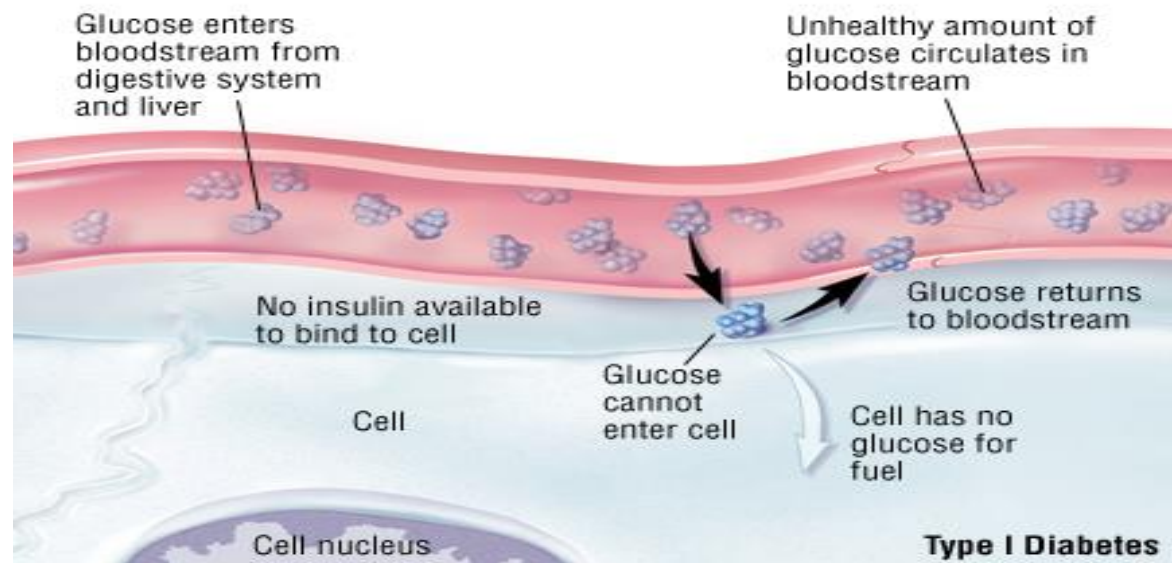


Figure 1.4: A scheme describes how glucose cannot enter the cell in the absence of insulin in type 1 diabetes.

**2. Type 2 diabetes:** Unlike type 1 diabetes, diabetes type 2 has late onset, it is much more common than type 1 and a result of insulin resistance in the cells, which means that the pancreatic beta cells function normally, but target cells refuse to respond to insulin, which results in accumulation of glucose in the blood stream like all the diabetes types (Shoback DG 2011) as shown in figure 1.5.

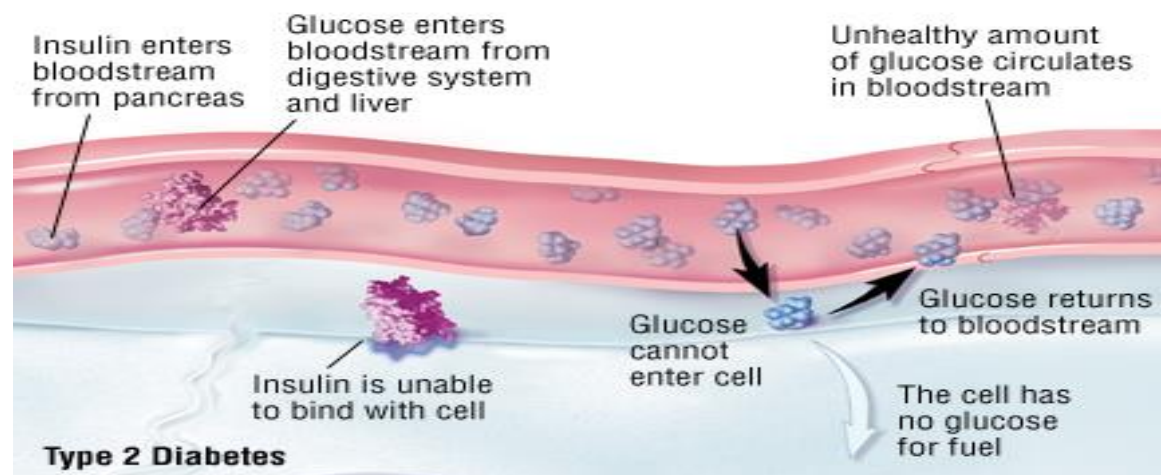


Figure 1.5: Type 2 diabetes as a result of insulin resistance.

**3. Gestational diabetes:** this type occur during pregnancy, and occupies 2-10% of the pregnancies in the world, it is caused by a combination of decreased insulin secretion and insufficient response of the cells (NDIC 2014).

**4. Mature onset diabetes of the young (MODY):** This type of diabetes is rare, usually the onset age of this type before 25 years. And inherited by genetic autosomal dominant mutation that lead to decrease insulin production (Kharroubi, A. T.2015 ,UA NIH 2017) figure 1.6 describe the various types of diabetes.

Stages  Aetiological process	Normoglycaemia	Hyperglycaemia			
	Normal glucose tolerance <i>(achieved without pharmacological agents)</i>	IGT and/or fasting hyperglycaemia	Not insulin requiring	Insulin requiring for control	Insulin requiring for survival
Islet cell destruction ◆ Autoimmune ◆ Idiopathic			Type 1 diabetes		
◆ Predominantly insulin resistance ◆ Predominantly insulin secretory defects			Type 2 diabetes		
Other specific disorders (eg MODY, endocrinopathies)			Other specific types of diabetes		
Pregnancy related defects			Gestational diabetes		

Figure 1.6: Classification of diabetes.

Patients who have partial destruction of beta cells may not require insulin if they control the glucose uptake by their diet because some of their beta cells can produce insulin and they can survive without medication. however patients who have excessive or complete beta cells destruction and no insulin secretion need insulin for their survival (American Diabetes Association 2017). Suitable glucose level control by

medications, along with diet control, body mass reduction and exercise will help to maintain the glucose level in the normal range (American Diabetes Association 2017).

### **1.3 Type 1 diabetes mellitus.**

Type 1 diabetes is the most common disease in early ages, but it also can be diagnosed during adulthood (Francesco C 2019). Seasonal coincidence may play a role in type 1 diabetes occurrence. This fact was proved by study which showed that the number of diabetic type 1 patients increase in children born in spring (Patterson CC 2009). What distinguishes it compared to the other diabetes types is that insulin secretion by beta cells in the pancreas is decreased or even not existed, caused by destruction of pancreatic beta cells. The cells destruction is affected by genetic factors or environmental factors (Khan HS 2009 ).

### **1.4 Epidemiology of diabetes type 1.**

Diabetes disease in general is one of the most expanded diseases worldwide. Figure 1.7 shows the prevalence of type 1 diabetes in different countries. Unlike China and India, where type 1 diabetes is uncommon, Finland has the highest disease occurrence rate among all countries with an incidence of 6 cases over 10,000 of the population every year, Sardinia came in the second place with an incidence rate of 4 per 10,000 annually. As for Venezuela the incidence rate was 0.9 - 1.8 per 10,000 of the population (Thunander M 2008). Germany, and Norway had the least incidence rate with 0.26 and 0.33 per 10,000 of the population respectively (Thunander M 2008, Eehalt S 2012), in Palestine 4.4% of the diagnosed subjects with diabetes are diagnosed with type 1 diabetes while the rest are diagnosed with type 2 diabetes ([www.worlddiabetesfoundation.org](http://www.worlddiabetesfoundation.org)).

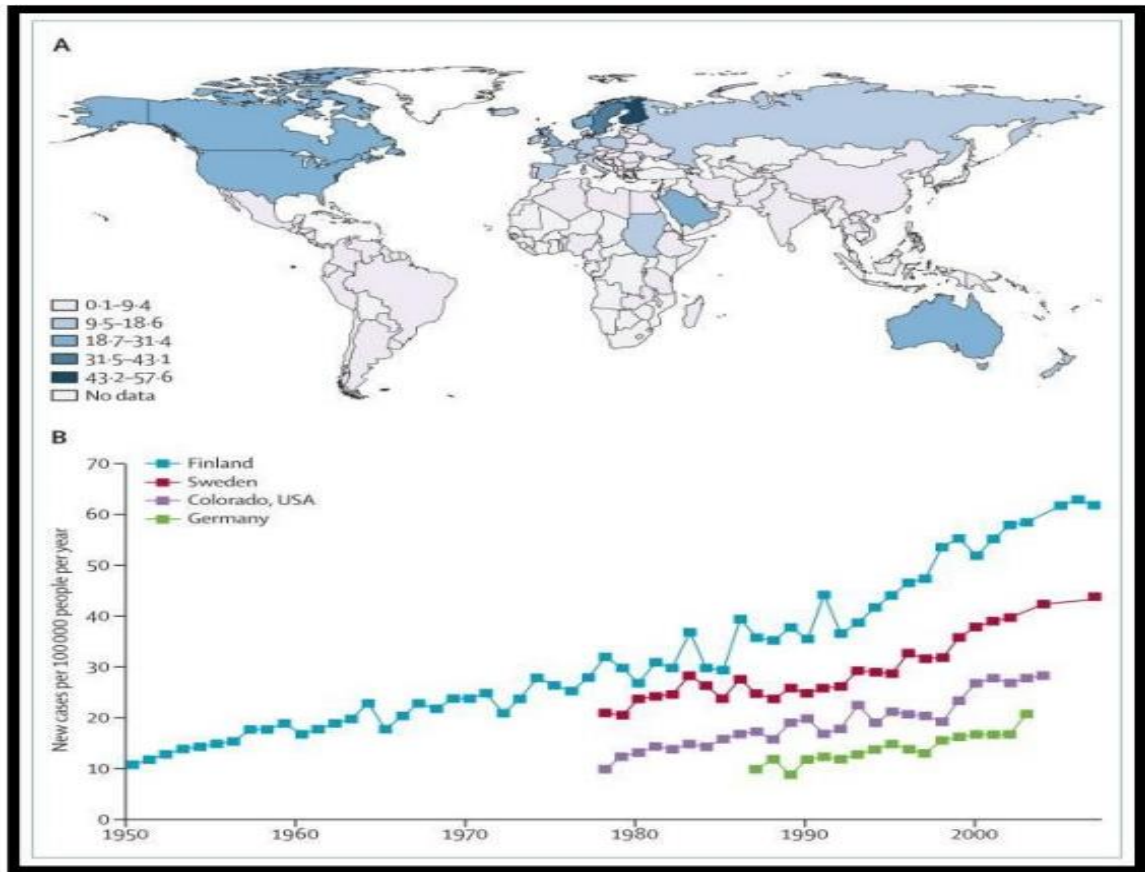


Figure 1.7: A. the prevalence of type 1 diabetes, B. the incidence of type 1 diabetes in different countries.

## 1.5 Subtypes of diabetes type 1:

**1. Autoimmune diabetes:** This type is caused by autoimmune response of T- cells to beta cells of the pancreas as a response to inflammation (Devendra D 2004) Detection of autoantibodies against beta cells of the pancreas in the patients serum is the main sign of autoimmune type 1 diabetes (Giugliano D 1996 ) Although 90% of the patients have autoantibodies against the islet cells in their serum the remaining 10% have negative autoantibodies in their serum (Hea Sang Lee 2019). These autoantibodies are diversified in multiple types: islet cells autoantibodies (ICA), insulin autoantibodies (IAA), protein tyrosine phosphatase (IA2) glutamic acid decarboxylase (GAD) and zinc transporter protein (ZnT8A) (Thunander M 2012).

Symptoms are like any other type of diabetes but more severely in children including polyuria, polyphagia, lack of energy, polydipsia, weight loss, infections, severe dehydration, slow wounds healing and ketoacidosis (Lamb MM 2009).

In addition to environmental factors which identified to play a role in the disease progression such as viruses including congenital rubella (Ginsberg-Fellner F 1985), enterovirus, rotavirus, herpesvirus, cytomegalovirus (Stene LC 2010). Genetic factors had been identified to play a role in disease progression like DR and DQ genes that are linked to HLA which present in autoimmune type 1 diabetes (Ginsberg-Fellner F 1985). Low vitamin D levels, feeding infants with cow milks instead of breast feeding, prenatal pollutants exposure, obesity in childhood, are all factors which increase the risk of diabetes type 1 incidence (Daneman D 2006).

**2. Idiopathic type 1 diabetes:** the cause of idiopathic type 1 diabetes is not identified yet. Patients with this type have episodic of ketoacidosis and varying insulin deficiency degree (Chinag JL 2014).

**3. Fulminant type 1 diabetes:** it is not autoimmune mediated type of type 1 diabetes just like the idiopathic type 1 (Yeung WC 2011 ). Patients suffer from ketoacidosis early after hyperglycemia occurrence with factors including viruses, genetic and environmental may lead to immune response that destroys the beta cells of the pancreas with no detection of autoantibodies in patients serum (Bach JF 2012).

### **1.6 Genetic association with diabetes type 1:**

Up to 50% of the risk factors that cause type 1 diabetes are genetic factors. The probability of identical twin to have type 1 diabetes is 25- 50 %, and for dizygotic twin is 6%, which is the same for siblings (Jahromi MM 2006). These numbers reflect the significant involvement of the genetic factors on diabetes occurrence.

**1. Human leukocyte antigen:** HLA gene is located in chromosome 6p21, which encodes for two classes of HLA: HLA class 1 and HLA class 2. The gene location and distribution is shown in figure 1.8 (Trowsdale J 2013, Hea Sang Lee 2019).

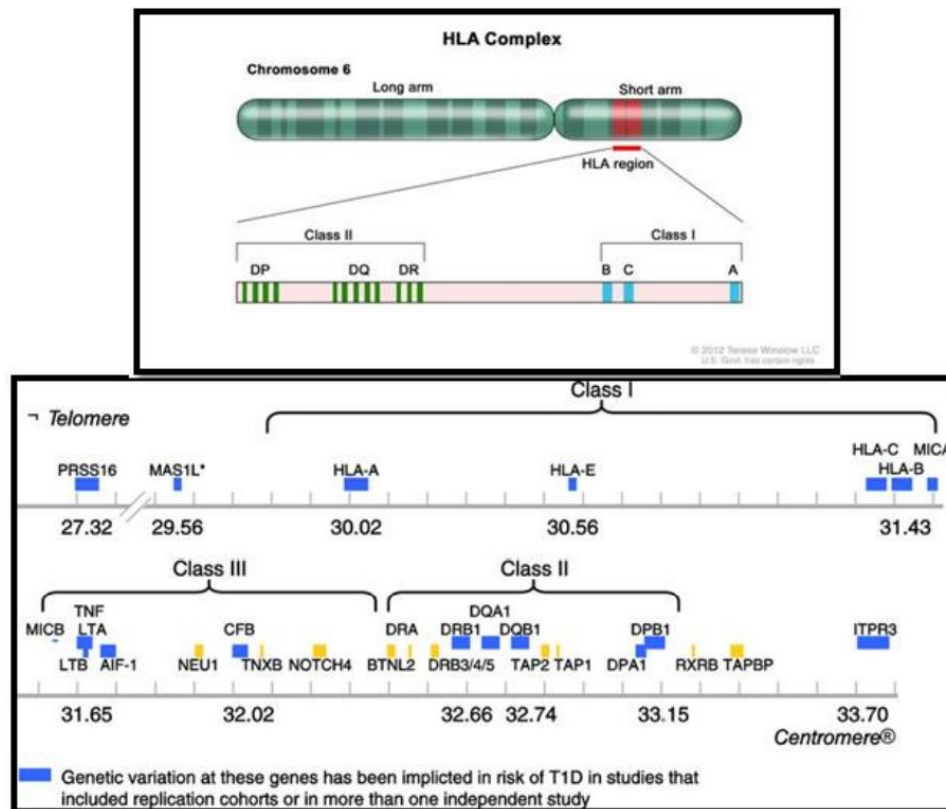


Figure 1.8: Human leukocyte antigen (HLA) location and distribution in chromosome 6.

For each class type of HLA antigen several subtypes are identified including: HLA class 1(HLA-A, HLA-B, HLA-C) HLA 2 class (HLA-DP, HLA DQ, HLA-DR) (Hea Sang Lee 2019). Class 1 HLA with its subtypes have more association with type 1 diabetes compared to HLA 2 class due to its interaction with HLA 2 gene classes. Among all class 2 HLA genes HLA-DR and HLA-DQ have more strong association with type 1 diabetes than the other classes (Valdes AM 1999). This is due to the variability of increasing and decreasing the affinity of binding to specific antigens related to type 1 diabetes (Stankov K 2013).

## 2. Non-HLA genes:

Even though diabetes type 1 is mainly an autoimmune disease which characterized with destruction of beta cells, however, More than 50 genes other than HLA genes are linked with type 1 diabetes such as AIRE gene which is an autoimmune regulator that affect the immune response to self-antigens and FOXP3 gene which results in defective regulation of T cells (Kharroubi, A. T.2015). Some of the genes are linked directly to diabetes like the insulin gene (INS) which is located in chromosome 11p15.5, while (rs2476601) polymorphism of protein tyrosine phosphatase non-receptor type 22 (PTPN22), cytotoxic T-lymphocyte antigen (CTLA-4), interleukin-2 receptor alpha subunit. Other genes may increase the risk of having diabetes type 1 in an indirect way (Hea Sang Lee 2019) some of Non-HLA genes are shown in figure 1.9.

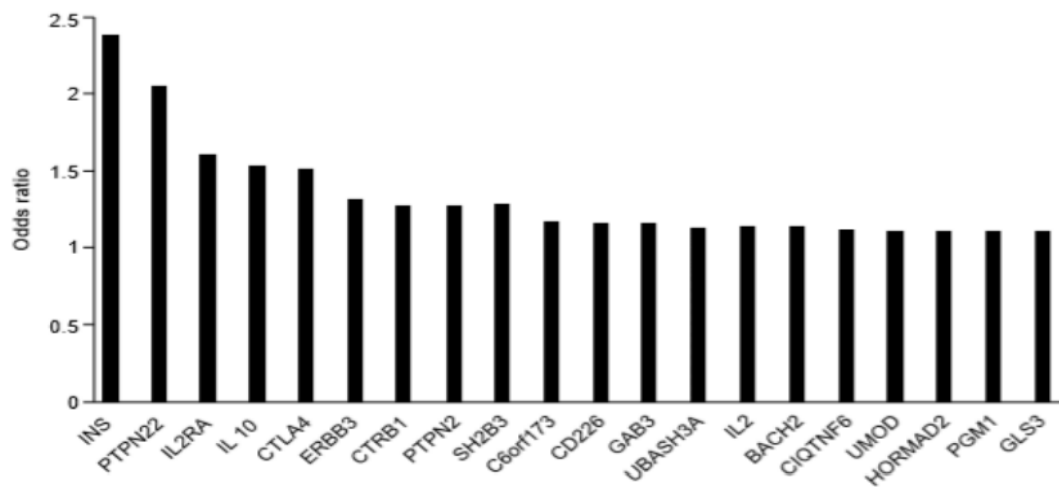


Figure 1.9: Association scale of non-HLA genes with type 1 diabetes.

## Osteoporosis

### 1.7 Osteoporosis

The skeleton is made up of bones which provide support, give shape to the body, and protects our organs from injuries. Bones are made of different layers of cells, the outer is the compact bone, the inner is the sponge bone because its less dense than the outer and it looks like a sponge as shown in figure 1.10. within the sponge bone cells, there are two types of functional cells, osteoclast which break down the bone cells, the second is the osteoblast which build up the bone cells using minerals including calcium and phosphate, a balance between both cells activity, must be preserved to maintain bone tissue health (Xiao Chen 2017). Hormones such as estrogen, growth hormones and testosterone are very important for keeping the activity of osteoblast higher than osteoclast, so the amount of bone formation will stay higher than the amount of bone resorption (Shun-ichi Harada 2003). Imbalance between osteoblast and osteoclast activity will cause osteoporosis which characterized by bone tissue breakage with clear fragility and increased prone to fracture as shown in figure 1.10 ( Consensus development conference 1993)

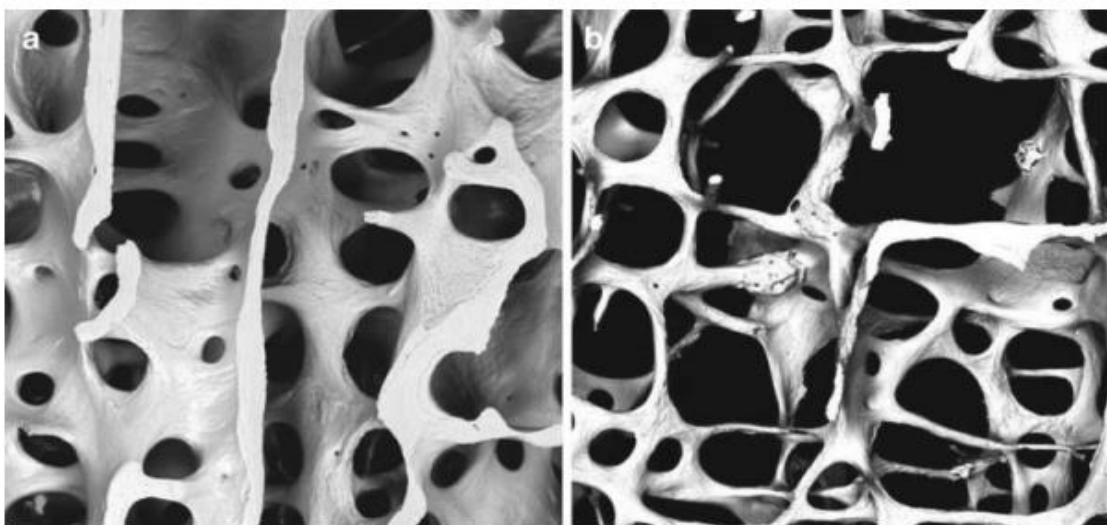


Figure 1.10: Microscopic structure of: a. normal bone tissue, b. osteoporotic bone tissue.

Fractures in patients mostly happen in specific parts of the bone skeleton including: vertebral (spine), proximal humerus, proximal femur (hip), distal forearm which is mostly where the first hit of osteoporosis occurs, followed by the proximal femur and vertebral (Rose SH 1982). Proximal femur (hip) fracture is the most dangerous fracture specially in old ages mostly in women and the probability of death from hip fracture is more than death from breast cancer (AGS 2007). In men the number of in-hospital deaths due to hip fracture is higher than deaths in women (Bukata SV 2011). Early detection for osteoporosis is very important because with early detection and treatment of the first fragility fracture hit, the possibility of the next fracture will be minimized to 50% which increase the public health (Bukata SV 2011).

### **1.8 Epidemiology of osteoporosis.**

Globally, 125 million people are suffering from osteoporosis, they are concentrated in USA and Canada, Oman, Iran, Taiwan, Argentina, Malta and several countries across Europe, since these countries having the highest percentages of osteoporotic cases worldwide (J. A. Kanis 2012). In Europe 22 million women, 5.5 million men were diagnosed with osteoporosis in 2010, among them 3.5 with fragility fractures, including 610 thousands hip fractures, 520 thousands spinal fractures, 560 thousands forearm fractures and 1.8 million with other types of fractures (Hernlund E 2013).

### **1.9 Osteoporosis classification:**

There are two main types of osteoporosis:

**1. Primary osteoporosis:** This type of osteoporosis occurs spontaneously, which include three subtypes.

**A. Type 1 (Postmenopausal) primary osteoporosis:** caused by decreasing the amount of estrogen hormone which regulate calcium deposition in the bone, and is very important for osteoblast function (Mathew B 1999).

**B. Type 2 (Senile) primary osteoporosis:** characterized by calcium and vitamin D deficiency specially in elderly people more than 70 years old, most old women which have osteoporosis have a combination of postmenopausal along with senile osteoporosis (Hannan MT 2000).

**C. Type 3 (Idiopathic) primary osteoporosis:** this type mainly is linked to children and young adults, which is rare and the cause is unknown. The hormonal and vitamin D level is normal and it is not clear why patients have weak bones (Juvenile osteoporosis 2015)

**2. Secondary osteoporosis:** this type is caused by other disease that is somehow affect the bone health like hyperthyroidism, hypogonadism, hyperparathyroidism, diabetes mellitus, or by drugs such as, barbiturates, anticonvulsants, corticosteroids (Juvenile osteoporosis 2015).

### **1.10 Risk factors.**

Several risk factors were identified to have strong association with osteoporosis including:

**1. Gender and age:** Regarding gender, osteoporosis is not identified as a gender related disease at any site (R Rizzoli 2001). Both genders loss around 0.5- 1 percent of bone mass every year following reaching the peak of bone mass at age 35 years in females and 40 years in males. The changes in bone mass during life span is shown in

figure 1.11 (Richard D 1989). Due to estrogen deficiency after menopausal osteoporosis incidence will increase enormously in women (Gambert 1995).

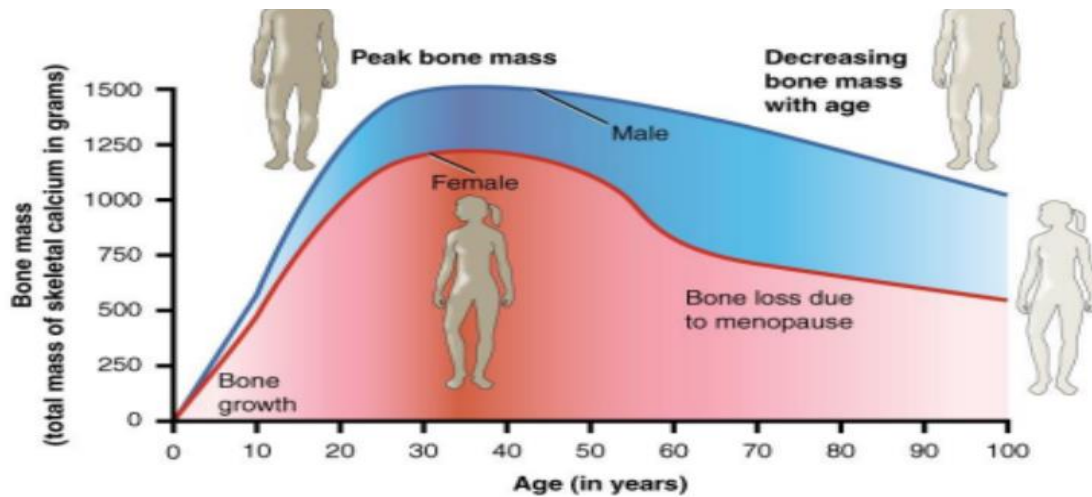


Figure 1.11: Change in bone mass during the human life span.

Bone turnover is the process where resorption and reformation of bone tissue occur. Osteoclast takes around 10 days to resorb old osteocyte, followed by 3 months of osteocyte formation by osteoblast (Sahana S 2016). To assess the turnover fidelity, different markers are produced during the process that can be measured (Carey JJ 2006) as shown in figure 1.12.

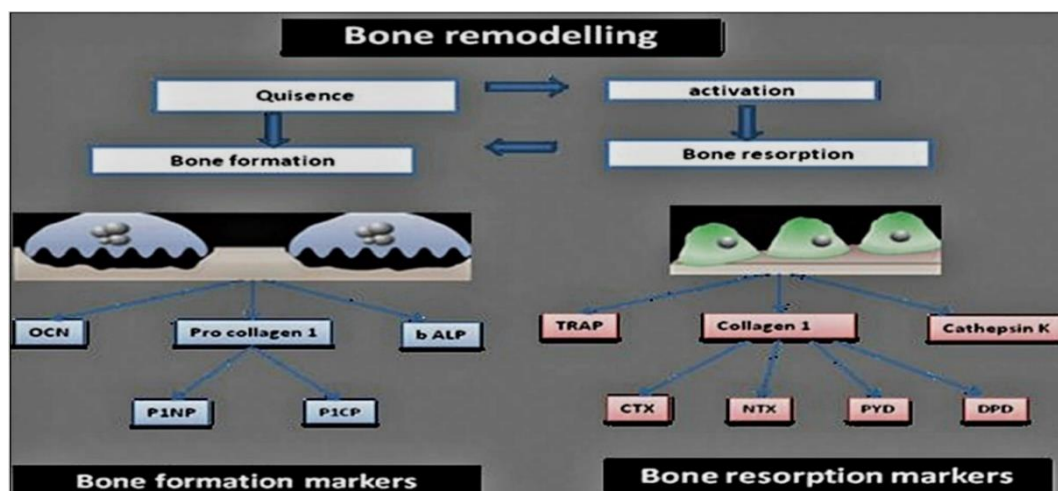


Figure 1.12: Bone turnover markers.

**2. body mass index (BMI):** In women, BMI is very effective on bone health (Beers 2004). Women with low BMI are at higher risk of developing osteoporosis. Since low BMI mean low fats and fats in turn help to store definite forms of estrogen, which is known to play a role in bone health (Beers 2004).

**3. family history and genetic factors:** Osteoporosis is multifactorial disease, genetic factors represent one of these significant factor which contribute to the development of the disease, A number of genes are linked to the maintenance of bone health (Guéguen R, Jouanny 2009). Several genes including Vitamine D receptor (VDR), Estrogen receptor (ER), Calcitonin (CT), other genes listed in table 1.1 (Andrew K. 2012).

**Table 1.1: Some of the candidate genes linked to osteoporosis. (Andrew K. 2012).**

Gene	Pathway/Function	Chromosome	Study conformation
VDR	Vitamin D endocrine pathway	12	CGAS, MA-C
ESR1	Estrogen endocrine pathway	6	CGAS, GWAS, MA-C, MA-G
LRP5	Wnt/ $\beta$ catenin pathway	11	CGAS, GWAS, MA-C, MA-G
SOST	Wnt/ $\beta$ catenin pathway	17	CGAS, GWAS,
TNFSF11	RANK/RANKL/OPG pathway	13	CGAS, GWAS, MA-G
COL1A1	Structural protein	17	CGAS, MA-C
SPP1	Bone remodeling	4	CGAS, MA-C
ITGA1	Structural protein	5	CGAS, MA-C
SP7	Transcription factor	12	GWAS, MA-G
SOX6	Transcription factor	11	GWAS, MA-G

**4. drug use:** It's known that bone health is affected by some drugs such as steroids, anticonvulsants and glucocorticoids especially when prolonged use of these drugs (Panday K 2014). Corticosteroids are part of glucocorticoid, with an anti-inflammatory function that reduce calcium absorption and increase parathyroid hormone secretion which increases receptor activator of nuclear factor kappa B ligand (RANKL) (Carpinteri R). In addition, it decreases osteoprotegerin (OPG) which in turn increases bone resorption by activation of osteoclast, which increases loss of calcium by urine and decreases osteoblast function by apoptosis. All these factors will affect the bone health (Paola I 2016) figure 1.13 demonstrates the collective effects of glucocorticoid on bone health status.

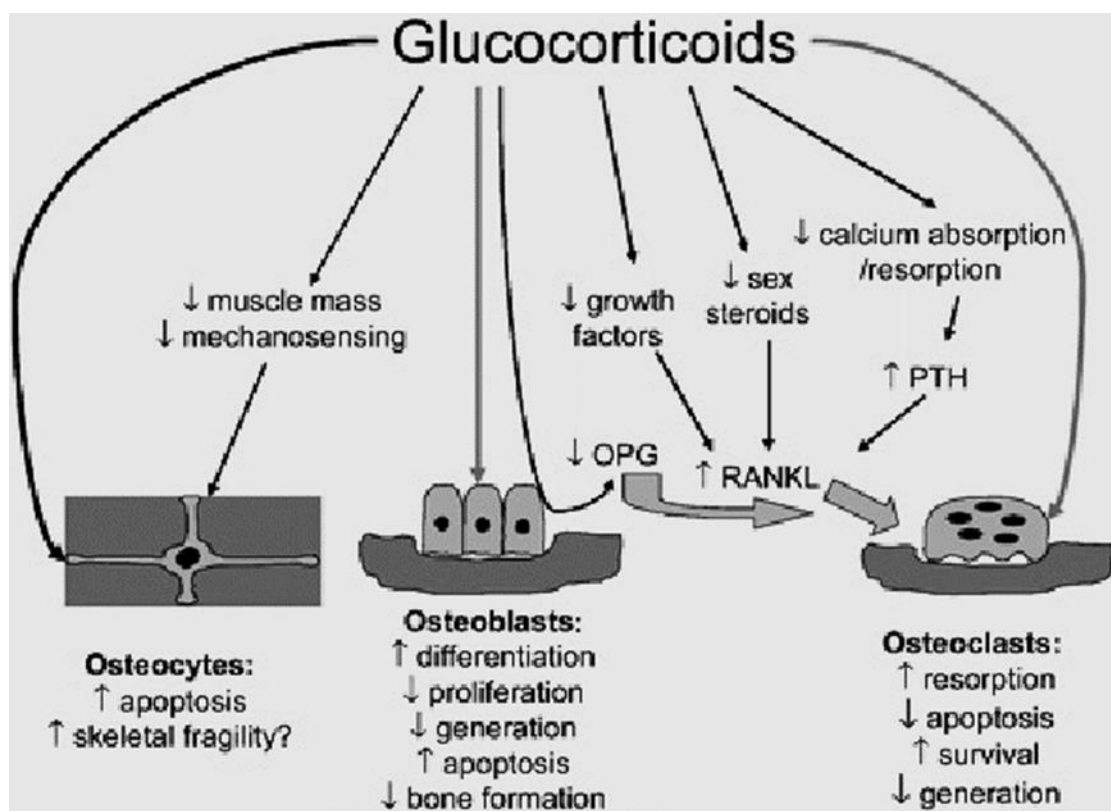


Figure 1.13: Glucocorticoid effect on bone health

**5. Menopause and estrogen decrease:** After menopause, a recognizable reduction in estrogen occurs which will decrease osteoblast activity that is responsible of replacing resorped bones (McNamara 2010). Both osteoblast and osteoclast have estrogen receptor ER alpha and ER beta. Estrogen increases osteoclast apoptosis by inhibiting its receptor, but when estrogen is absent in menopause hematopoietic progenitors of osteoclast will increase, and osteoclast apoptosis will be inhibited which results in bone resorption (L. M. McNamara 2010) and decrease the bone formation by osteoclast due to osteoblast apoptosis due to estrogen deficiency (Tatsumi S 2007) as described in figure 1.14.

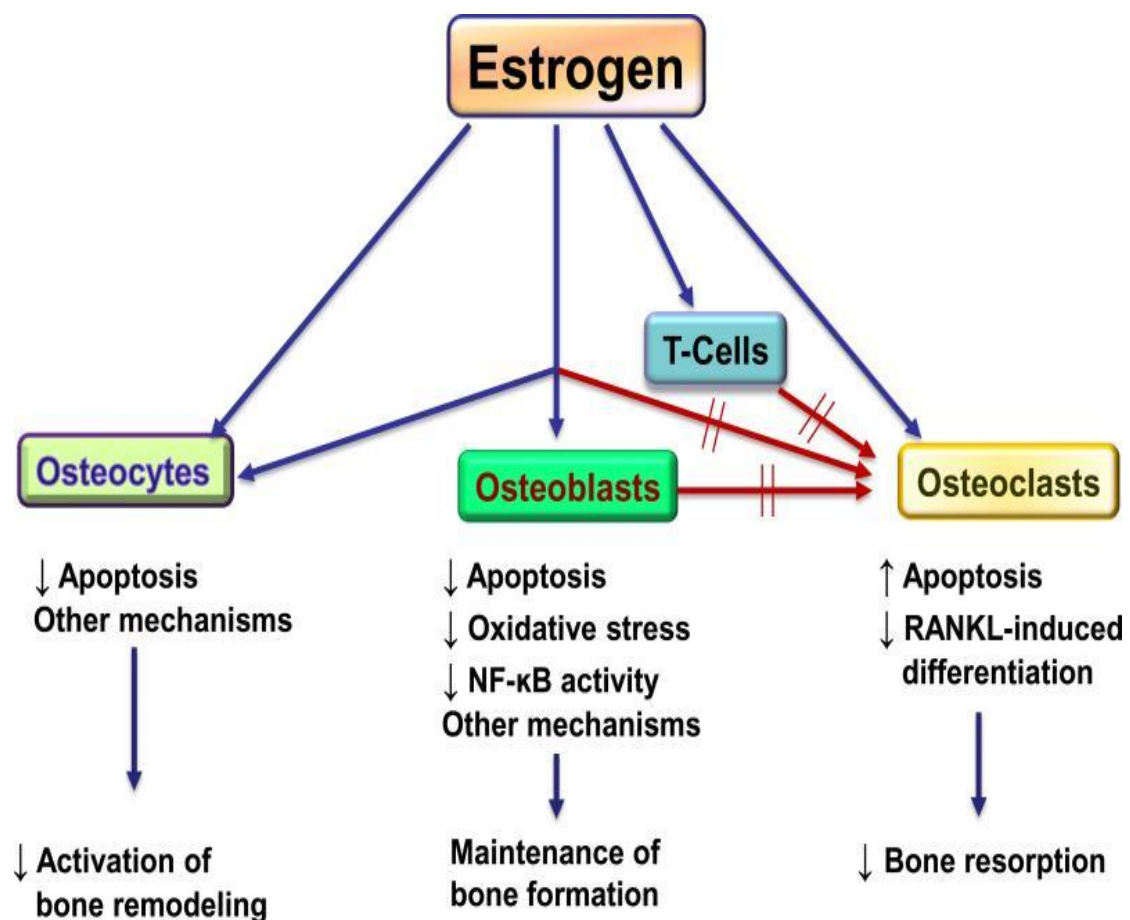


Figure 1.14: Estrogen effect on bone health.

## 6. Physical activity.

Physical activity is very important in maintaining bone health. Activity and mechanical loading on bone skeleton will prevent bone loss. At the molecular level, physical activity increases OPG/RANKL ratio which increase bone formation and resorption inhibition (Paola C 2016) as described in figure 1.15.

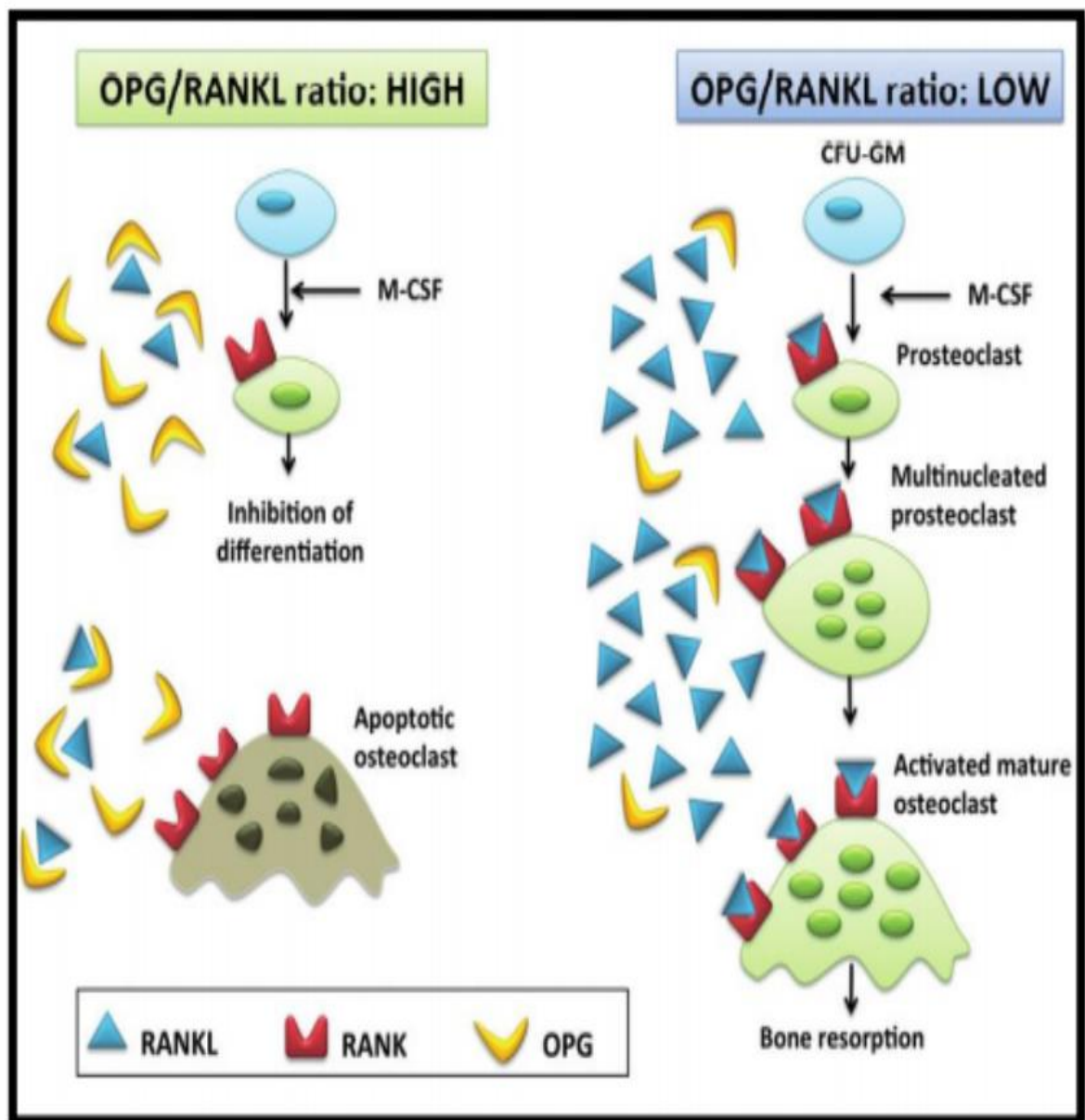


Figure 1.15: Effect of OPG/ RANKL ratio in osteoblast, osteoclast activity.

### 1.11 Osteoporosis diagnosis:

Bone mineral density (BMD) is the gold standard test for osteoporosis diagnosis, BMD test is done using Dual-Energy X-ray absorptiometry (DEXA). Two X-rays energies used in this test to measure bone density in two dimensions. It is fast, and with low patients exposure to radiation. BMD is calculated by measuring the minerals amount on specific site divided by this site space (  $\text{g/cm}^2$ ). DEXA very useful for diagnosis and monitoring of treatment efficiency (Audran 2004). World Health Organization (WHO) has established detailed normal ranges for bone health examination based on results generated from DEXA (Cummings, S.R 2002) as described in table 1.2.

**Table 1.2: WHO normal ranges recommendations of BMD score generated from DEXA examination.**

BMD T-score	Diagnosis
T-score $\geq -1$ SD	Normal
$-1 > \text{T-score} > -2.5$ SD	Low bone mass/ Osteopenia
T-score $\leq -2.5$ SD	Osteoporosis
T-score $\leq -2.5$ SD with existing fracture	Sever osteoporosis

## **1.12 Association of diabetes type 1 with bone health.**

Previous reports showed that patients with diabetes type 1 and low bone density are at high risk of fracture incidence (WHO 1994, Tayyab s. 2015). Maintenance of bone health is achieved by a balance between formation of new osteocytes and osteocyte resorption by osteoclast. Therefore, the imbalance between formation and resorption will lead to abnormality in bones mainly osteoporosis (Schwartz AV 2017). Bone remodeling balance in diabetic patients usually weak, due to decrease in bone formation compared with bone resorption, in response to insulin loss in diabetes type 1 patients which regulate the turnover and bone formation by signaling, which lead to lower BMD score and bone quality causing accumulation of microfractures that can't be healed (Krakauer JC 2006). During bone formation the body needs a specific amount of energy which is generated from glucose metabolism. Insulin is the most important hormone needed to enter glucose inside cells to generate energy needed for different biological processes in the body including bone formation (McKenna MJ 2015). In addition to the direct effect of diabetes type 1 on bone formation, complications in other organs due to diabetes type 1 are substantial in increasing fractures, such as neurologic and cardiovascular complications by increasing the risk of fall, and nephropathy by subsequently parathyroid hormone metabolism and derangements in vitamin D (Tayyab s. 2015).

## Vitamin D receptor

### 1.13 Vitamin D receptor (VDR).

The term vitamin D, also known as *cholecalciferol* is generated when UV light energy from the sun contacts the body skin which plays an important role in different biological processes inside the body including the immune response, and skeletal metabolism (Haussler 1998). It is generated from a precursor molecule 7-dehydrocholesterol, which is metabolized within the body to the hormonally-active form to start its biological function. This transformation occurs in two steps, as shown in figure 1.16 (Bikle 2004)

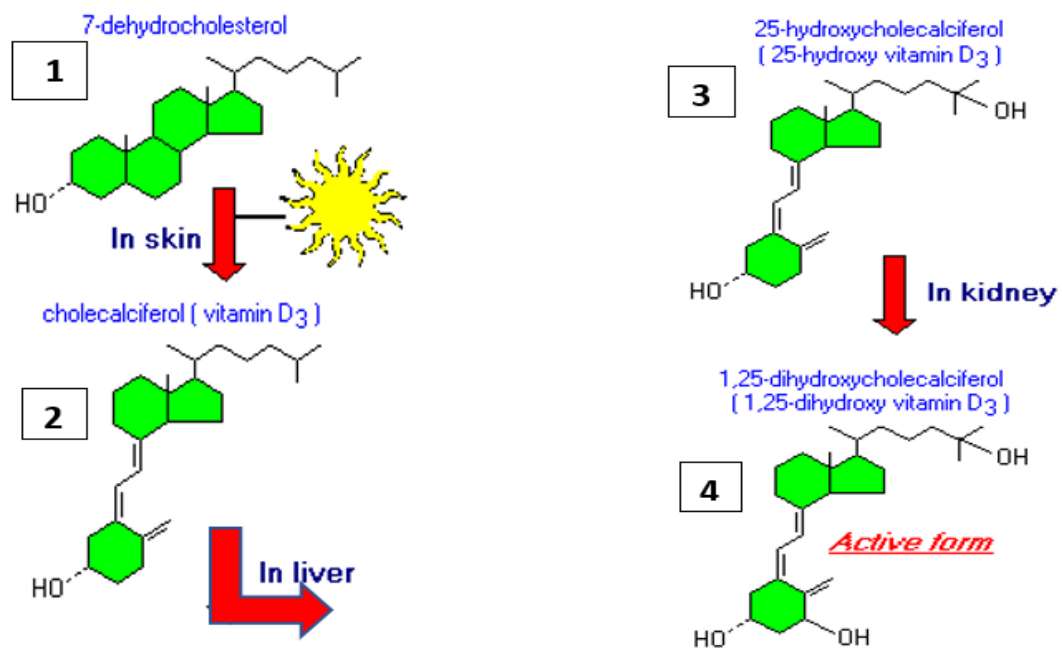


Figure 1.16: Transformation process of vitamin D to its active form.

The hormonal activities of 1,25-(OH)<sub>2</sub>D<sub>3</sub> are mediated through its intracellular receptor—the vitamin D receptor (VDR). VDR is a transcription factor that needs vitamin D stimulation to start its activity of transcribing genes (DeLuca&Schnoes,1983).

### 1.14 Vitamin D effect on biological activities:

The  $1,25(\text{OH})\text{D}_3$  is a hormone involved in mineral metabolism and bone growth. It promotes the differentiation of osteoblasts and regulates the production of proteins including collagen, alkaline phosphatase, and osteocalcin that are involved in bone formation. It also induces RANK, a membrane protein in osteoclasts to stimulate the formation and activity of osteoclasts. Thus, vitamin D hormone regulates both bone formation and bone resorption. (Bikle,2004).

Vitamin D also effect bone by maintaining intracellular and extracellular calcium concentrations within a physiologically acceptable range, and providing the proper balance of calcium and phosphorus to support mineralization in coordination with parathyroid hormone as explained in figure 1.17 (Scientific Committee on Food 2002).

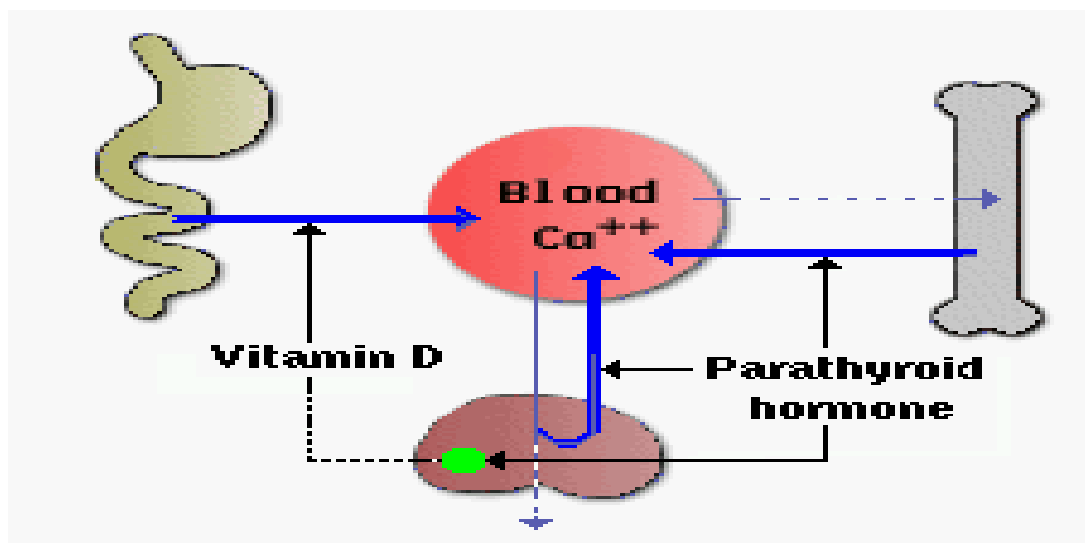


Figure 1.17: Physiological effect of vitamin D on calcium absorption and use by the bone.

### 1.15 Vitamin D receptor (VDR):

The vitamin D receptor (VDR) is a transcription factor. It is about 50-60kDa and distributed in almost all cells in the body (Daniel 2004, Rachez et al. 2000). It is activated after binding with  $1,25(\text{OH})\text{D}_3$ . The heterodimer VDR-RXR activated after binding with  $1,25(\text{OH})\text{D}_3$ , which results in activation of gene expression of responsive genes, which mediate the observed biological functions of Vitamin D (Bikle 2004) figure 1.18.

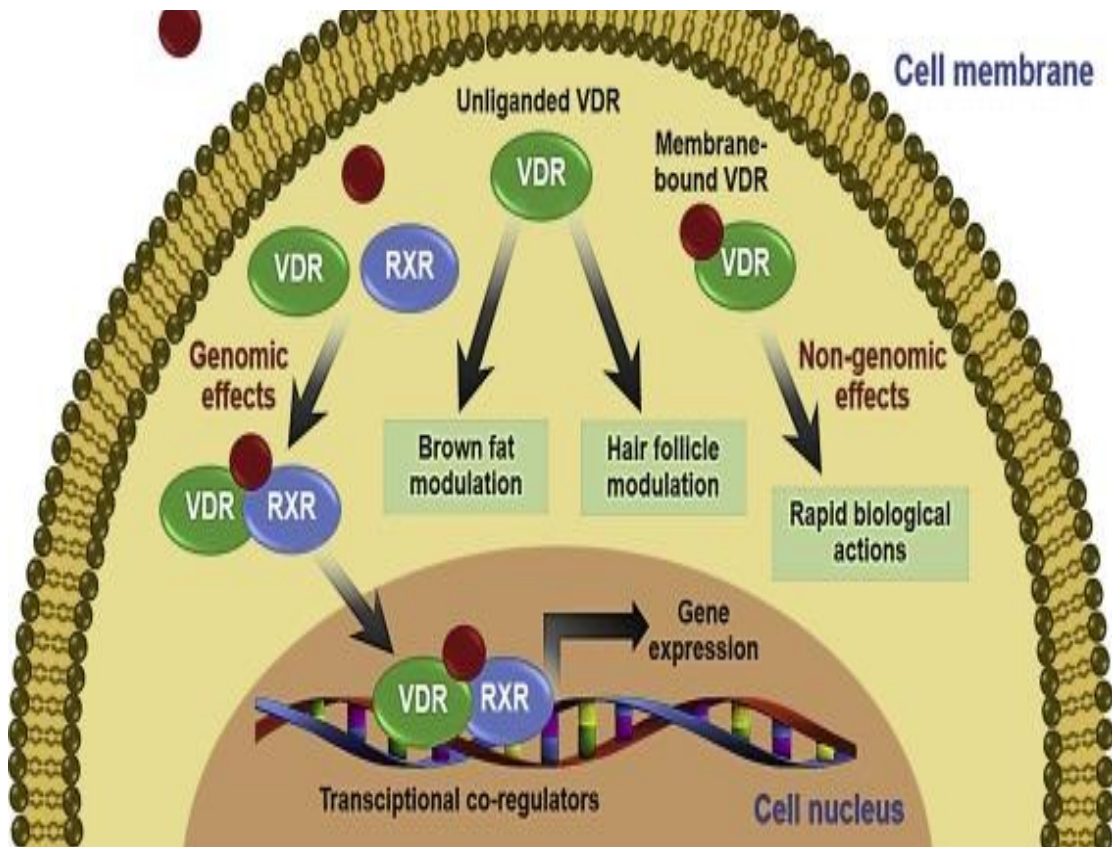


Figure 1.18: Gene expression by VDR after binding with vitamin D.

### 1.16 VDR gene.

The VDR gene is located on chromosome 12q13.11 (Taymans,1999) figure 1.19. It is approximately 100kb long and contains 14 exons; three exons (1A,1B and 1C) are found in the 5`non-coding sequence . Another 3 exons (1d,1e,1f) were discovered later, and 8 exons (II – IX) encode for the VDR protein structure (Uitterlinden etal,2004). VDR gene is the first candidate gene to be studied in osteoporosis due to its acts as regulator of calcium metabolism and bone cell functionx (Ralston,2003 , Uitterlinden,2002).

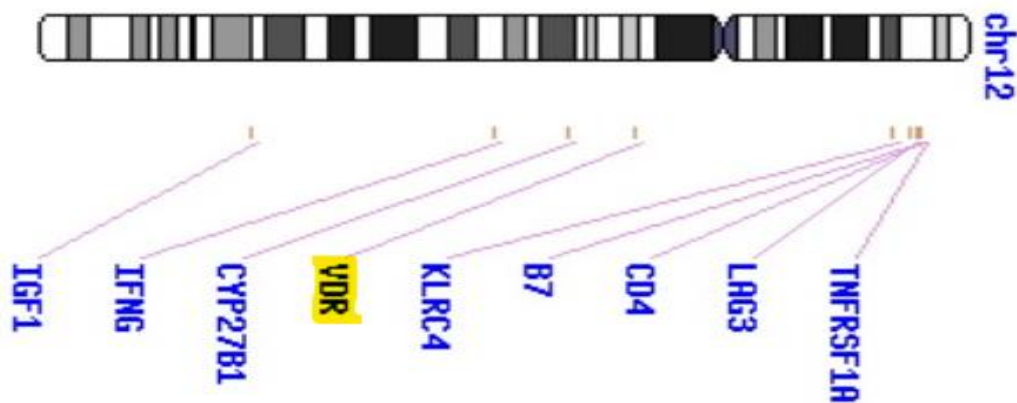


Figure 1.19: Human chromosome 12.

### 1.17 polymorphism in the VDR gene.

One of the most substantial studies in bone-related genetic research in the past few years was the discovery of new polymorphisms in the VDR gene and their association with bone mineral density (BMD) and osteoporosis (Haussler etal,1998). About, 63 polymorphisms across 22 kb of sequences were discovered in the VDR gene and some of them are shown in figure 1.20 (Uitterlinden, 2004) table 1.3.

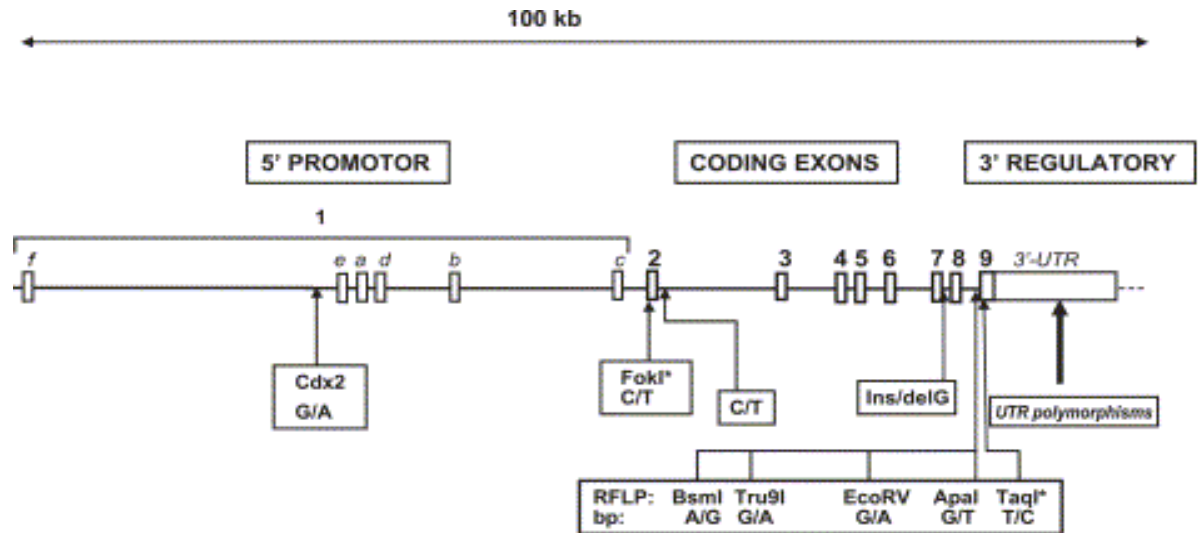


Figure 1.20: Location of known polymorphisms in the VDR gene.

**Table 1.3: Major VDR polymorphisms with their location and nucleotide variation.**

Polymorphism	Location	Codon	Amino acid change	Nucleotide Variant	References
CdX2	1e promoter: binding site for an intestinal-specific transcription factor	–	–	G/A	*Arai et al. 2001 *Uitterlinden et al. 2004
FokI (Alleles F/f)	Exon 2	ATG Codon 1 (start codon)	Yields 3- aa shorter protein	C/T	Arai et al. 1997
BsmI (Alleles B/b)	Intron 8 (between exon 8+9)	–	–	A/G	Morrison et al. 1992
Tru 9I	Intron 8 (between exon 8+9)	–	–	G/A	Ye et al. 2000
Eco RV	Intron 8 (between exon 8+9)	–	–	G/A	Morrison et al. 1992
ApaI (Alleles A/a)	Intron 8 (between exon 8+9)	–	–	G/T	Faraco et al. 1989
TaqI (Alleles T/t)	Exon 9	Codon 352	Silent mutation	T/C	Morrison et al. 1994



## 2. rs1544410 (BsmI B > b) polymorphism:

BSMI polymorphism is located between exon 8 and 9 in intron 8 (Morrison 1992).

while some studies by (Morrison et al 1992,1994,1997) suggested that rs1544410

BsmI polymorphism site is associated with low BMD risk, other studies didn't find any

effect (Uitterlinden et al.2002). Hustmyer et al in his paper argued that the results

obtained from Morrison`s work may be linked to another gene that involved in bone

density regulation other than VDR, or that this genetic linkage may be obscured by

additional environmental factors which differs between population to another

(Hustmyer et al,1994). Table 1.4 and 1.5 concluded studies and findings associated

with BSM-I and FOK-I polymorphisms in the VDR gene.

**Table 1.4: Some studies and findings that confirms the association of BSM-I and FOK-I polymorphisms in the VDR gene with bone health.**

Marker locus	n	Population characteristics	Phenotype and effect	P -value	Reference
<b>BsmI</b>	250	Normal healthy white twins	BMD of LS, FN	LS (0.000054, FN (0.038)	Morrison et al. 1994
<b>BsmI</b>	202	Normal healthy premenopausal Japanese women	BMD (Bb<bb) Bone turnover: levels of osteocalcin, alkaline phosphate. 1,25-dihydroxyvitamin D (Bb>bb) FN BMD (bAT<other haplotypes)		Tokita et al. 1996
<b>BsmI</b>	100	Normal prepubertal American girls of Mexican descent	Femoral BMD (bb>BB) Lumbar vertebral BMD (bb>BB)	0.04 (for bb>BB) 0.03 (for bb>BB)	Sainz et al. 1997
<b>FokI</b>	154	Premenopausal American women (72 black and 82 white) 20-40 yr	Whole-group FN BMD (ff<FF) White: total body BMD, FN BMD (ff<FF)	0.015 0.042 (total body BMD) 0.001 (FN)	Harris et al. 1997
<b>FokI</b>	100	Postmenopausal Mexican-	LS BMD (ff<Ff<FF) Decrease in BMD at	0.01 0.005	Gross et al. 1996

		American white women	FN in 2 yr (ff>FF)		
<b>FokI</b>	72	Healthy children aged 7-12 yr	BMD (FF>Ff>ff) Calcium absorption (FF>Ff>ff)	0.02 0.04	Ames et al. 1999
<b>BsmI</b>	558	Non-obese postmenopausal women (BMI<30 kg/m <sup>2</sup> )	FN BMD (bb>BB)	0.04	Vandevyver et al. 1997
<b>BsmI</b>	88	Japanese hemodialysed patients	Decrease of whole-body and FN BMD during 18 months (BB>Bb, bb)	<0.02	Karkoszka et al. 1998
<b>FokI</b>	98	Postmenopausal osteoporotic French women aged 45-75 yr	FN BMD (ff<FF, Ff)	Significant	Lucotte et al. 1999
<b>BsmI</b>	90	Healthy white males	Height at birth (BB<Bb, bb), growth from birth to age 16.9 0.3 (BB<Bb, bb), height during (age 16.9 0.3) and after puberty (age 19.3 0.7) (BB<Bb, bb), bone area of humerus, femur and total body (BB<Bb,bb)	0.01 (height at birth) 0.01 (growth after birth) 0.005-0.008 (height during and after puberty) <0.05 (bone area)	Lorentzon et al. 2000
<b>BsmI</b>	99	Healthy white girls, aged 16.9 1.2 yr	LS BMD (Bb>bb)	0.02	Lorentzon et al. 2001
<b>FokI</b>	118	Healthy Irish adults aged 16-67 yr	Serum osteocalcin (ff>Ff>FF), urinary pyridinoline and deoxypyridinoline (ff>Ff>FF)		Sheehan et al. 2001
<b>FokI</b>	163	Postmenopausal Chinese women in Taiwan	LS BMD (Ff>ff)	0.29	Chen et al. 2002
<b>BsmI</b>		Postmenopausal Korean women	BMD		Kim et al. 2003

**Table 1.5: Studies and findings that confirms that there is no association of BSM-I and FOK-I polymorphisms in the VDR gene with bone health.**

Marker locus	n	Population characteristics	Phenotype	Reference
<b>FokI</b>	174	Premenopausal French women	BMD, calcium, parathyroid hormone, vitamin D, osteocalcin, alkaline phosphatase	Eccleshall et al. 1998
<b>BsmI</b>	163	Postmenopausal women	BMD	Boschitsch et al. 1996
<b>BsmI</b>	268	Chinese, 155 men aged 22-88, 113 premenopausal women aged 40-53 yr	BMD, BMC, markers of bone turnover	Tsai et al. 1996
	69	Premenopausal white women	BMD	Alahari et al. 1997
<b>BsmI</b>	723	Danish women	BMD at LS, hip, and forearm	Jorgensen et al. 1996
	110		Early postmenopausal bone loss (age 51-53 yr)	
	108		Late postmenopausal bone loss (age 63-69 yr)	
	109		Long-term postmenopausal bone loss (age 51-69 yr)	
<b>FokI</b>	182	Postmenopausal women with sporadic primary HPT	Serum calcium, serum PTH, BMD, parathyroid tumor weight, VDR and PTH mRNA levels, Ca -PTH set points	Correa et al. 1999
<b>FokI</b>	124	Postmenopausal osteoporotic French women aged 45-90 yr	Age, yr since menopause, height, weight, BMD at LS and FN	Lucotte et al. 1999
<b>BsmI</b>	191	Postmenopausal Japanese women	LS baseline BMD	Kikuchi et al. 1999
	393	Women aged 45-53 yr	BBA, SOS	Gregg et al. 1999
<b>BsmI</b>	200	Healthy perimenopausal Danish white women	Lumbar and femoral baseline BMD, bone loss rate, biochemical markers of bone metabolism (bone specific alkaline phosphatase, urinary hydroxyproline, serum osteocalcin)	Hansen et al. 1998

<b>BsmI</b>	114	Postmenopausal Czech women aged 62.5 8.9 yr	BMD	Zajickova et al. 2002
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### **1.18 Study problem.**

No previous information is currently available regarding the status of bone health in Palestinian type 1 diabetes mellitus patients and its probable causes. Investigating the association between the indicated VDR gene polymorphisms, which affect gene activity and expression with T1DM and BMD levels will provide vital information regarding the important consideration of bone health management inclusion of additional factors regarding the importance of proper management and care in T1DM patients.

### **1.19 Aim of the study.**

The major aims of this investigation are to directly study the association between the indicated VDR gene polymorphisms as a potential factor affecting BMD in T1D and its significance in the pathophysiology of T1D and provide an important tool to control this disease and its complications especially regarding the preservation of or bone health or other unrelated effects.

## Chapter 2

### Materials and methods

#### 2.1 Study subjects:

Eighty six 86 diabetic type 1 patients of both genders, were recruited from various clinician centers. Their ages range between 18-30 years. The subjects were diagnosed with diabetes type 1 based on specific parameters as shown in table (2.1).

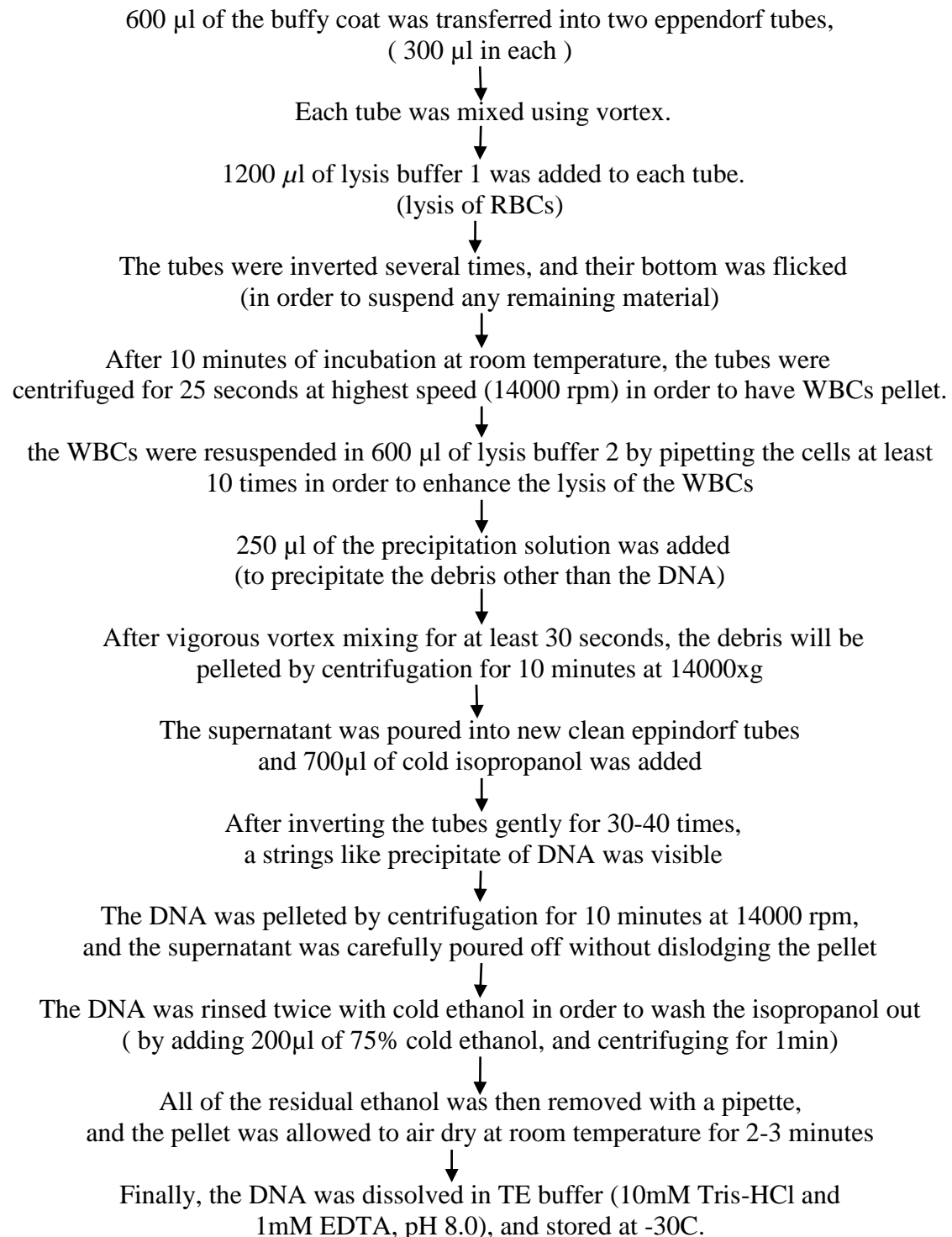
The 88 healthy control subjects with the same age range were recruited from Arab American university students and community. All subjects signed a written informed consent to participate in the study. The diabetic patients subjects suffer from type 1 diabetes with no other known chronic or acute diseases. And the control subjects were healthy with no known chronic or acute diseases that may affect the analysis. Obese subjects and subjects who suffer from chronic and acute diseases were excluded from the study.

**Table (2.1): Lab test Parameters of diabetes type 1 of the study subjects.**

Parameter	Result
Glucose level (mg/dl)	450 +- 50
C-peptide	0.1 +- 0.32
Hemoglobin A1c (%)	10.5 +- 0.55
Autoantibody	Positive

## 2.2 DNA extraction.

Blood samples (3-5ml) were collected in EDTA tubes, centrifuged for 15 minutes at 2000 rpm. Then DNA was extracted using commercially available kit (Master Pure™ Genomic DNA Purification Kit, Epicenter Technology Co. Cat.No.MG71100). The extraction procedure steps were as follows:



### **2.3 DNA quantification**

The concentration and purity of the extracted DNA were examined using a photometer (Nanodrop). 1 µl of pure water (nucleotide free water) was used as a blank to the photometer, followed by 1 µl of each sample DNA. The absorbance ratio A<sub>260</sub>/A<sub>280</sub> was used in the analysis of each sample which indicates the ratio between DNA to proteins that reflects the purity of the samples and a ratio of >1.6 was considered with high purity. The results were assessed as follows:

- Absorbance at 230nm: represent the salt concentration.
- Absorbance at 260nm: represent the DNA concentration.
- Absorbance at 280nm: represent the protein concentration.

### **2.4 Polymerase chain reaction (PCR)**

The PCR, mixture was prepared in 25 µl total volume as follows: 12.5 µl 2X ready master PCR mix (Promega). 1 µl DNA template (approximately 200 ng), 1 µl (150 ng/µ) of both forward and reverse primers (the primers sequence used to amplify the DNA sequence containing the indicated polymorphisms are described in table (2.2)) 9.5 µl water, The PCR protocols used to amplify both DNA fragments from exon 2 and intron 8 of the VDR gene to test for the polymorphisms rs10735810 and rs1544410 are described in table (2.3)).

**Table 2.2: Primers of rs10735810 (exon 2) and rs1544410 (intron 8) used in the study.**

Gene	Polymorphism	Primer type	5'-3' Primer Sequence	Product size (bp)	Reference
VDR	rs10735810 (exon 2) polymorphism	Forward	AGCTGGCCCTG	250	(Tofteng etal, 2002)
		Reverse	GCACTGACTCT G TCTTCTCCCTCC CTTCCACTG		
VDR	rs1544410 (intron 8) polymorphism	Forward	AACCAAGACTA	820	(Tofteng etal, 2002)
		Reverse	CAAGTACCGCG TCAGTGA AACCAGCGGGA AGAGGTCAAGG G		

**Table 2.3: PCR protocol for amplification of the DNA fragments including rs10735810 (exon 2) and rs1544410 (intron 8).**

PCR Conditions	VDR (rs1544410 intron 8)		VDR (rs10735810 exon 2)	
Initial denaturation	94 C, 2min		94 C, 5min	
2 <sup>nd</sup> Denaturation	94° C,30sec	30 cycles	95° C, 1min	30 cycle
Annealing	60° C,45sec		56° C,1min	
Extension	72 °C,1min		72° C,2min	
Final extension	72° C, 10min		72° C, 5min	

## 2.5 Restriction fragments length polymorphism (RFLP)

The principle of RFLP is based on the treatment of PCR amplified DNA product with a restriction enzyme that cuts the DNA at specific known site. If a single nucleotide haplotype variation is present the enzyme will either cut the DNA or amplified DNA fragment will remain intact. This method will show an aberrant pattern if polymorphisms exist in restriction sites where the enzyme cuts (Tauata et al, 2000). The digestion contained mixture: 1.5 10x buffer, 0.15  $\mu$ l BSM-1 (10000  $\mu$ /ml source) or 0.3  $\mu$ l FOK-1(5000  $\mu$ /ml source) enzyme, 10 $\mu$ l of PCR product, 3.35  $\mu$ l H<sub>2</sub>O for the BSM-1 or 3.2  $\mu$ l H<sub>2</sub>O for the FOK-1. The digestion mixture were incubated overnight at 37 °C, followed by electrophoresis in 2% agarose gel to analyze the enzyme digestion products as described ( one intact DNA fragment indicate: homozygous noncut, two smaller fragments indicate: homozygotic cut and three fragments indicate: heterozygotic haplotype). As described in detail for both enzymes in figure 2.1

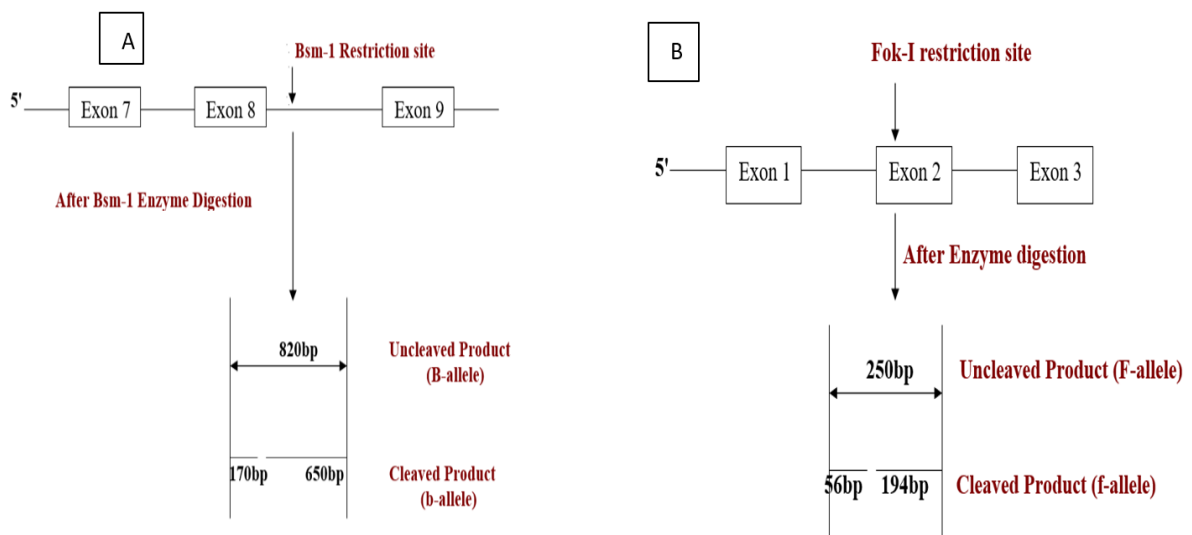


Figure 2.1: RFLP product for A. BSM-1 and B. FOK-1 after digestion of the PCR amplified DNA fragments.

## **2.6 Agarose gel preparation.**

2% (g/ml) agarose VDR polymorphism was used. 2g agarose (GIBCO, Invitrogen Corporation , Cat No. 15510-019) were dissolved in 100L 1x Tris Acetate EDTA (TAE) working buffer. The mixture was boiled until all agarose was dissolved , allowed to cool to ~60°C, followed by adding 10µl/ml of ethidium bromide to the mixture, followed by mixing until the mixture is completely homogeneous. The warm agarose was poured evenly into a tray containing 2 combs to produce wells when the gel is completely cooled. After approximately 15 min, the tray was placed into an electrophoresis tank (Bio-Rad, sub-cell®GT) filled with 1X TAE gel loading buffer, and 5 µl of the digested DNA products were loaded into the gel after PCR and enzyme incubation. For bands size determination, 5 µl of ready-made ladder marker was loaded and the gels were allowed to run at 120 volt for approximately 30-45minutes.

## **2.7 BMD evaluation.**

Patients and control subjects were referred to Alrazi hospital in Jenin and Aberih medical center (who possess DEXA screening facility) for BMD screening using DEXA at three locations: femoral neck, Total hip, and lumber spine L1-L4. The BMD T and Z scores are shown in table 2.4.

**Table 2.4: BMD, T and Z score range of total hip, femur neck and L1.L4 spine in normal (A) and osteoporosis (B)**

**A**

**Bone Density:** Exam date 01/09/2020

Region	BMD (g/cm <sup>2</sup> )	T-score	Z-score	Classification
AP Spine(L1-L4)	1.056	0.1	0.1	Normal
AP Spine(L2-L4)	1.079	0.0	0.1	Normal
Femoral Neck(Left)	0.811	-0.3	-0.3	Normal
Total Hip(Left)	0.870	-0.6	-0.6	Normal
Whole Body(Total)	1.074	-0.4	-0.4	

*World Health Organization criteria for BMD impression classify patients as Normal (T-score at or above -1.0), Osteopenia (T-score between -1.0 and -2.5), or Osteoporosis (T-score at or below -2.5).*

**B**

**Bone Density:** Exam date 02/04/2020

Region	BMD (g/cm <sup>2</sup> )	T-score	Z-score	Classification
AP Spine(L1-L4)	0.656	-3.6	-3.6	Osteoporosis
AP Spine(L2-L4)	0.611	-4.3	-4.2	Osteoporosis
Femoral Neck(Left)	0.577	-2.5	-2.4	Osteoporosis
Total Hip(Left)	0.591	-2.9	-2.9	Osteoporosis
Whole Body(Total)	0.868	-3.2	-3.2	

*World Health Organization criteria for BMD impression classify patients as Normal (T-score at or above -1.0), Osteopenia (T-score between -1.0 and -2.5), or Osteoporosis (T-score at or below -2.5).*

## 2.8 Statistical analysis

Statistical analysis was performed using SPSS software including descriptive analysis, correlations between variables, distribution frequency of all genotypes, ROC test, and association between variables, multi-variate analysis. A p-value > 0.05 was accepted as a significant result.

## Chapter 3

### Results

#### 3.1 Description of the study subjects

Table 3.1 shows the demographic description of the study subjects (diabetic and control) including mean and standard deviation of quantitative variables including number, weight, height, age, BMI and related clinical data. The table shows 86 diabetic patients and 88 control subjects who participated in the study. Both groups have similar age range, BMI, height and weight with no significant difference. Slight variation in the sex ratio between two groups is indicated. The table clearly demonstrates significant difference between both groups in relation to blood glucose level, HbA1c, C-peptide and autoantibody as expected. Figure 3.1 shows representative BMD data measured by DEXA scan for normal spine (3.1 A), femur neck and total hip (3.1 B), osteoporotic spine (3.1 C) and femur neck and total hip (3.1D). The evaluation of the indicated BMD values are based on international standards as shown in table 1.2 (see methods section). Table 3.2 shows means and standard deviation of BMD expressed as Z-score values between control and diabetic subjects. The data indicate significant difference between both groups in the three indicated sites where diabetic patients have lower Z-score values (-0.14 total hip, -0.17 femur neck, -0.32 L1/L4 spine) compared to nondiabetic subjects (0.28 total hip, 0.27 femur neck, 0.30 L1/L4 spine) indicating osteoporotic status in the three sites for diabetic patients.

**Table 3.1: Characteristics of the study subjects quantitative variables**

	Case status	N	Mean	Std. Deviation	p-value
age	Control	88	22.60	2.8	0.160
	Patient	86	23.4	4.0	
Glucose level (mg/dl)	Control	88	95.67	+4.08	<0.001
	Patient	86	450.44	+50.3	
C-peptide (ng/ml)	Control	88	2.85	+0.7	<0.001
	Patient	86	0.1	+0.32	
Hemoglobin A1c (%)	Control	88	5.71	+0.64	<0.001
	Patient	86	10.5	+0.55	
Autoantibody	Control	88	Negative		
	Patients	86	Positive		
Body Mass Index	Control	88	23.8	4.9	0.728
	Patient	86	24.1	4.2	
Height	Control	88	168.1	10.1	0.024
	Patient	86	164.6	10.0	
Weight	Control	88	68.6	19.4	0.148
	Patient	86	65.0	12.8	
			Control	Patient	
Sex	male		33	45	0.049
	female		55	41	

**Table 3.2: Z- score difference between control and diabetic subjects.**

	Case status	N	Mean	Std. Deviation	p-value
Total Hip Z1	Control	88	0.28	0.93	0.005
	Patient	86	-0.14	1.07	
Femur neck Z1	Control	88	0.27	1.01	0.005
	Patient	86	-0.17	1.06	
L1-L4 Z1	Control	88	0.30	1.04	<0.001
	Patient	86	-0.32	1.18	

**A**

**ASL**  
AL-Birah street  
AL-Birah

Telephone: 022413223

E-Mail: s.endocrine@gmail.com

Name: \_\_\_\_\_  
Patient ID: \_\_\_\_\_  
DOB: 07 November 1994

Sex: Female  
Ethnicity: White

Height: 155.0 cm  
Weight: 75.0 kg  
Age: 25

Referring Physician:

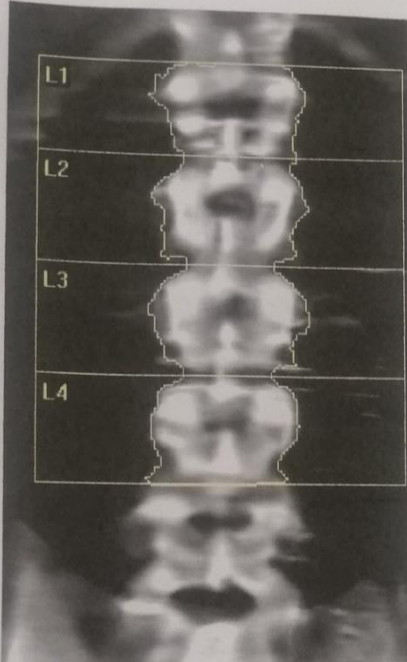


Image not for diagnostic use  
116 x 131

**Scan Information:**

Scan Date: 09 January 2020 ID: A01092009

Scan Type: f Lumbar Spine

Analysis: 09 January 2020 13:22 Version 13.6.0.5  
Spine

Operator:

Comment:

**DXA Results Summary:**

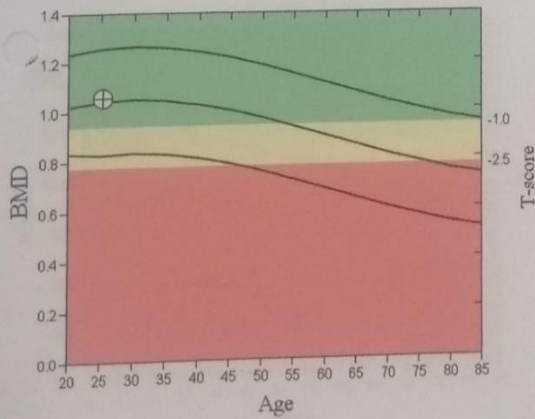
Region	Area (cm <sup>2</sup> )	BMC (g)	BMD (g/cm <sup>2</sup> )	T-score	Z-score
L1	12.27	11.94	0.973	-0.2	-0.1
L2	14.31	15.10	1.055	0.2	0.3
L3	14.43	15.70	1.088	0.0	0.1
L4	14.30	15.65	1.094	0.3	0.4
<b>L1-L4</b>	<b>55.31</b>	<b>58.38</b>	<b>1.056</b>	<b>0.1</b>	<b>0.1</b>
L2-L4	43.04	46.44	1.079	0.0	0.1

Total BMD CV 1.0%

WHO Classification: Normal

Fracture Risk: Not Increased

**L1-L4**



**Comment:**

T-score vs. White Female. Source:2012 BMDCS/Hologic Z-score vs. White Female.  
Source:2012 BMDCS/Hologic

**B**

**ASL**  
AL-Birah street  
AL-Birah

Telephone: 022413223

E-Mail: s.endocrine@gmail.com

Name:  
Patient ID:  
DOB: 07 November 1994

Sex: Female  
Ethnicity: White

Height: 155.0 cm  
Weight: 75.0 kg  
Age: 25

Referring Physician:

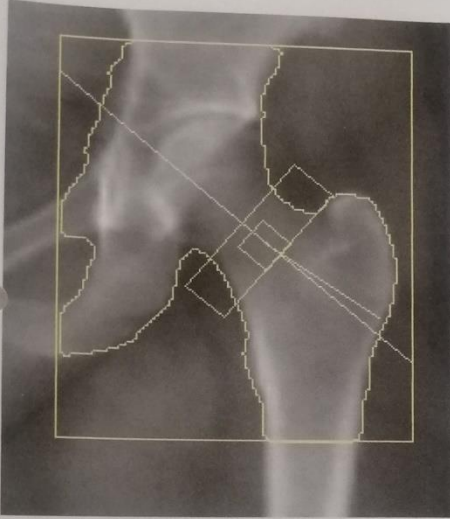


Image not for diagnostic use  
101 x 119  
NECK: 49 x 15

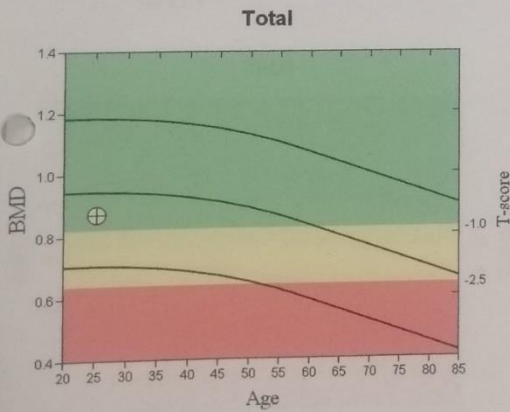
**Scan Information:**

Scan Date: 09 January 2020 ID: A0109200A  
Scan Type: f Left Hip  
Analysis: 09 January 2020 13:24 Version 13.6.0.5  
Hip  
Operator:  
Comment:

**DXA Results Summary:**

Region	Area (cm <sup>2</sup> )	BMC (g)	BMD (g/cm <sup>2</sup> )	T - score	Z - score
Neck	4.43	3.59	0.811	-0.3	-0.3
<b>Total</b>	<b>28.15</b>	<b>24.50</b>	<b>0.870</b>	<b>-0.6</b>	<b>-0.6</b>

Total BMD CV 1.0%  
WHO Classification: Normal



**10-year Fracture Risk**

**FRAX not reported because:**

All T-scores for Spine Total, Hip Total, Femoral Neck at or above -1.0

**Comment:**

T-score vs. White Female. Source:2012 BMDCS/NHANES White Female. Z-score vs. White Female. Source:2012 BMDCS/NHANES White Female.

**C**

**ASL**  
AL-Birah street  
AL-Birah

Telephone: 022413223

E-Mail: s.endocrine@gmail.com

Name:	Sex: Female	Height: 159.0 cm
Patient ID:	Ethnicity: White	Weight: 66.0 kg
DOB: 05 May 1990		Age: 29

Referring Physician:

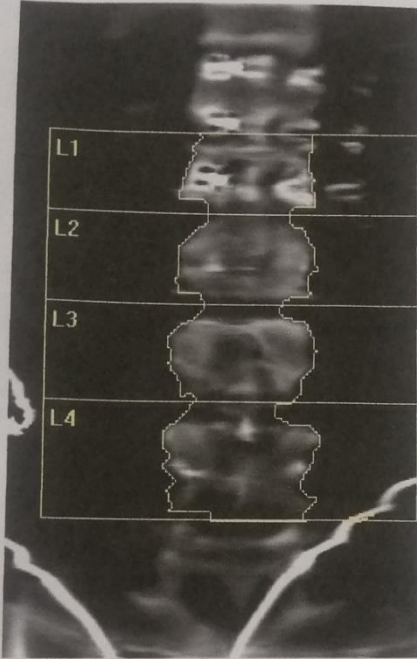


Image not for diagnostic use  
116 x 120

**Scan Information:**

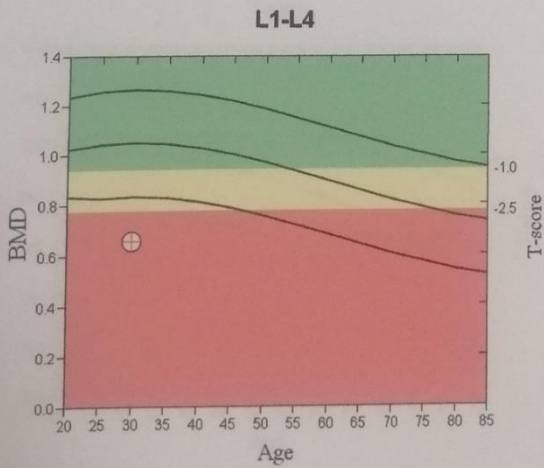
Scan Date: 04 February 2020 ID: A02042008  
Scan Type: f Lumbar Spine  
Analysis: 04 February 2020 14:48 Version 13.6.0.5  
Spine (auto low density)

Operator:  
Comment:

**DXA Results Summary:**

Region	Area (cm <sup>2</sup> )	BMC (g)	BMD (g/cm <sup>2</sup> )	T-score	Z-score
L1	9.54	7.96	0.834	-1.4	-1.4
L2	10.67	7.40	0.694	-3.0	-3.0
L3	11.93	7.63	0.640	-4.0	-4.0
L4	15.47	8.23	0.532	-4.8	-4.8
L1-L4	47.60	31.23	0.656	-3.6	-3.6
L2-L4	38.06	23.27	0.611	-4.3	-4.2

Total BMD CV 1.0%  
WHO Classification: Osteoporosis  
Fracture Risk: High



**Comment:**

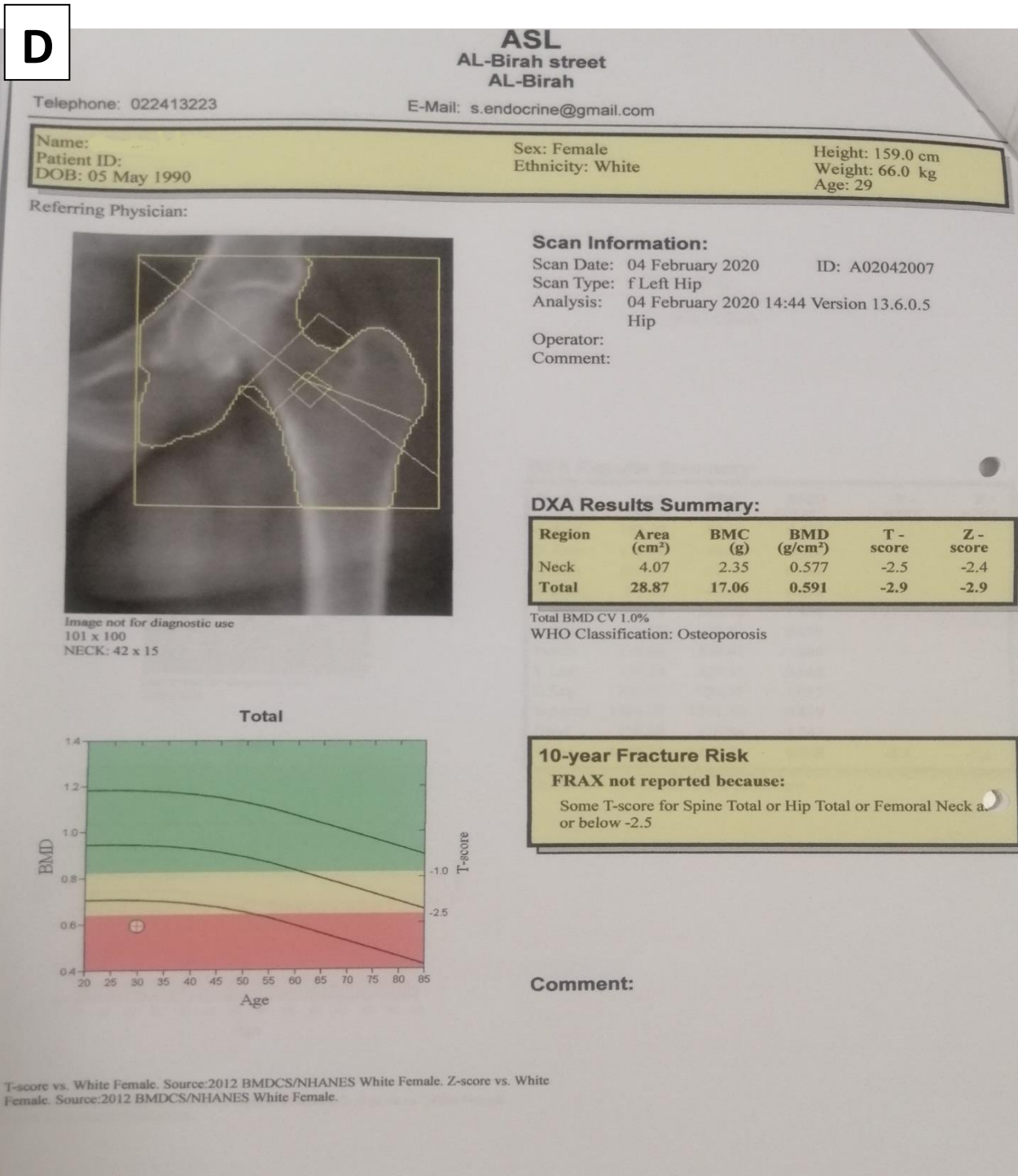


Figure 3.1: Representative DEXA scan results showing BMD, T and Z score in L1.L4 spine, total hip and femur neck in normal (A,B) and osteoporotic (C,D ) samples.

### **3.2 Detection of rs10735810 (exon 2) and rs1544410 (intron 8) in the VDR gene among study subjects.**

The various genotypes in the two indicated SNP's in exon 2 (rs10735810) and intron 8 (rs1544410) were detected by restriction fragment length polymorphism (RFLP). Figure 3.2 shows a representative PCR amplified 820 bp DNA fragment from intron 8 with the rs1544410 (upper panel) and PCR amplified 250 bp DNA fragment from exon 2 with the rs10735810 (lower panel). No other DNA bands were detected which indicate the specificity of the reaction. The enzyme digestion of the two PCR amplified fragments using BSM1 and FOK1 endonucleases is shown in figures 3.3 and 3.4 respectively. Three different DNA fragments were detected from both digestions (820 bp, 650 bp and 170 bp) using BSM1 enzyme and (250 bp, 194 bp and 56 bp) using FOK1 enzyme. The detection of one band after BSM1 or FOK1 digestion at 820 bp and 250 bp represents homozygous GG and CC respectively. The detection of three bands following BSM1 digestion (820 bp, 650 bp and 170 bp) or FOK1 digestion (250bp, 194 bp and 56 bp) indicate heterozygous GA and CT genotype respectively, while the detection of only two bands after BSM1 digestion (650bp and 170bp) and FOK1 digestion (194bp and 56 bp) indicate homozygous AA and TT genotypes respectively.

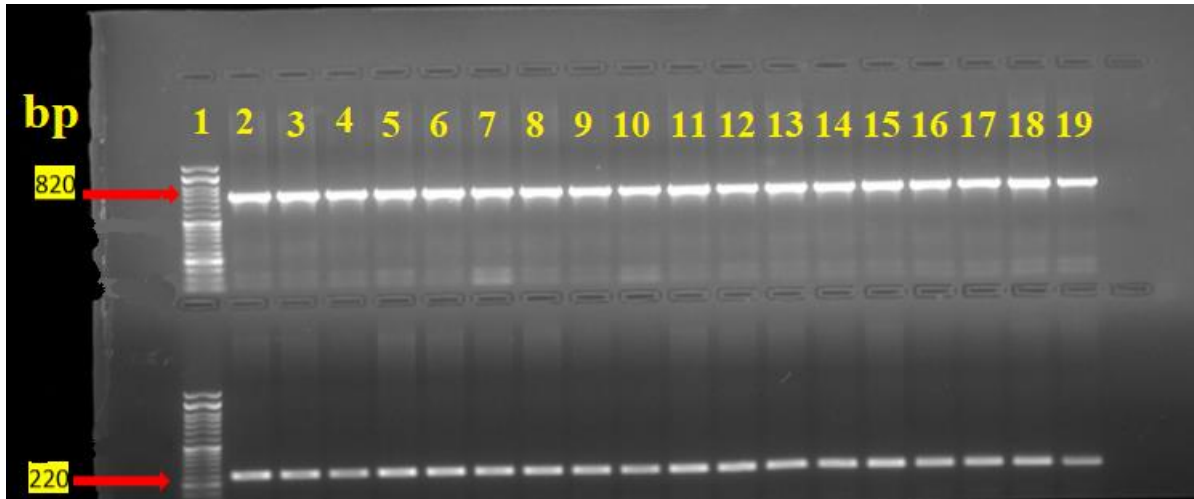


Figure 3.2: Representative gel electrophoresis showing the 820 bp PCR amplified DNA fragments of the VDR gene including rs1544410 in intron 8 (upper lane 2-19) and the 220 bp DNA fragment rs10735810 in exon 2 (lower lane 2-19). Lane 1 shows 50bp ladder in the upper and lower parts of the gel.

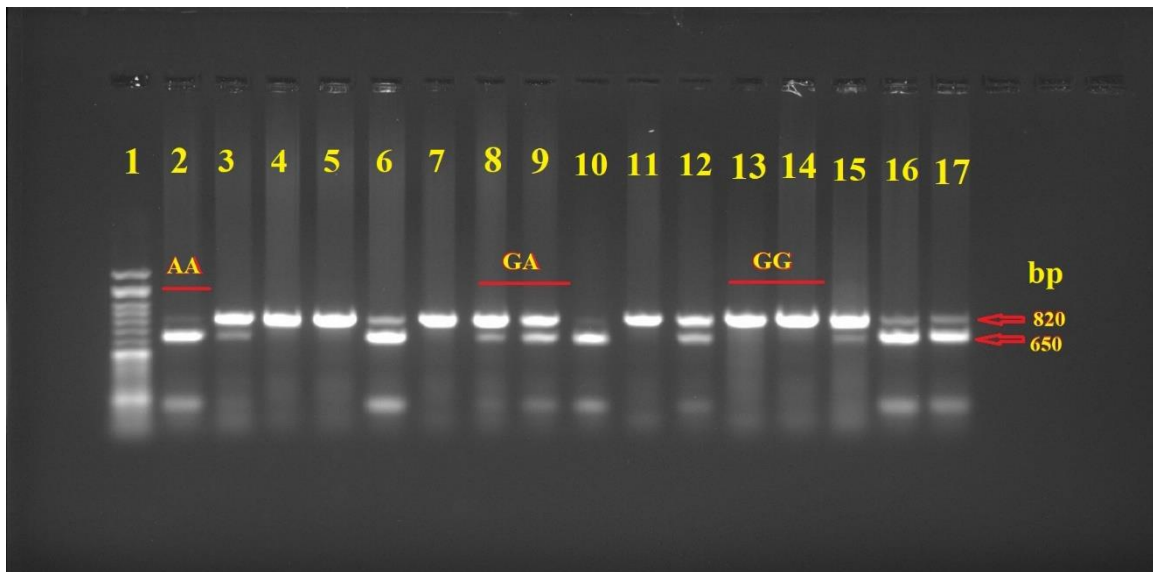


Figure 3.3: Representative gel electrophoresis showing results of BSM1 enzyme digestion of the 820 bp PCR product. A single 820 bp band represents homozygous GG (lanes 4,5,7,11,13,14). The presence of three fragments at 820 bp, 650 bp, 170 bp represents heterozygous GA genotype (lanes 3,6,8,9,12,15). The detection of two fragments at 650 bp and 170 bp indicate homozygous AA genotype (lanes 2,10,16,17). 50bp ladder at lane 1

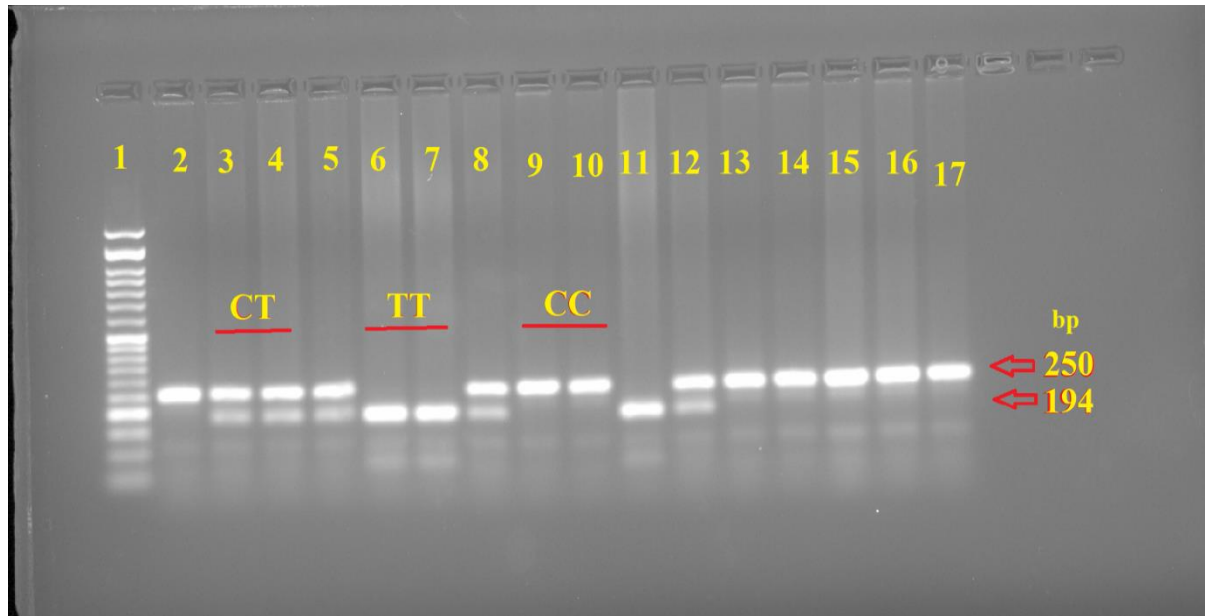


Figure 3.4: Representative gel electrophoresis showing results of FOK1 enzyme digestion of the 250 bp PCR product. A single 250 bp band represents homozygous CC (lanes 2,9,10,13-17). The presence of three fragments at 250 bp, 194 bp, 56 bp represents heterozygous CT genotype (lanes 3,4,5,8,12). The detection of two fragments at 194 bp and 54 bp indicate homozygous TT genotype (lanes 6,7,11). Lane 1 shows 50 bp ladder.

**Genotype and haplotype distribution of rs10735810 (exon 2) and rs1544410 (intron 8) in the VDR gene among diabetic patients and controls.**

Table 3.3 shows the genotypes and haplotypes distribution of the VDR gene rs10735810 (exon 2) and rs1544410 (intron 8) among diabetic patients and controls. The data shows no significance difference in rs10735810 genotypes (CC, TT and CT) and rs1544410 genotypes (GG, AA and GA ) between both study groups. Similarly, no significant difference could be detected in rs10735810 haplotypes (CC+CT vs TT+CT ) and rs1544410 haplotypes (GG+GA vs AA+GA) distribution among the two groups.

**Table 3.3: Comparison of genotype and allele frequency distribution of rs10735810 (exon 2) and rs1544410 (intron 8) among control and diabetic subjects.**

			Case status		Total	p-value
			Patient	Control		
rs10735810 exon 2	CT	N	32	41	73	0.57
		% within Case status	37.2%	46.6%	42.0%	
	CC	N	16	6	22	
		% within Case status	18.6%	6.8%	12.6%	
	TT	N	38	41	79	
		% within Case status	44.2%	46.6%	45.4%	
CC/CT vs CT/TT	CC, CT	N	64	53	117	0.22
		% within Case status	37.2%	30.1%	33.6%	
	CT, TT	N	108	123	231	
		% within Case status	62.7%	69.8%	66.3%	
rs1544410 intron 8	GA	N	37	49	86	0.24
		% within Case status	43.0%	55.7%	49.4%	
	GG	N	22	17	39	
		% within Case status	25.6%	19.3%	22.4%	
	AA	N	27	22	49	
		% within Case status	31.4%	25.0%	28.2%	
GA/GG vs GA/AA	GA, GG	N	81	83	164	0.64
		% within Case status	47.0%	47.1%	47.1%	
	GA, AA	N	91	93	184	
		% within Case status	52.9%	52.8%	52.8%	

### 3.3 Association of BMD with the study variables

#### 3.3.1 Quantitative variables

Pearson correlation coefficient ( $r$ ) was calculated to evaluate the correlation between several variables in the study subjects including age and BMD (total hip, femur neck, L1/L4 spine), body mass index, height and weight. Pearson correlation coefficient ( $r$ ) is a linear correlation coefficient that returns a value between 0 and 0.19 indicating very low correlation, 0.2 and 0.39 indicates low correlation, 0.4 and 0.59 indicates moderate correlation, 0.6 and 0.79 indicates high correlation and a value between 0.8 to 1.0 indicates very high correlation (Nurfatihah Zamani, 2020). The results in table 3.4 shows no significant association between total hip BMD with age and height while a significant difference is observed and low correlation with body mass index ( $r=0.307$ ,  $p<0.001$ ) and weight ( $r=0.226$ ,  $p<0.001$ ). Moreover, no significant association was evident between femur neck BMD with age. However, significant association was detected with a low correlation with body mass index ( $r=0.363$ ,  $p<0.001$ ), with low correlation with height ( $r=0.239$ ,  $p<0.001$ ) and with moderate correlation with weight ( $r=0.410$ ,  $p<0.001$ ). L1/L4 spine site BMD showed no significant difference and a very low correlation with the four indicated variables.

**Table 3.4: Correlations of quantitative variables with BMD at the tree sites**

		Age	Body mass index	Height	Weight
Total hip	Pearson Correlation	-0.061	0.307**	0.065	0.226
	P-value	0.428	<0.001	0.391	<0.001
Femur neck	Pearson Correlation	-0.071	0.363**	0.239**	0.410**
	P-value	0.353	<0.001	<0.001	<0.001
L1/L4 spine	Pearson Correlation	0.019	0.042	-0.081	-0.006
	P-value	0.807	0.579	0.289	0.934

### 3.3.2 Qualitative variables

Tables 3.5, 3.6 and 3.7 show the association between BMD in the three indicated sites with qualitative variables including rs10735810 (exon 2), rs1544410 (intron 8) genotypes and gender. No association was shown between BMD in the three indicated sites with rs10735810 genotypes as shown in table 3.5 while the association between rs1544410 genotypes and BMD showed significant difference with only femur neck BMD (  $P=0.034$ ) showing that GG genotype have lowest BMD score as shown in table 3.6. The Association between femur neck and L1/L4 spine BMD with gender was significant ( $P=0.035$  and  $P=0.005$  respectively) showing that female have lower BMD score in femur neck and male have lower BMD score in L1/L4 spine compared to female, however, no association was evident between gender and total hip BMD as shown in table 3.7.

**Table 3.5: Association of rs10735810 (exon 2) with BMD at all sites in all subjects**

Exon 2		N	Mean	Std. Deviation	
Total Hip Z1 score	CT	73	-0.01	0.99	0.174
	CC	22	-0.17	1.18	
	TT	79	0.22	0.99	
	Total	174	0.07	1.02	
Femur neck Z1 score	CT	73	0.06	1.03	0.270
	CC	22	-0.27	1.24	
	TT	79	0.13	1.02	
	Total	174	0.05	1.05	
L1.L4 Z1 Score	CT	73	0.04	1.19	0.092
	CC	22	-0.50	1.21	
	TT	79	0.08	1.07	
	Total	174	-0.008	1.15	

**Table 3.6: Association of rs1544410 (intron 8) with BMD at all sites in all subjects**

	Intron 8	N	Mean	Std. Deviation	p-value
Total Hip Z1 score	GA	86	.2523	.99773	0.064
	GG	39	-.1641	.96341	
	AA	49	-.0551	1.08302	
	Total	174	.0724	1.02553	
Femur neck Z1 score	GA	86	.2547	1.06679	0.034
	GG	39	-.0487	1.00887	
	AA	49	-.2184	1.03091	
	Total	174	.0534	1.05877	
L1-L4 Z1 Score	GA	86	.1326	1.07292	0.258
	GG	39	-.2051	1.18209	
	AA	49	-.1000	1.25946	
	Total	174	-.0086	1.15483	

**Table 3.7: Association of gender with BMD at all sites in all subject**

	Gender	N	Mean	Std. Deviation	P-value
Total hip z score	Male	78	0.17	1.16	0.221
	Female	96	-0.01	0.89	
	Total	174	0.07	1.02	
Femur neck z score	Male	78	0.30	1.19	0.005
	Female	96	-0.14	0.89	
	Total	174	0.05	1.05	
L1-L4 Z1 Score	Male	78	-0.21	1.13	0.035
	Female	96	0.15	1.15	
	Total	174	-0.008	1.15	

### 3.4. Association of osteoporosis with the study variables

To study the effects of different variables on osteoporosis status, control and diabetic subjects were divided into osteoporotic and normal subjects based on their BMD Z-score results. The association of the study variables including diabetes as an independent variable with osteoporosis was analyzed.

#### 3.4.1 Association with quantitative variables

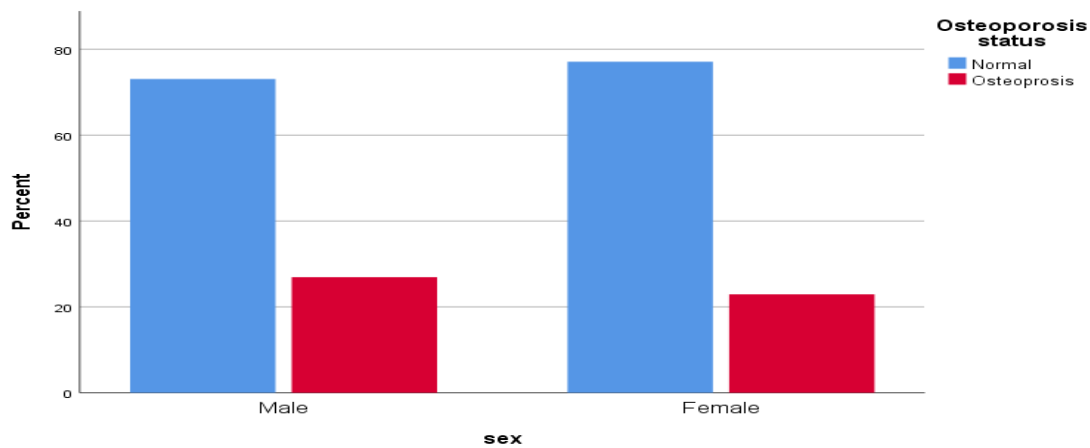
Univariate analysis comparing means and standard deviation of quantitative variables including age, BMI, height, weight between osteoporotic and normal subjects is shown in table 3.8. The data shows no significant difference was detected between osteoporosis and age, height and BMI, however, weight was significantly lower among osteoporotic subjects compared to controls  $(p=0.034)$

**Table 3.8: Association of the quantitative variables with osteoporosis among all subjects in the study**

Variable	Status	N	Mean	Std. Deviation	p-value
age	Normal	131	22.9	3.3	0.418
	Osteoporosis	43	23.4	3.9	
BMI	Normal	131	24.4	4.4	0.054
	Osteoporosis	43	22.8	4.7	
Height	Normal	131	166.5	10.1	0.788
	Osteoporosis	43	166.0	10.7	
Weight	Normal	131	68.2	17.0	0.034
	Osteoporosis	43	62.5	14.4	

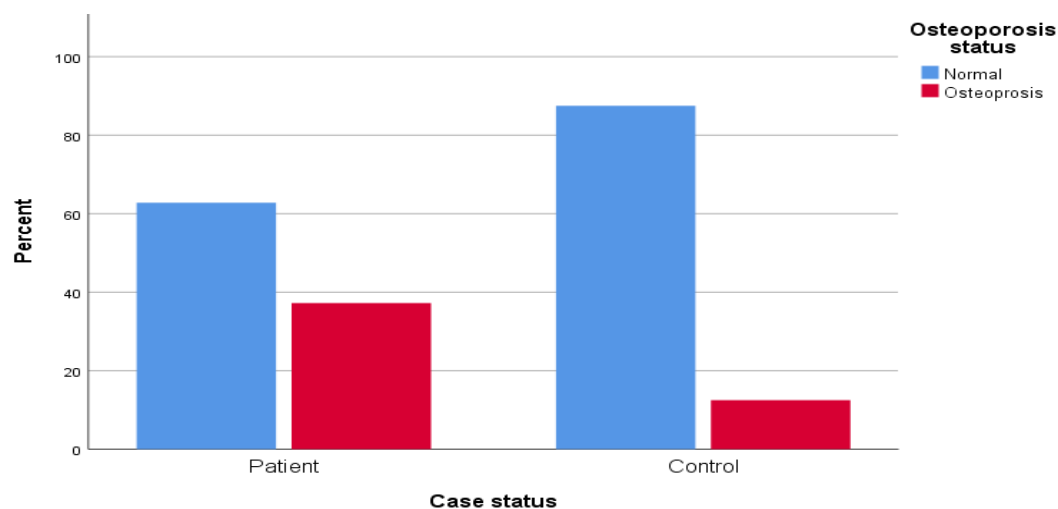
### 3.4.2 Association of osteoporosis with the qualitative variables

The association of osteoporosis with sex and diabetic status among the study subjects is shown in figures 3.5 and 3.6 respectively. The data shows no significant difference with sex distribution as shown in figure 3.5 while a significant association in osteoporosis was detected with diabetic status compared to normal non-diabetic control subjects (38% vs 12%  $p=0.001$ ) as shown in figure 3.6 these results clearly indicate that osteoporosis is a complication of diabetes this could be a result of genetic factors including VDR gene haplotypes, environmental or both .



Chi-square  $P=0.54$

Figure 3.5: Osteoporosis by gender.



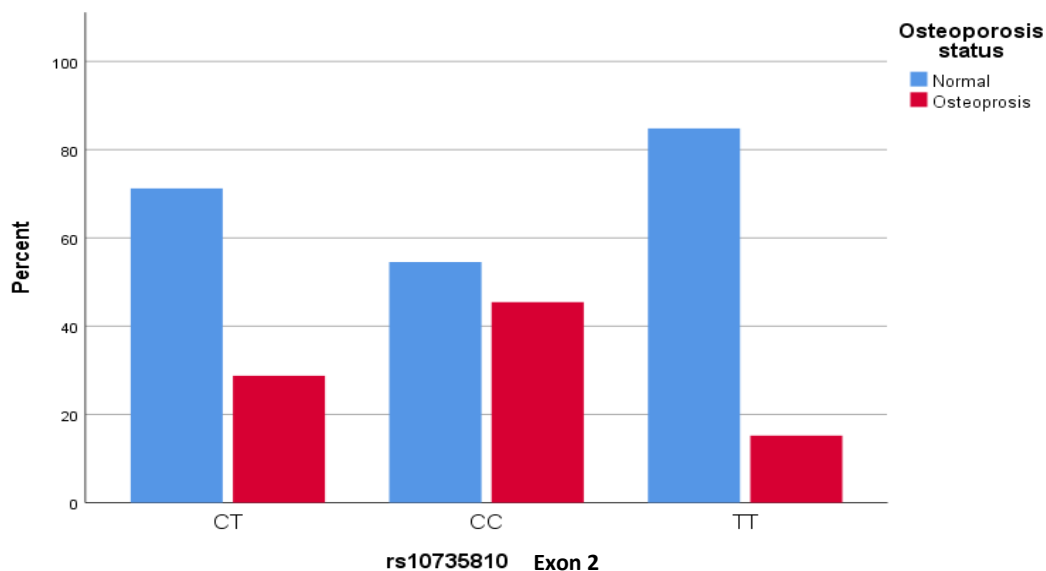
Chi-square  $P<0.0001$

Figure 3.6: Osteoporosis status association with diabetic status among study subjects

### 3.5 Association of the indicted VDR exon 2 and intron 8 SNPs genotypes with osteoporosis

#### 3.5.1 Association of rs10735810 (exon 2) with osteoporosis:

Figure 3.7 shows the relative frequency of rs10735810 (exon 2) genotype for osteoporotic and normal subjects irrespective of site. A significant difference is detected between the variants genotypes and osteoporosis ( $p=0.008$ ). The result shows that the genotype CC was associated with higher percent of osteoporotic subjects compared to CT and TT. The distribution of homozygous CC genotype was higher in normal 54.5% compared to osteoporotic 45.5%. However, the heterozygous CT genotype was significantly lower in osteoporosis 28.8% compared to normal 71.2%, while, the homozygous TT genotype was significantly lower in osteoporosis 15.2% compared to normal 84.8%. This data clearly indicate the C haplotype is significantly associated with osteoporosis while the T haplotype is associated with normal bone status.



$P=0.008$

Figure 3.7: Association of osteoporosis with rs10735810 (exon 2) genotypes among the study subjects.

## Association between osteoporosis in the indicated three sites and exon 2 rs10735810

The association between osteoporosis in the indicated 3 sites and rs10735810 genotypes (total hip, femur neck, L1/L4 spine) was analyzed. A significant association between rs10735810 genotypes and osteoporosis in total hip was detected ( $p=0.049$ ) as shown in table 3.9 and in femur neck ( $p=0.045$ ) as shown in table 3.10 whereas table 3.11 shows no significant association is evident between rs10735810 genotypes and osteoporosis in L1/L4 spine, homozygous C genotype was shown to have more osteoporosis than CT and TT genotypes in the three indicated sites. The association of osteoporosis with rs10735810 haplotypes in the three indicated sites showed significant difference between total T (CT+TT) compared to total C (CT+CC) and osteoporosis in all sites, total hip ( $p=0.01$ ), femur neck ( $p=0.01$ ) and L1/L4 spine ( $p=0.04$ ) (data not shown). The C haplotype seems correlated with osteoporosis in all 3 sites, since the number of osteoporotic subjects with homozygous C is significantly higher than heterozygous CT and homozygous TT.

**Table 3.9: Association of osteoporosis with exon 2 in total hip**

			Total Hip		Total	p-value
			Normal	Osteoporosis		
rs10735810 exon 2	CT	N	55	18	73	0.049
		% within rs10735810 exon 2	75.3%	24.7%	100.0%	
	CC	N	14	8	22	
		% within rs10735810 exon 2	63.6%	36.4%	100.0%	
	TT	N	68	11	79	
		% within rs10735810 exon 2	86.1%	13.9%	100.0%	
Total	N	137	37	174		
	% within rs10735810 exon 4	78.7%	21.3%	100.0%		

**Table 3.10: Association of osteoporosis with exon 2 in femur neck**

			Femur Neck		Total	p-value
			Normal	Osteoporosis		
rs10735810 exon 2	CT	N	462	11	73	0.045
		% within rs10735810 exon 2	84.9%	15.1%	100.0%	
	CC	N	16	6	22	
		% within rs10735810 exon 2	72.7%	27.3%	100.0%	
	TT	N	73	6	79	
		% within rs10735810 exon 2	92.4%	7.6%	100.0%	
Total		N	151	23	174	
		% within rs10735810 exon 2	86.8%	13.2%	100.0%	

**Table 3.11: Association of osteoporosis with exon 2 in L1/L4 Spine**

			L1/L4 Spine		Total	p-value
			Normal	Osteoporosis		
rs10735810 exon 2	CT	N	58	15	73	0.135
		% within rs10735810 exon 2	79.5%	20.5%	100.0%	
	CC	N	16	6	22	
		% within rs10735810 exon 2	72.7%	27.3%	100.0%	
	TT	N	70	9	79	
		% within rs10735810 exon 2	88.6%	11.4%	100.0%	
Total		N	144	30	174	
		% within rs10735810 exon 2	82.8%	17.2%	100.0%	

Table 3.12 show the association between rs10735810 (exon 2) genotypes and osteoporosis at all sites in diabetic patients compared with control subjects. No significant association between osteoporosis with diabetic or control subjects was observed, however, a significant association was shown between osteoporosis and normal subjects ( $P=0.008$ ) regardless to their diabetic status. When osteoporosis was

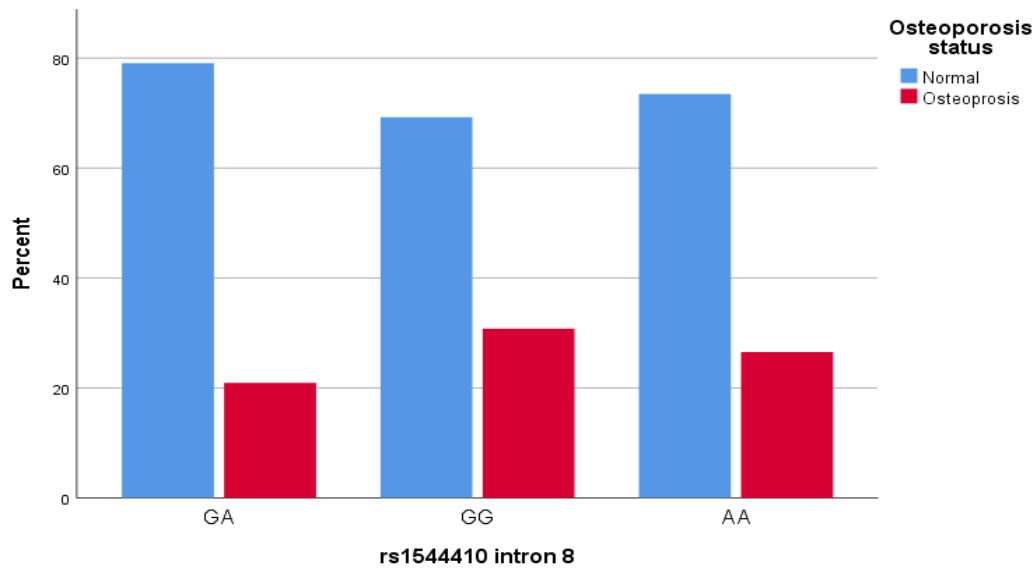
compared with exon 2 based on haplotype in total subjects, CT+TT, CT+CC show significant difference  $p=0.001$  with osteoporosis in all subjects (data not shown).

**Table 3.12: Association of rs10735810 (exon 2) with osteoporosis at all sites in diabetic patients compared with control subjects.**

			Normal	Osteoporotic		p-value
Diabetic	CT	N	19	13	32	0.102
	CC	N	7	9	16	
	TT	N	28	10	38	
	Total	N	54	32	86	
Control	CT	N	33	8	41	0.128
	CC	N	5	1	6	
	TT	N	39	2	41	
	Total	N	77	11	88	
Total	CT	N	52	21	73	0.008
	CC	N	12	10	22	
	TT	N	67	12	79	
	Total	N	131	43	174	

### 3.5.2 Association of rs1544410 (intron 8) with osteoporosis

Figure 3.8 shows the relative frequency of rs1544410 (intron 8) genotype between osteoporotic and normal subjects irrespective of site. Contrary to the site in exon 2, a non-significant differences is evident between the various genotypes frequency (20.9% for GA, 30.8% for GG and 26.5% for AA) at this DNA site and osteoporosis ( $P=0.468$ ).



p-value 0.468

Figure 3.8: Distribution of osteoporosis with rs1544410 (intron 8) genotypes among the study subjects.

### **The association between rs1544410 (intron 8) with osteoporosis by site**

Table 3.13, 3.14 and 3.15 shows the association between rs1544410 (intron 8) genotypes with osteoporosis at the 3 indicated sites (total hip, femur neck, L1.L4 spine) in all subjects. As shown in table 3.13, 3.14 and 3.15, there is no significant association between rs1544410 (intron 8) genotypes and osteoporosis in the three indicated sites. The association of osteoporosis in the three indicated sites was also tested with rs1544410 haplotypes. No significant difference was observed between total G (GA+GG) compared to total A (GA+AA) and osteoporosis in femur neck (p=0.39), total hip (p=0.76) and L1/L4 spine (p=0.51) (data not shown).

**Table 3.13: Association of osteoporosis with intron 8 in femur neck**

			Femur Neck		Total	p-value
			Normal	Osteoporosis		
rs1544410 intron 8	GA	N	79	7	86	0.116
		% within rs1544410 intron 8	91.9%	8.1%	100.0%	
GG	N	N	33	6	39	
		% within rs1544410 intron 8	84.6%	15.4%	100.0%	
AA	N	N	39	10	49	
		% within rs1544410 intron 8	79.6%	20.4%	100.0%	
Total	N	N	151	23	174	
		% within rs1544410 intron 8	86.8%	13.2%	100.0%	

**Table 3.14: Association of osteoporosis with intron 8 in total hip**

			Total Hip		Total	p-value
			Normal	Osteoporosis		
rs1544410 intron 8	GA	N	74	12	86	0.059
		% within rs1544410 intron 8	86.0%	14.0%	100.0%	
GG	N	N	27	12	39	
		% within rs1544410 intron 8	69.2%	30.8%	100.0%	
AA	N	N	36	13	49	
		% within rs1544410 intron 8	73.5%	26.5%	100.0%	
Total	N	N	137	37	174	
		% within rs1544410 intron 8	78.7%	21.3%	100.0%	

**Table 3.15: Association of osteoporosis with intron 8 in L1/L4 Spine**

			L1/L4 Spine		Total	p-value
			Normal	Osteoporosis		
rs1544410 intron 8	GA	N	74	12	86	0.450
		% within rs1544410 intron 8	86.0%	14.0%	100.0%	
GG	N	N	32	7	39	
		% within rs1544410 intron 8	82.1%	17.9%	100.0%	
AA	N	N	38	11	49	
		% within rs1544410 intron 8	77.6%	22.4%	100.0%	
Total	N	N	144	30	174	
		% within rs1544410 intron 8	82.8%	17.2%	100.0%	

Table 3.16 shows the association between rs1544410 (intron8) genotypes with BMD at all sites in diabetic patients compared to control subjects. The results show no association between rs1544410 (intron 8) genotype with BMD among both groups.

**Table 3.16: Association of rs1544410 (intron 8) with BMD at all sites in diabetic patients compared with control subjects.**

			Normal	Osteoporotic	Total	p-value
Diabetic	GA	N	25	12	37	0.728
	GG	N	13	9	22	
	AA	N	16	11	27	
	Total	N	54	32	86	
Control	GA	N	43	6	49	0.723
	GG	N	14	3	17	
	AA	N	20	2	22	
	Total	N	77	11	88	
Total	GA	N	68	18	86	0.468
	GG	N	27	12	39	
	AA	N	36	13	49	
	Total	N	131	43	174	

### 3.6 Scatter plot

Scatter plots was performed to investigate the relationship between BMD and BMI at the three sites ( Total hip, L1.L4 spine, femur neck ). Positive correlation (r) indicates that higher BMI is correlated with higher BMD as shown before (Kharroubi A 2017). The result showed BMI was positively correlated with BMD in all subjects at the three sites with R=0.094 for total hip, R=0.132 for femur neck and R=0.002 for L1-L4 spine (see appendix).

### 3.7 Association of the study variables with BMD and osteoporosis

#### (multivariate analysis)

Multivariate analysis was done to evaluate the relative effect and predictive ability of each variable in the study on BMD in the three sites and osteoporosis after controlling the effect of the other variables. As table 3.17 shows sex, age, body mass index and diabetes can be predictors and significantly associated with low total hip BMD score (B=-0.094 p=0.001, B=-0.006 p=0.050, B=0.015 p<0.001, B=0.042 p=0.046) respectively. Table 3.18 shows that body mass index and diabetes are significantly associated predictors for low femur neck score (B=0.083 p<0.001, B=0.452 p=0.004) respectively, while diabetes status is significantly associated and a good predictor for low L1/L4 spine score (B=0.636 p=0.001) as shown in table 3.19. Table 3.20 shows that body mass index, rs10735810 exon 2 genotypes and diabetes status are significantly associated with osteoporosis status and they can be a good predictors for it (B=-0.114 p=0.042, B=-0.463 p=0.030, B=-1.66 p<0.001) respectively.

**Table 3.17: Multivariate analysis of the study variables with total hip BMD**

Total hip	B	t	P-value
Sex	-0.094	-3.316	0.001
Age	-0.006	-1.977	0.050
Body Mass Index	0.015	6.915	<0.001
Height	0.000	-0.219	0.827
rs10735810 exon 2	0.013	1.269	0.206
rs1544410 intron 8	-0.005	-0.440	0.660
Diabetes	0.042	2.007	0.046

**Table 3.18: Multivariate analysis of the study variables with femur neck BMD**

Femur neck	B	t	P-value
Sex	-0.337	-1.600	0.111
Age	-0.039	-1.805	0.073
Body Mass Index	0.083	5.102	<0.001
Height	0.004	0.346	0.730
rs10735810 exon 2	0.023	0.302	0.763
rs1544410 intron 8	-0.116	-1.348	0.179
Diabetes	0.452	2.885	0.004

**Table 3.19: Multivariate analysis of the study variables with L1/L4 spine BMD**

L1/L4 spine	B	t	P-value
Sex	0.151	0.602	0.548
Age	0.009	0.340	0.734
Body Mass Index	0.012	0.601	0.549
Height	-0.012	-0.943	0.347
rs10735810 exon 2	0.030	0.322	0.748
rs1544410 intron 8	-0.118	-1.163	0.246
Diabetes	0.636	3.415	0.001

**Table 3.20: Multivariate analysis of the study variables with osteoporosis at all sites**

Osteoporosis	B	P-value	Odds ratio	95% C.I	
				Lower	Upper
Sex	0.228	0.688	1.256	0.412	3.829
Age	0.076	0.166	1.079	0.969	1.202
Body Mass Index	-0.114	0.024	0.892	0.808	0.985
Height	0.020	0.467	1.021	0.966	1.078
rs10735810 exon 2	-0.463	0.030	0.629	0.414	0.957
rs1544410 intron 8	0.086	0.702	1.090	0.702	1.691
Diabetes	-1.665	<0.001	0.189	0.077	0.467

## Chapter 4

### Discussion

TDM1 diabetes Miletus is a metabolic disorder characterized by lack of insulin secretion or resistance, which in turn leads to increase accumulation of glucose concentration (Voet D 2011). Diabetes is one of the most prevalent disorders worldwide, and its prevalence keeps rising up every year. It was the ninth leading cause of death in 2019 ([www.who.int](http://www.who.int)) and the mechanism (s) in the disease development is far from understood. Therefore, continued investigation in the field is highly needed in order to understand its molecular and pathophysiological mechanisms to control the disease expansion among the various populations. Diabetes is known to cause several medical complications including osteoporosis since bone remodeling depends on energy which depends on glucose metabolism (McKENNA mj 2015). Decreased levels of insulin in type 1 diabetes in turn will lead to an imbalance between bone formation and resorption. This imbalance will lower bone mineral density and decrease bone quality causing microfractures accumulation (Krakauer JC 2006), lack of insulin directly activates many bone formation markers including alkaline phosphatase, collagen synthesis and osteocalcin (formed as a result of RUNX-2 pathway activation and have an essential role in the interaction between bone and glucose metabolism(Fulzele K 2011). Several studies showed reduction in osteoprotegrin (which a soluble receptor for RANKL 1) in diabetes mice models which inhibits osteoclast differentiation leading to an increase in bone resorption and increase risk of fractures (Silva J. A 2012).

Several molecules suspected to be involved the complications of type 1 diabetes received special attention including vitamin D which have a significant effect on the pathogenesis of type 1 diabetes through vitamin D receptor (VDR) gene (Ban Y 2001)

(Penna-Martinez M 2017). Furthermore, some studies suggested deficient vitamin D is connected to the autoimmune destruction of  $\beta$ -cells and the onset of type 1 diabetes resulted by loss of immune regulation (Z Rheumatol 2000) ( Mathieu C 2004). Since vitamin D exerts its effect via a nuclear vitamin D receptor (VDR).( Lemos MC 2008) Therefore, the VDR gene can be considered as a candidate gene for type 1 diabetes pathogenesis.

Several previous studies revealed the importance of several genes that are linked to osteoporosis, including vitamin D receptor gene (VDR) which are suspected to maintain bone health linked to osteoporosis, more specifically in rs1544410 (intron 8) and rs10735810 (exon 2) and rs1544410 (intron 8) (Haussler et al 1998). rs1544410 (intron 8) SNP is suspected to cause reduced protein expression, whereas rs10735810 (exon 2) leads to 3 amino acids shorter protein with decrease in its activity (Arai et al 1997, Morrison 1992).

VDR is a transcription factor which is involved in the regulation of several genes including osteocalcin, osteopontin and RANKL (Bikle 2004). Therefore, variation in the VDR gene is projected to affect bone quality through affecting the expression of genes responsible for bone structure and differentiation (Bikle 2004).

The association between selected VDR gene polymorphisms and BMD score was investigated in several previous studies specially with postmenopausal women. Some studies confirmed positive association (Mohammed. G. A. Ansari 2021), while other studies suggested there is no association between the VDR polymorphisms and BMD score (Uitterlinden et al 2002, Horst-Sikorska W 2013). These studies clearly indicate the association between VDR polymorphisms and BMD differ from one population to another (Hustmyer et al, 1994). This study aimed to investigate the association between the indicated VDR gene variants (rs10735810 (exon 2) and rs1544410 (intron 8) ) with

BDM in Palestinian type 1 diabetes mellites patients. The association between BMI with BMD at the three indicated sites (femur neck, total hip and L1/L4 spine) revealed positive correlation as expected indicating higher BMI is linked to higher the BMD, since Body mass index (which is an indicator of obesity) is a common factor that affects BMD (Kharroubi A 2017, Areej Zraiqi MSc thesis AAUP 2020). The positive correlation between BMD in the three locations: total hip, femur neck and lumbar spine L1-L4 is in agreement with the pathophysiology of bone health where higher BMI value represents a protective indicator of bone (table 3.4), which is in agreement with other studies (Wynn A 1995, Kharroubi A 2017).

Our data showed significant difference in BMD between controls and diabetic subjects at the three locations (table 3.2) and in total osteoporosis (figure 3.6) indicating diabetic patients have lower BMD compared to control. These results confirmed the association of type 1 diabetes with osteoporosis. Same investigation was observed by (Zhu,Q 2021). However no difference between osteoporosis status could be detected based on gender in the study subjects.

Our data showed no association between rs10735810 (exon 2) and rs1544410 (intron 8) haplotypes and genotypes with diabetes in the Palestinian population (table 3.3) Similar results were reported among the Turkish population ( Yavuz D 2011) Portuguese (Lemos M 2008), finnish (Turpeinen H 2003) , on the contrary, rs1544410 (intron 8) was found to be associated with diabetes type 1 among Sudanese population (KE Khalid 2016), japan (Motohashi Y 2003) and Saudi children (Ali R 2018).

Our data shows no association between BMD and gender or age at the three indicated sites. However, differential link was evident between BMD at the three sites and other quantitative variables. Femur neck showed positive association with BMI, height and

weight while total hip was associated with BMI and weight, in contrast, L1/L4 spine showed no association with any of the tested quantitative or qualitative variables (tables 3.4, 3.7). BMD at the three indicated sites was not associated with rs10735810 (exon 2) haplotypes. Association between femur neck and rs1544410 (intron 8) was shown. However, no association was detected between VDR haplotype with total hip and L1/L4 spine score. Similar to Indian postmenopausal women study (Ahmad I 2018) which concluded that rs10735810 (exon 2) was associated with BMD at the three sites and the rs1544410 (intron 8) is associated with total hip and L1/L4 spine with no association with femur neck (tables 3.5, 3.6).

Our data showed significant association of rs10735810 (exon 2) C allele as a risk of osteoporosis and T as protective allele with no association between the various alleles in rs1544410 (intron 8) and osteoporosis (figures 3.7, 3.8). The association of osteoporosis with rs10735810 (exon 2) genotypes was similar to the results observed in Chinese elderly osteoporotic men study (Zhang HH 2006). In addition, rs10735810 (exon 2) is associated with osteoporosis in total hip and femur neck, with no association with osteoporosis in L1/L4 spine (tables 3.9, 3.10, 3.11). The rs10735810 (exon 2) total C haplotypes (CT+CC) compared to total T (CT+TT) showed strong association with osteoporosis at the three indicated sites. The number of osteoporotic patients increased with homozygous TT genotype which was higher than TC heterozygous while the least number of osteoporotic patients was observed with the CC homozygous genotype compared to the other genotypes. The Iranian osteoporotic postmenopausal study reported the homozygous CC genotype was higher among all subjects in their study and the majority of subjects with CC genotype were healthy while osteoporotic subjects remain at basal status in genotype distribution (Hossein-zhad A 2009). Which indicate that T allele and the 3 amino acids shorter VDR

protein may also affect the osteoporotic status and that the C allele might be more advantageous for bone health. No association between rs1544410 (intron 8) genotypes and haplotypes ( total G (GA+GG) and total A(GA+AA)) with total osteoporosis status or with osteoporosis at the three indicated sites was detected (tables 3.13, 3.14, 3.15). The same results was observed in Turkish osteoporotic postmenopausal women study which showed that intron 8 rs1544410 variant had no association with osteoporosis (Uysal AR 2008). ). The distribution of rs1544410 alleles in the current study remain at basal status between healthy and osteoporotic patients. These findings emphasizes the important effect of rs10735810 (exon 2) on bone health more than rs1544410 (intron 8). Moreover, in the Chinese population meta-analysis study (An, M 2017) reported that rs1544410 was not associated with osteoporosis but have a protective effect against osteoporosis.

Our data showed both rs10735810 (exon 2) and rs1544410 (intron 8) in the VDR gene represent the causative factor of low BMD and osteoporosis but they are not linked to osteoporosis in diabetes type 1 patients (tables 3.12, 3.16), similar to the finding in the Turkish population (Dilek Gogas 2011). The cause of osteoporosis in diabetic type 1 patients might be due to other genetic factors than the VDR gene in addition to environmental factors including vitamin D status and other nutritional factors.

**In conclusion**, our data confirmed a strong association between type 1 diabetes, BMD, and total osteoporosis. The data evidently showed that (rs10735810 (exon 2) but not rs1544410 (intron 8) was associated with lower BMD and osteoporosis status at the indicated sites. The data also showed no association between rs10735810 (exon 2) and rs1544410 (intron 8) haplotypes and genotypes with diabetes. However, it showed significant association of rs10735810 (exon 2) C allele as a risk of osteoporosis and the T as protective allele with negative between the various alleles in rs1544410

(intron 8) and osteoporosis. Furthermore, the data showed rs10735810 (exon 2) but not rs1544410 (intron 8) alleles represent causative factor of low BMD and osteoporosis but is not linked to osteoporosis in diabetes type 1 patients, The cause of osteoporosis in diabetic type 1 patients might be due to other genetic factors and/or to environmental factors including vitamin D status and other nutritional factors.

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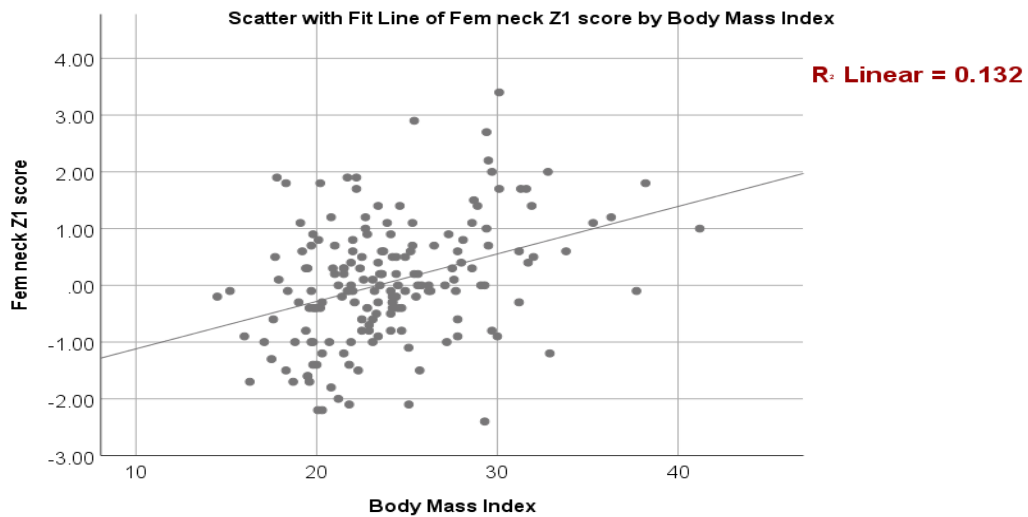
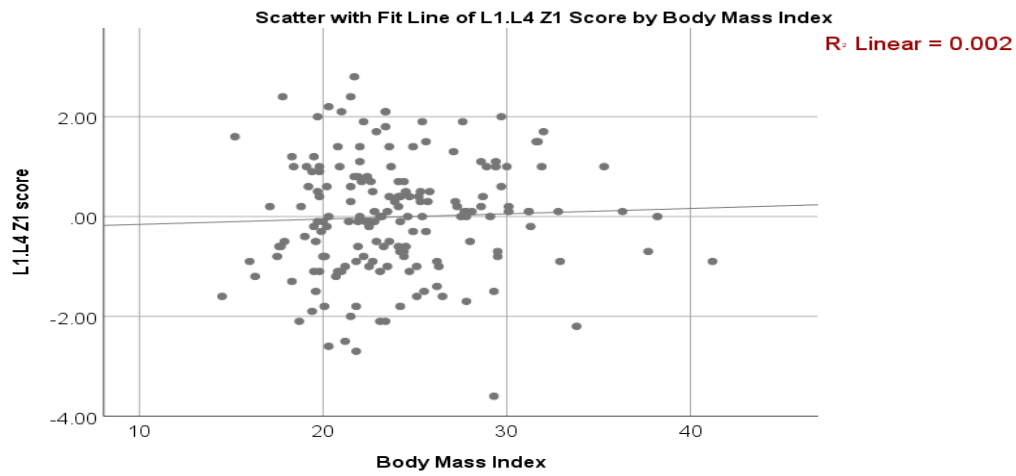
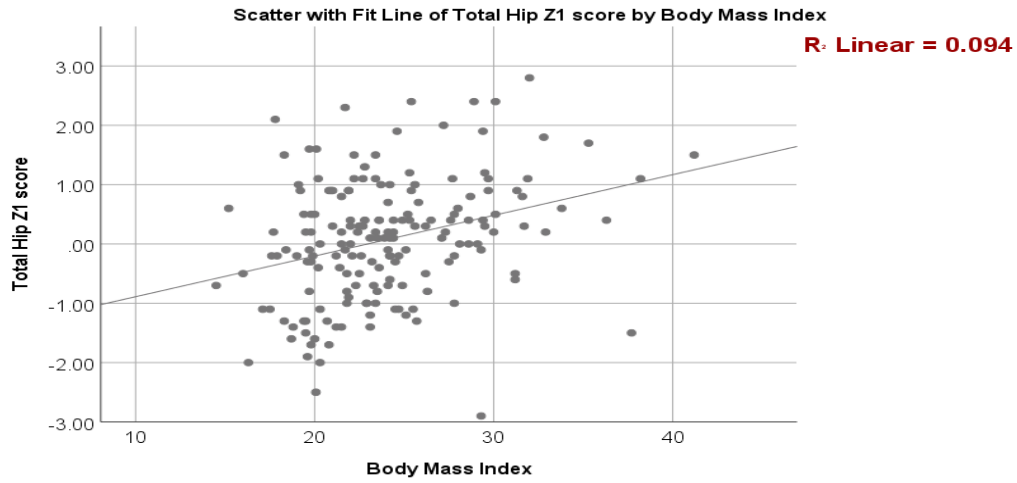
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## Appendix

### Scatter plot:



## الملخص

**الهدف:** الهدف الرئيسي للبحث هو دراسة العلاقة بين طرز جينية مختارة في جين مستقبل الفيتامين د وهشاشة العظام عند مرضى السكري الفلسطينيين نوع الأول.

**المواد والأدوات:** انضم لهذا البحث 88 مشتركين اصحاء و 86 مرضى سكري نوع 1، تتراوح

اعمار كلا المجموعتين من 18-30 سنة، تم قياس كثافة العظام لكلا المجموعتين بواسطة

تصوير امتصاص الاشعة ثنائية الطاقة في ثلاثة مواقع ( الحوض، عنق الفخذ، الفقرة الأولى

والرابعة من فقرات العمود الفقري القطنية)، تم جمع الدم من كلا المجموعتين واستخراج

الحمض النووي منه، ومن ثم باستخدام تفاعل البلمرة المتسلسل و تقييد طول القطعة متعددة

الاشكال بالمختبر تم التعرف على الاشكال الموروثة للطرازات المختارة في جين مستقبل

الفيتامين للإحصاء. SPSS د، تم تحليل النتائج بواسطة برنامج

**النتائج:** أظهرت النتائج ان هناك علاقة بين هشاشة العظام وبين الطرز الجيني 10735810

في المخرج 2 في الجين مقارنة بالمشاركين الاصحاء، بينما الطرز 1544410 في المدخل 8

لا يوجد لها علاقة بهشاشة العظام مقارنة بالمشاركين الاصحاء، بالنسبة للطرز الجيني

10735810 تختفي العلاقة بينه وبين هشاشة العظام عندما يتم تقسيم المشاركين الى

مرضسكري ومشاركين اصحاء، اما عن الطرز 1544410 فوجد علاقة بينه وبين هشاشة

العظام في عنق الفخذ بشكل خاص عندما يتم جمع المشاركين كمجموعة واحدة، لكن لا يوجد

علاقة بينه وبين معدل هشاشة العظام العام لا عند مرضى السكري ولا عند المشاركين بشكل

عام .

**الملخص:** لا يوجد علاقة بين الطرازات الجينية المختارة في جين مستقبل الفيتامين د وبين

هشاشة العظام عند مرضى السكري الفلسطينيين في أماكن القياس الثلاث في الجسم، هناك حاجة

لتوسيع البحث وإمكانية دراسة طرز أخرى في جين مستقبل الفيتامين د لها علاقة بهشاشة العظام

وقياس مستوى الهرمون الناتج من فيتامين د وهرمونات أخرى لها علاقة بصحة العظام.