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Genetic Marker Polymorphisms in The VDR & MTHFR Genes Among Osteoporotic and Normal Palestinian Women in Bethlehem District

By
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Al-Quds University
2005

Dedicated to ……. my mother

my husband: Samer Samman

my cute children:

Ahmad & Widad

my lovely sisters: Razan, Linda, & Amar

I certify that this thesis submitted for the degree of master is the result of my own research, except where otherwise acknowledged, and that this thesis (or any part of the same) has not been submitted for a higher degree to any other university or institution.

…….

Riham Walid Saleem Smoom

May,2005

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Abstract

Osteoporosis is a complex skeletal disease, characterized by low bone mineral density (BMD) and microarchitectural deterioration of bone tissue, with a consequent increase in bone fragility, leading to higher susceptibility to fractures. Many recent studies indicated that BMD is – at least in part – genetically determined. However, the most common candidate genes appearing to be closely linked with variation in BMD and high risk to develop osteoporosis, are vitamin D receptor (VDR) and methylene tetrahydrofolate reductase (MTHFR) genes. The hypothesis that commonly occurring polymorphisms in the VDR and MTHFR genes affect BMD has raised great interest. Several studies have confirmed this relationship– although some are contradictory –, while others showed negative findings.

In our present study, we have analyzed the *Bsm-I* and *Fok-I* polymorphisms in the VDR gene using RFLP-PCR, and the (C677T) *MTHFR* polymorphism using ARMS-PCR, in association with BMD in 344 (165 osteoporotic, 93 osteopenic, and 86 normal) Palestinian women in Bethlehem district. Secondly, we also investigated whether this association is affected by gene-gene interaction, or gene-environmental interaction including years since menopause, height, and weight.

The results of these analysis indicate clearly the presence of significant associations between the b-allele of the Bsm-I VDR gene and high BMD, as well as significant associations between the CT genotype of the MTHFR gene and decreased BMD compared to the TT genotype. On the contrary, the Fok-I VDR RFLP alone was a weak predictor of BMD. Interestingly, the gene-gene interactions, and gene-environmental interactions results indicate significant association between them and predicting the BMD level.

ملخص

العلامات الجينية ذات التغير الطبيعي في سلسلة الحامض النووي الرايبوزي منزوع الأكسجين (DNA) في الجينين (MTHFR &VDR) بين النساء الفلسطينيات المصابات بهشاشة العظام والسليمات في محافظة بيت لحم

هشاشة العظام هو مرض يصاب به الهيكل العظمي للإنسان، من خصائصه: نقص في كثافة العظم وكتلته وبالتالي زيادة الهشاشة والليونة في العظم وزيادة احتمال كسرها نتيجة أي حادث بسيط.

هناك عدة عوامل مسببة في حدوث هذا المرض: حيث أشارت العديد من الدراسات الحديثة إلى وجود عوامل جينية وراثية تساهم في زيادة احتمال حدوث المرض. ومن أهم الجينات التي ثبت أن لها علاقة بهشاشة العظام هي (MTHFR & VDR). حيث وجد أن هناك تغير طبيعي في سلسلة الحامض النووي منزوع الأكسجين (DNA) في كل من هذين الجينين، يؤدي إلي إيجاد صبغات وراثية متضادة الصفات (alleles). وقد أثبتت بعض الدراسات الحديثة أن وجود هذه الصبغات الوراثية له علاقة بكثافة العظم عند السيدات، بينما بعضها الآخر أنكر هذه العلاقة، أو أوجد علاقة عكسية.

ان الهدف من هذه الدراسة هو بحث توزيع هذه الصبغات الوراثية متضادة الصفات في النساء الفلسطينيات السليمات والمصابات بهشاشة العظام في محافظة بيت لحم، ثم معرفة إن كان هناك علاقة بين هذه الصفات (alleles) وكثافة العظم، بالإضافة إلى معرفة تأثير ارتباط هذه الصبغات المختلفة للجينين معا بكثافة العظم، وارتباطها بالعوامل البيئية الأخرى مثل الوزن، الطول، والعمر بعد انقطاع الدورة .

لقد تم تحليل ثلاثة من هذه التغيرات الطبيعية لسلسلة الحامض النووي : (Bsm-1 + Fok-1) للعامل الوراثي (VDR)، و(C-T)) للعامل الوراثي(MTHFR) باستخدام تقنية تفاعلات البلمرة المسلسلة: (RFLP-PCR)) للعامل الوراثيVDR و(ARMS-PCR)) للعامل الوراثيMTHFR عند ٣٤٤ سيدة (١٦٥مصابة + ١٧٩ سليمة) من مناطق محافظة بيت لحم.

وبعد معرفة كيفية توزيع هذه الصبغات على العينة المدروسة، تم استخدام برنامج SPSS في تحليل النتائج لمعرفة إذا كان هناك علاقة بين أي من هذه الصبغات وكثافة العظم - في مناطق العمود الفقري، والحوض، وأعلى الفخذ - ؛ ومدى تأثير العوامل البيئية الأخرى على هذه العلاقة.

نتيجة لهذه الدراسة، وجد أن هناك علاقة بين هذه الصبغات ومعدل كثافة العظم. وان هذه العلاقة ليست بسيطة، بل مرتبطة بعوامل أخرى مثل الطول، الوزن، والعمر بعد انقطاع الدورة. و تكمن أهمية هذه الدراسة في أن أي فتاة، إذا قامت بوقت مبكر بعمل فحص تحليلي لهذه الجينات لمعرفة إن كان لديها قابلية عالية لحدوث هشاشة عظام فيما بعد. وبالتالي تأخذ احتياطاتها للوقاية من هذا المرض قبل حدوثه، أملا في التخفيف من انتشار هذا المرض بين النساء الفلسطينيات، لما له من تأثيرات سلبية على الصحة العامة.

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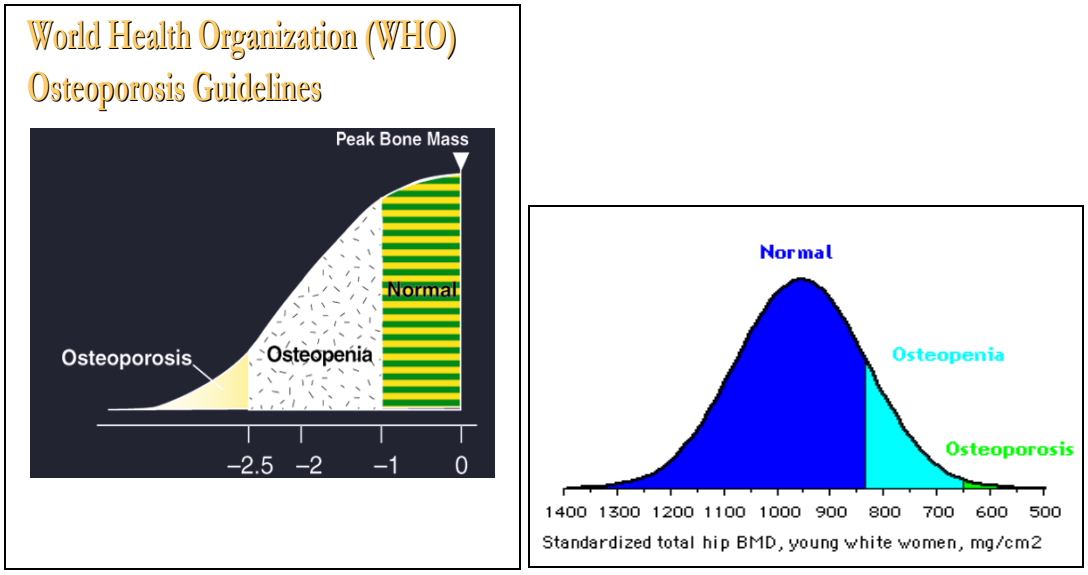
1.1. What is osteoporosis?

1.1.1. Definition:

Osteoporosis (or porous bones) is a complex systemic multifactorial skeletal disease, characterized by low bone mass and microarchitectural deterioration of bone tissue, with a consequent increase in bone fragility, leading to a higher susceptibility to fractures.(Levi etal.2002 , National Institutes of Health,2000, Maria,2001).

Osteoporosis was only officially defined as a disease in 1994 – while now it is acknowledged as a high priority health concern . In 1994 , the World Health Organization **(WHO)** produced a definition of osteoporosis based on low Bone Mineral Density (BMD), as shown in figure 1.1. , as a value of BMD 2.5 SD or more below the mean value for young adults (i.e. a T score of less than -2.5). Severe osteoporosis was also defined by a T score below -2.5 in addition to one or more fractures. Individuals with T score between -1.0 and -2.5 were defined as having osteopenia. Consequently, for example, using standardized bone density measurements of the total hip, normal bone has greater than 833mg/cm^2,osteopenia is between 833 and 648 mg/cm^2,osteoporosisis is lower than 648mg/cm^2, while severe osteoporosis is established in association with fragility fractures.

These definitions are very important because, in additional to their use in medical practice, they are related to thresholds for therapeutic intervention, since entry to drug trials is usually based on these values. (Health topics,2005 , Russell,2003 , All about bones,2001).



©2004 J. Wexler *(All About Bones ,2001)*

Figure 1.1:
WHO, Guidelines for Preclinical Evaluation and Clinical Trials in Osteoporosis

1.1.2. Pathogenesis:

The human body contains over 200 bones. All contain a mix of both cortical (the outer compact bone, representing 75% of the bone in the body, and is found in high percentage in the long shafts of bones in our forearms and legs (i.e. the femoral neck)) and trabecular bone (the inner cancellous spongy bone , representing the remaining 25%, found in high percentage in the vertebrae, pelvic

bones, and the ends of the long bones of the arms and legs) – see figure 1.2 . Therefore, the proportioned mix defines the strength of each bone (Brown,2000 , Nelson & Wernick,2000 , Health topics,2004 , McCarthy& Frassica,1998), while the growth of the bony skeleton determines the size and proportions of the body (Martini etal.2001).

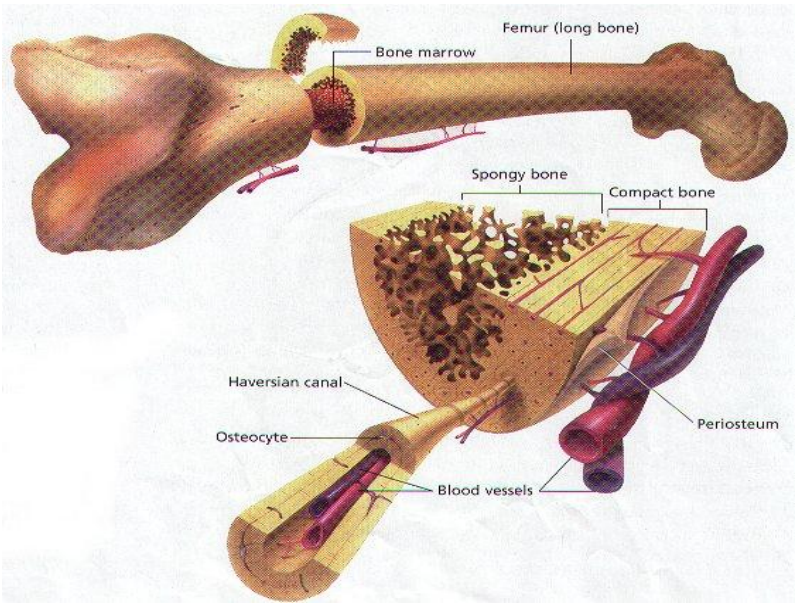


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Figure 1.2: Bone macroscopic structure

Like all living tissue, bones constantly repair and renew themselves in a process called **bone remodeling** . The purpose of remodeling is to allow the bone to adapt to changes in distribution of mechanical stress and to repair microdamage which can occur in response to repeated loading (Russel,2003).Bone cells that are responsible for demolition are the osteoclasts. These cells secrete an acid that dissolves old bone martix, resulting in calcium and other minerals' release from the

dissolved bone into the blood stream. Therefore, when the body needs calcium, it signals the osteoclasts to dissolve bone. Most of this material is recycled later in the remodeling process, but some is used to perform other functions in the body.

As shown in figure 1.3, the remodeling process begins when osteoclasts dissolve enough bone to create a tiny cavity, and die . In the second stage of remodeling, the osteoblasts (bone cells responsible for rebuilding bones) line the cavity with collagen (soft, sticky substance that forms the framework for bone), then they draw calcium and other minerals from the blood, forming crystals on the collagen. After that, the collagen and minerals harden into bone tissue. As the osteoblasts finish their work, they transform into mature bone cells (osteocytes) and become part of the new bone. These cells stay alive but are no longer active. At the end of the remodeling cycle, the cavity is refilled with new bone (Nelson &Wernick,2000):

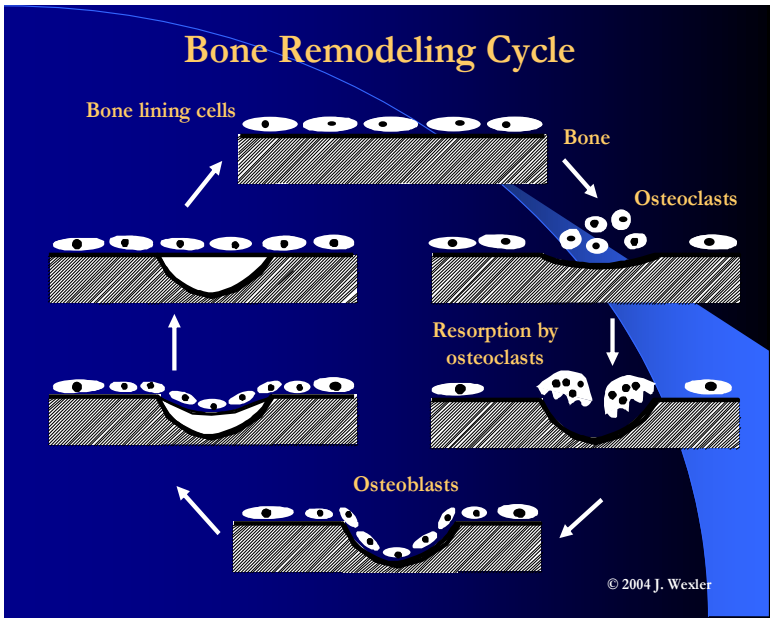


Figure 1.3:The remodeling process

In normal healthy adults, a complete remodeling cycle lasts approximately 4 months ,while, in individuals with osteoporosis ,a complete cycle may last 2 years (Lawson,2001).Only about 5 to 20% of the bone surface undergoes remodeling at any given time, the rest remain in a quiescent phase. In a healthy person, 5-10% of bone substance is replaced annually (Roche Facets,2005).

Under normal conditions , the amount of bone made corresponds very closely to the amount removed , so that, in any remodeling cycle , the total amount of bone tends to remain constant. In osteoporosis, and as a result of different factors, net bone loss results from an imbalance between the two components of the bone renewal process (i.e. bone resorption and bone formation). **This is the fundamental basis of osteoporosis.** More specifically, the numbers of bone remodeling sites increase, the extent of resorption may be greater, and the amount of bone replaced is smaller (Russel , 2003). Since the trabecular bones break down and rebuild faster than the cortical portion, they are more vulnerable to the effects of osteoporosis. Consequently, hip, spine, and wrist (having high percentage of trabecular bones) are the most common to break due to osteoporosis (Osteoporosis Dictionary-Miacalin,2005). The term "microarchitectural deterioration" refers to the thinning of the trabeculae and the loss of intertrabecular connections in bone, however, the incomplete replacement of osteons in cortical bone creates tunnels (see figure 1.4). These changes in bone mass and structure, reduce the overall

strength of the bone and make it prone to fracture (National Institutes of Health,2000 , Russel,2003).

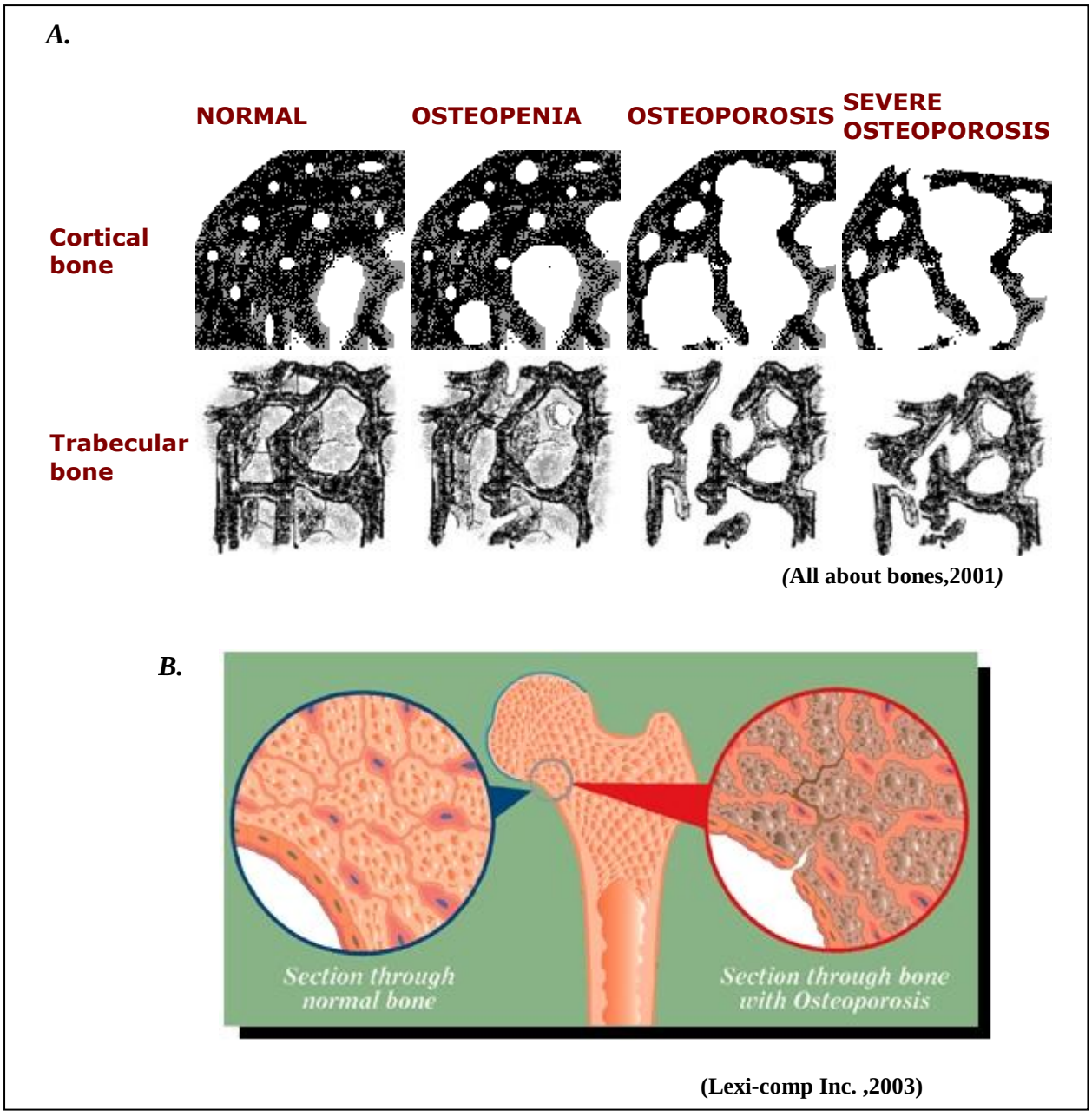


Figure 1.4: Effect of osteoporosis on bones

1.1.3. Classification:

There are two main types of osteoporosis:

1. Primary osteoporosis: occurring spontaneously, and has three subtypes:

a -Type I (postmenopausal) osteoporosis: caused by a lack of estrogen, the main female hormone, which helps to regulate the incorporation of calcium in women bone .

b-Type II (Senile) osteoporosis : probably results from an age-related calcium deficiency or a vitamin D deficiency. Senile means the condition occurs only in older people. It usually affects people older than 70 years and is twice as common in women as in men. Most older women with osteoporosis have a combination of postmenopausal and senile osteoporosis.

c- Type III (Idiopathic) osteoporosis : is a rare type of osteoporosis; the word idiopathic simply means that the cause is unknown. This type of osteoporosis occurs in children and young adults who have normal hormone levels, normal vitamin levels, and no obvious reason to have weak bones.

2. Secondary osteoporosis: Is caused by another disease (as chronic kidney failure and hormonal disorders (especially Cushing's disease, hyperthyroidism, hyperparathyroidism, , hypogonadism, and diabetes mellitus)) or by drugs (as corticosteroids, barbiturates, and anticonvulsants). (Beers et al, 2003, 2004).

1.1.4. Symptoms & diagnosis:

At first, osteoporosis produces no symptoms because bone density loss occurs very gradually. **Some people never develop symptoms.** Eventually, however, bone density may decrease enough for bones to collapse or fracture, producing severe sudden pain or gradually developing aching bone pain and deformities (Beers etal.2003,2004). The earliest symptom of osteoporosis is often an episode of acute back pain caused by a pathologic vertebral compression fracture, or an episode of groin or thigh pain caused by a pathologic hip fracture (Glaser & Kaplan, 1997).

A doctor may suspect osteoporosis in anyone, especially older woman, who breaks a bone with little or no force. Bone mineral density testing can be used to detect or confirm suspected osteoporosis, even before a fracture occurs. A number of rapid screening techniques are available to measure bone density at the wrist or the heel; however, the most useful test is the **D**ual-**E**nergy **X**-ray **A**bsorptiometry **(DEXA)** , system that uses two x-ray energies to measure bone density with a low patient exposure and a fast scan time. DEXA measures bone mineral density in two dimensions only. It is calculated by dividing the mineral content of the site of bone being assessed by the area of bone and is represented by the units of g/cm^2. Bone density is measured at the sites where major fractures are likely to occur: the spine and hip. This test is painless and can be performed in about 5 to 15 minutes.

It is useful for people at high risk of developing osteoporosis and for those in whom the diagnosis is uncertain. It is also useful for monitoring the response to treatment. (Beers etal.2003,2004, Cummings etal.2002).

1.1.5. Epidemiology:

Osteoporosis is a major public health problem, occurring in every population and geographic area studied (Klotzbuecher, 2000) . In the next 60 years, it is estimated that the number of osteoporotic fractures will quadruple worldwide (Dubey, 1998). Unless decisive steps are taken now to prevent the occurrence of osteoporosis, a serious global epidemic appears inevitable. (Riggs & Melton III, 1995a).

1.1.6. Risk Factors:

Many people are not aware of the risk factors and the consequences of osteoporosis (Klotzbuecher,2000). Given that there are no symptoms before fracture, it is important that physicians and patients recognize the following risk factors:-

a)Gender and Age:

Before puberty, there is no consistent gender-related differences in bone mass at any skeletal site (Glastre etal.1990, Bonjour etal.1991,Geusens etal.1991).This similarity in bone mass between males and females is maintained until the onset of pubertal maturation (Gilsanz etal.1991),where girls exceed boys by 10-15%, while boys increase during and after puberty(Aloia,1993).

The graph shown below , in figure 1.5, demonstrates the changes in bone mass throughout an individual's lifetime (Wasnich etal.1989). Peak bone mass is achieved in the mid-to-late twenties. At about 35 years old, an imbalance in bone turnover leads to the beginning of a gradual loss of bone (approximately 0.5 to 1 percent per year) in both men and women.With the onset of menopause in females, bone loss accelerates dramatically (especially trabecular bone) as a result of estrogen deficiency (Gambert etal.1995). In the menopausal and immediate postmenopausal years, approximately 2 to 4 percent of bone is lost per year (Meier,1990). The reason for this increased rate of loss is not well-elucidated, but it may be due to changes in homeostasis or hormonal modulation of bone turnover (Birge,1993). There is no corresponding acceleration in bone loss seen in men. However, testicular function does decline somewhat with age and may contribute to age-related bone loss in males. After age 55 to 60, bone loss again slows to premenopausal rates.

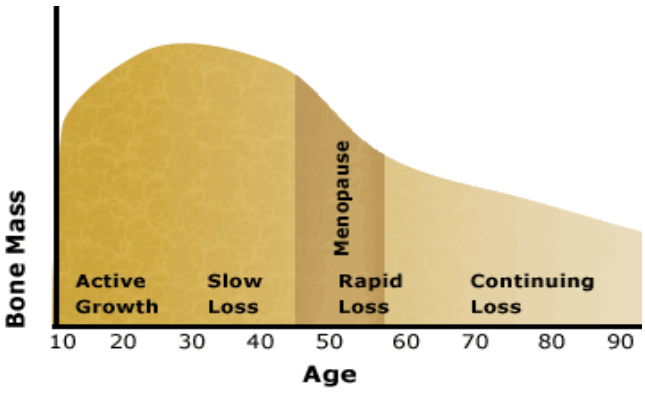


Figure 1.5: Bone Mass Changes with age

The reasons for age-related bone loss are complex and poorly understood and are influenced by gonadal hormone status, calcium intake and bioavailability, vitamin D status, physical activity, and hormones such as parathyroid hormone, corticosteroids, thyroid hormone, growth hormone, and calcitonin (Meier,1990).

b) Low body weight (or having small thin body frame): This may be due to the fact that thin women usually have lower body fat than do heavier women, so their estrogen levels are lower , because Fat tissue activates certain forms of estrogen (Beers etal.2033,2004).

c) Family history of osteoporosis: The genetics of osteoporosis is discussed in the next section.

d) A history of any prior fracture (Klotzbuecher,2000)

e) Prolonged use of medicines like steroids, antiacids,& anticonvulsants

f) Caucasian or Asian race

g) Early menopause

h) Inactive lifestyle, smoking, excessive alcohol intake, caffeine intake

i) Low calcium and vitamin D diet, high in salt and protein

j) Low testosterone levels in men

(Audran,2004 , Bunker,1994 , Balbona,2000, Frederic,1997)

1.2.Genetics of osteoporosis:

The genetics of osteoporosis represents one of the most active areas of research in bone biology (Liu etal,2003). Given the complex biology of the skeleton, it is likely that bone mass is under the control of a large number of genes, many of which exert relatively small effects on BMD and few of which contribute substantially to the variation in this trait. It is also likely that complex gene - environment interactions exist (Brandi etal.2001). Indeed, Interactions among genetic, hormonal and lifestyle factors are complex and in general poorly determined (Raya,2004).

1.2.1. What is the evidence for genetic contribution to osteoporosis?

It has been shown that daughters of osteoporotic women have low BMD (Seeman etal.1989). A study comparing peak bone mass at both the lumbar spine and femoral neck in young adult daughters from peri- and early postmenopausal women with decreased BMD and in daughters of women with normal BMD showed a decreased BMD among the former group (Barthe etal.1998).

In order to investigate the proportion of the BMD variance across the population explained by genetic factors, which is known as the heritability (Kelly etal. 1995), mainly two human models have been used: Parent-offspring (family) model, and twin model. In the twin model, correlations for BMD are compared between monozygotic (MZ) twins (sharing 100% of their genes) and dizygotic (DZ) twins, (having 50% of their genes in common). Stronger correlation coefficients among

adult MZ as compared with DZ twins are indicative of the genetic influence on peak bone mass (Rizzoli etal.2001). Indeed, studies in twins and families indicate that genetic factors play an important role in the regulation of BMD and other determinants of osteoporotic fracture risk. For example, the heritability of BMD has been estimated to lie between 50% and 85% in twin studies, with the strongest effect in the axial skeleton (Smith etal.1973, Pocock etal.1987, Christian etal.1989, Slemenda etal. 1991, Flicker etal.1995). Family-based studies also yielded strong heritability estimates for BMD (Gueguen etal.1995), with effects that are maximal in young adults and persist even after adjusting for lifestyle factors that are known to regulate BMD (Krall & Dawson-Hughes 1993).

To date, among the genetic strategies commonly employed for the dissection of complex traits, the analysis of the genetic determinants of BMD largely relied on association studies, in which a polymorphism in a candidate gene is analyzed in untreated affected and unaffected individuals from a given population (Brandi et al, 2001).

1.2.2.How is Osteoporosis Inherited?

Osteoporosis is a complex disease, thought to be mediated by an interaction between environmental factors and several different genes that individually have modest effects on BMD and other aspects of fracture risk (Gueguen etal. 1995). In rare instances, however, osteoporosis is inherited in a simple Mendelian manner. Examples of this include osteogenesis imperfecta (Rowe,1991), and osteoporosis

associated with inactivating mutations in the aromatase gene (Morishima etal.1995) and estrogen receptor α gene (Smith etal. 1994).

1.2.3. Why Define the Genes in Osteoporosis?

The proposed benefits of defining the genes causing osteoporosis are: (1) the ability to identify those who are at risk and (2) an understanding of the disease pathophysiology, which will facilitate the identification of novel therapeutic or preventative targets. Whether genetic tests are actually going to be of value in the prediction of those who are at risk of developing osteoporosis is quite uncertain. In theory, if all of the genes that cause the disease can be identified, and their interaction with each other and with non-genetic factors understood, then heritability figures from twin studies and family studies suggest that this information will be useful in predicting those who are at risk (Matthew,1999).

1.2.4. Candidate Genes for Osteoporosis:

The genetic study of osteoporosis has been based largely on research into candidate genes relevant to bone metabolism (Liu,,2003)(see table 1.1). However, the most common candidate genes appearing to be closely linked with variation in BMD and with high risk to develop osteoporosis, are the **v**itamin **D** **r**eceptor **(VDR)** and the **m**ethylene **t**etra**h**ydro**f**olate **r**eductase **(MTHFR)** genes discussed in the following two sections.

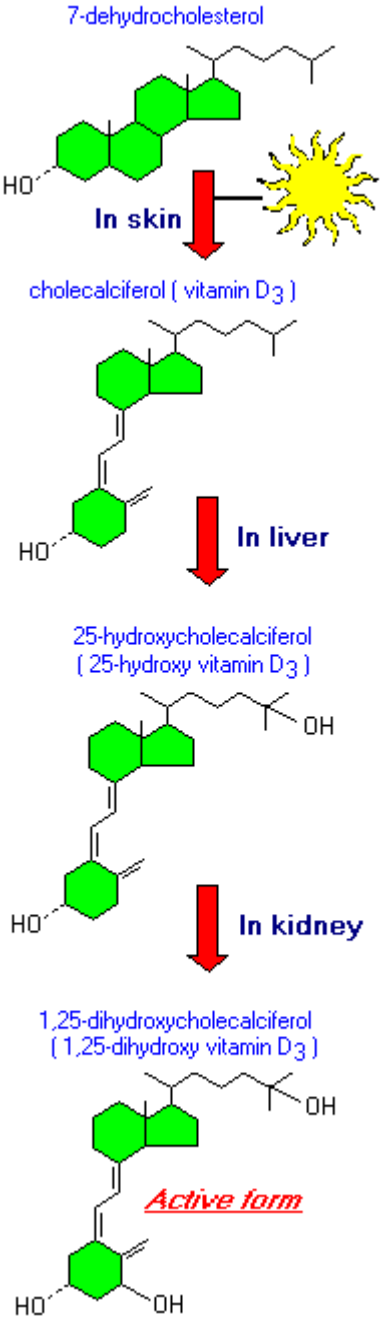
Table 1.1: Major candidate genes showing association to bone mass variation

Biological classification	Candid-ate gene	Protein	Chromosome location	Reference
Calciotropic hormones and receptors	VDR	Vitamin D receptors	12q12-14	Morrision et al. 1994
	ER-	Estrogen receptor-	6q25	Sano et al. 1995
	ER-	Estrogen receptor-	14q22-24	Ogawa et al. 2000
	CT	Calcitonin	11p15	Miyao et al. 2000a
	CTR	Calcitonin receptor	7q21	Masi et al. 1998
	PTH	Parathyroid hormone	11p15	Hosoi et al. 1999
	PTHR1	Parathyroid hormone receptor 1	3p22-21	Minagawa et al. 2002
	CYP19	Aromatase	15q21	Masi et al. 2001
	GCCR	Glucocorticoid receptor	5q31	Huizenga et al. 1998
	CaSR	Calcium-sensing receptor	3q13-21	Tsukamoto etal.2000a
	AR	Androgen receptor	Xq11-12	Sowers et al. 1999
Cytokines, growth factors and receptors	TGF- 1	Transforming growth factor- 1	19q13	Langdahl et al. 1997
	IL-6	Interleukin-6	7p21	Murray et al. 1997
	IGF-1	Insulin-like growth factor I	12q22-24	Miyao et al. 1998
	IL-1ra	Interleukin-1 receptor antagonist	2q14	Keen et al. 1998
	OPG	Osteoprotegerin	8q24	Arko et al. 2002
	TNF-	Tumor necrosis factor-	6p21	Fontova et al. 2002
	TNFR2	Tumor necrosis factor receptor 2	1p36	Spotila et al. 2000
Bone matrix proteins	COLIA1	Collagen type I 1	17q21-22	Grant et al. 1996
	COLIA2	Collagen type I 2	7q22	Suuriniemi et al. 2002
	BGP	Osteocalcin	1q25-31	Dohi et al. 1998
	MGP	Matrix Gla protein	12p13-12	Tsukamoto etal.2000b
	AHSG	-2-HS-glycoprotein	3q27	Dickson et al. 1994
Miscellaneous	ApoE	Apolipoprotein E	19q13	Shiraki et al. 1997
	MTHFR	Methylenetetrahydrofolate reductase	1p36	Miyao et al. 2000b
	P57(KIP 2)	Cyclin-dependent kinase inhibitor 1c	11p15	Urano et al. 2000
	HLA-A	Major histocompatibility complex, class I, A	6p21	Tsuji et al. 1998
	PPAR-	Peroxisome proliferator-activeated receptor-	3p25	Ogawa et al. 1999
	FRA-1	Fos-related antigen-1	11q13	Albegha et al. 2002
	RUNX-2	Runt-related transcription factor-2	6p21	Vaughan et al. 2002
	Klotho gene	Klotho protein	13q12	Kawano et al. 2002
	WRN (Werner syndrom e gene)	Werner helicase	8p12-11	Ogata et al. 2001

1.3. Vitamin D &Vitamin D Receptor (VDR) :

The steroid hormone vitamin D, its receptor(VDR), and the metabolizing enzymes involved in the formation of the biologically active form of the hormone, together are major players in the **vitamin D endocrine system.** This system plays an important role in skeletal metabolism. It also seems to play an important role in other metabolic pathways such as those involved in osteoarthritis, the immune response and cancer (Haussler etal.1998).

Figure1.6:Vitamin D



*The term vitamin D actually refers to a group of steroid molecules.Vitamin D_3, also known as *cholecalciferol*, is generated in the skin of animals when light energy is absorbed by a precursor molecule 7-dehydrocholesterol.The plant form of vitamin D is called vitamin D_2 or ergosterol.Vitamin D (as either D_3 or D_2) does not have significant biological activity. Rather, it must be metabolized within the body to the hormonally-active form.This transformation occurs in two steps, as seen in figure 6:

1.Within the liver, cholecalciferol is hydroxylated to 25-hydroxycholecalciferol by the enzyme 25-hydroxylase.

2.Within the kidney, 25-vitamin D serves as a substrate for 1-α-hydroxylase, yielding1,25-

dihydroxycholecalciferol , the biologically active form of vitamin D. Each of the forms of vitamin D is hydrophobic and is transported in blood bound primarily to vitamin D binding protein(DBP) (85-88%) and albumin (12-15%) (Colorado S.U.2001, Bikle, 2004). However, the hormonal activities of 1,25-$(OH)_2D_3$ are mediated through its intracellular receptor-the vitamin D receptor **(VDR)** (DeLuca&Schnoes,1983).Vitamin D receptor binds several forms of cholecalciferol. Its affinity for 1,25-dihydroxycholecalciferol is roughly 1000 times that for 25-hydroxycholecalciferol, which explains their relative biological potencies (Colorado S.U.,2001).

1.3.1.Physiological Effects of Vitamin D:

Vitamin D is well known as a hormone involved in mineral metabolism and bone growth. It promotes the differentiation of osteoblasts and regulates the production of proteins such as collagen, alkaline phosphatase, and osteocalcin thought to be important in bone formation. It also induces RANKL, a membrane bound protein in osteoblasts which enables osteoblasts to stimulate the formation and activity of osteoclasts. Thus, vitamin D regulates both bone formation and bone resorption. (Colorado S.U.2001, Bikle,2004).

However, **the principle function of vitamin D is to maintain intracellular and extracellular calcium concentrations within a physiologically acceptable range**, through the action on regulating calcium and phosphorus metabolism in the intestine and bone (in concert with parathyroid hormone and calcitonin) (figure

1.7). Actually, in the absence of vitamin D, dietary calcium is not absorbed at all efficiently.

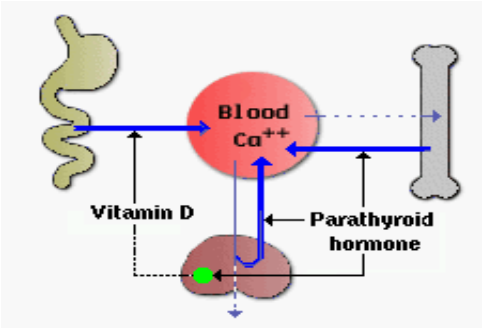


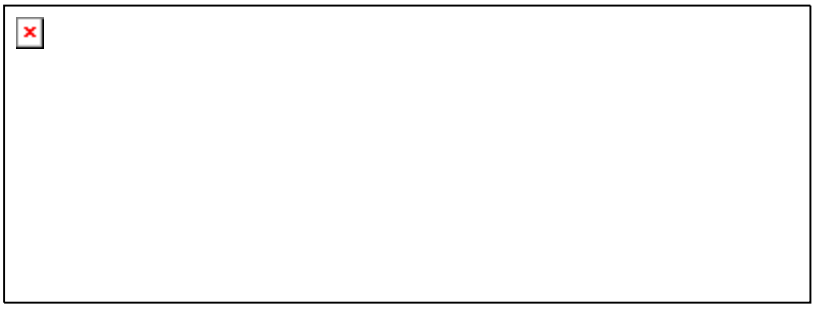
FIG. 1.7: A representation of the classic physiological actions of vitamin D: The hormonal form of vitamin D functions in the intestine, bone, and the distal renal tubule to mobilize calcium. In the case of the intestine, 1,25-(OH)2Da serves to mobilize phosphorus into the plasma. Saturating levels of calcium and phosphorus support mineralization in the skeleton and neuromuscular junction activity (Colorado S.U.2003, Litwack,1994)

Vitamin D stimulates the expression of a number of proteins involved in transporting calcium from the lumen of the intestine, across the epithelial cells and into blood. The best-studied of these calcium transporters is *calbindin*, an intracellular protein that ferries calcium across the intestinal epithelial cell. Thus, the crutial effect of vitamin D on bone is to provide the proper balance of calcium and phosphorus to support mineralization (Colorado S.U.2001&2003, Scientific Committee on Food,2002).

1.3.2. Vitamin D Receptor (VDR):

The main mechanism of action of vitamin D is the interaction of 1,25$(OH)_2$D with the nuclear vitamin D hormone receptor (Brown&Duncan, 1999).VDR was discovered in 1969 (Haussler&Norman,1969), and was eventually cloned and sequenced in 1987 (McDonnell etal.1987, Baker etal,1988). It is a protein of

approximately 50-60kDa depending on species. Figure 1.8 explains the basic structure of VDR (Daniel, 2004, Rachez et al. 2000):



<u>Figure 1.8. Model of the vitamin D receptor (VDR):</u> The VDR is unusual in that its N- terminal region is short relative to other steroid nuclear hormone receptors. This region is followed by two zinc fingers which constitute the principal DNA binding domain(the most conserved domain in VDR among the nuclear receptors in general). Nuclear localization signals (NLS) are found within and just C-terminal to the DNA binding domain. The ligand binding domain (where vitamin D binds)makes up the bulk of the C-terminal half of the molecule, with the major activation domain (AF2)comprising the most C-terminal region. The AF2 domain is largely responsible for binding to co-activators . Regions on the second zinc finger and within the ligand binding domain facilitate heterodimerization with the retinoid X receptor (RXR),while the proximal (N-terminal) zinc finger confers specificity for DNA binding to the vitamin D response elements (VDREs) , as seen later.

The VDR is widely distributed in most , if not all the cells in the body (Colorado S.U.2001). Actually, it is a transcription factor, mediating the effects of vitamin D on gene expression. The mechanism by which the vitamin D hormone enter a cell

and regulate DNA transcription , is summarized in the next figure below (Bikle,2004):

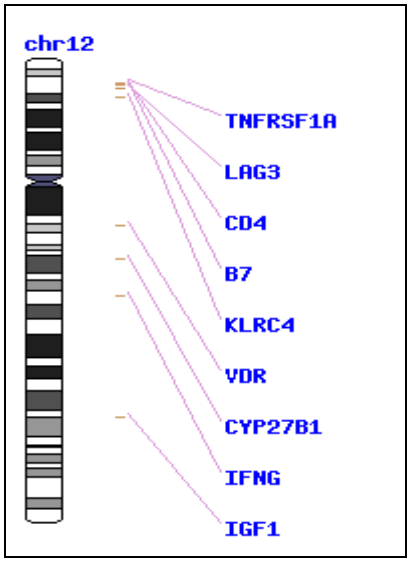
Figure 1.9: 1,25$(OH)_2$D-initiated gene transcription: 1,25$(OH)_2$D enters the target cell and binds to its receptor, VDR. The VDR then heterodimerizes with the retinoid X receptor (RXR). This increases the affinity of the VDR/RXR complex for the vitamin D response element (VDRE), a specific sequence of nucleotides in the promoter region of the vitamin D responsive gene. Binding of the VDR/RXR complex to the VDRE attracts a complex of proteins termed coactivators to the VDR/RXR complex. The coactivator complex spans the gap between the VDRE and RNA polymerase II and other proteins in the initiation complex centered at or around the TATA box (or other transcription regulatory elements). Transcription of the gene is initiated to produce the corresponding mRNA, which leaves the nucleus to be translated to the corresponding protein.

1.3.3 The VDR Gene:

The gene encoding the VDR was the first candidate gene to be studied in osteoporosis. It actually initiated the osteoporosis' molecular genetics, chosen on the basis that it acts as an important regulator of calcium metabolism and bone cell function (Ralston,2003 , Uitterlinden,2002). The hVDR gene is located on the "q"

arm of human chromosome 12 (figure 1.10) , precisely at chromosome 12cen-q12(Taymans,1999).

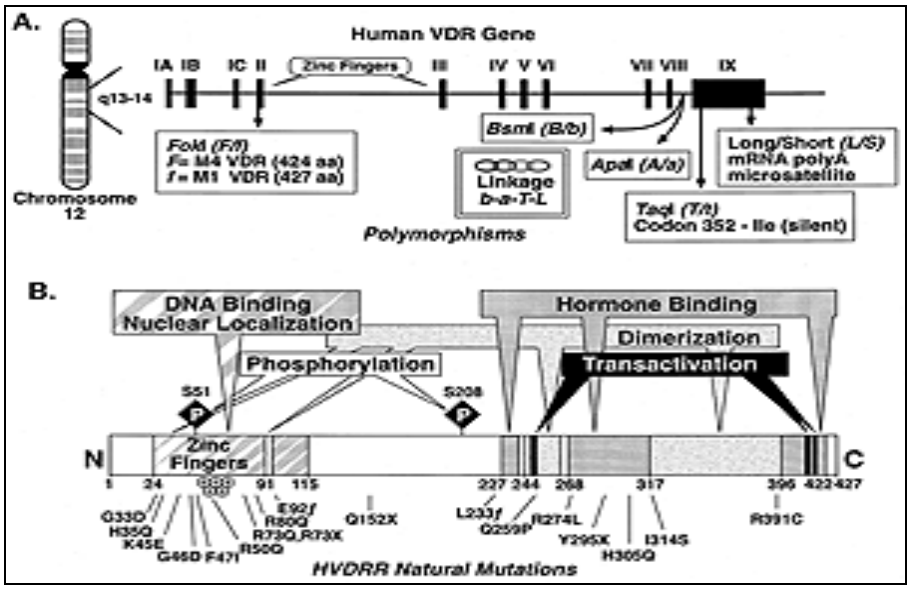
Figure 1.10:Human Chromosome 12 (Warranty & Disclaimer , 2001-2005)



The hVDR gene is approximately 100kb long and is made up of 14 exons together with intervening introns. As illustrated in figure 1.11, three exons (1A,1B and 1C) are found in the 5`non-coding sequence . Another 3 exons (1d,1e,1f) were discovered later (Uitterlinden etal,2004)**)** and 8 exons (II – IX) encode for the structural portion of the product. As in any other gene, different exons encode for different protein domains.The 3` boundaries of the exons that code for the two zinc fingers are highly conserved within the nuclear receptor family of genes (each of the two zinc fingers is encoded by separate exons (II and III),as in other nuclear receptor genes). An intron segment found in the 3` of exon 1c shows retinoic acid responsivity to induce transcription, while its GC- rich promoter (which doesn't contain a TATA box) contains binding sites for transcriptional factors .The

presence of a polymorphic sequence in exon II determines the presence or absence of an alternative translation start site (see discussion of *Fok*I polymorphism below). A unique feature of the hVDR gene is the presence of an additional exon (V) that is not found in other nuclear receptor genes ; it resides near the center of the gene and encodes residues 155–194 in hVDR. This region of the VDR protein is more expansive than the corresponding segment in other nuclear receptors (Miyamato etal.1997/Faraco, 1989/Laudet,1991/Haussler etal,1998/Uitterlinden etal,2004/ Crofts etal.1998).

Figure 1.11: Human VDR Gene (Haussler etal,1998)



1.3.4. Polymorphisms In The VDR Gene:

One of the most intriguing, yet controversial, areas of bone-related genetic research in the past few years has been the discovery of common polymorphisms in

the hVDR gene and their potential relationship to bone mineral density (BMD) and the pathophysiology of osteoporosis (Haussler etal,1998). In figure 1.12, and table 2, a number of the currently known VDR polymorphisms are depicted. However, apart from those polymorphisms, a number of novel polymorphisms were found in the promoter areas including in and around exons 1f-1c, in and around exons 2-9,and in the 3.2 kb 3′ untranslated region(UTR). Actually, 63 polymorphisms across 22 kb of sequences were discovered in the VDR gene (Uitterlinden, 2004).

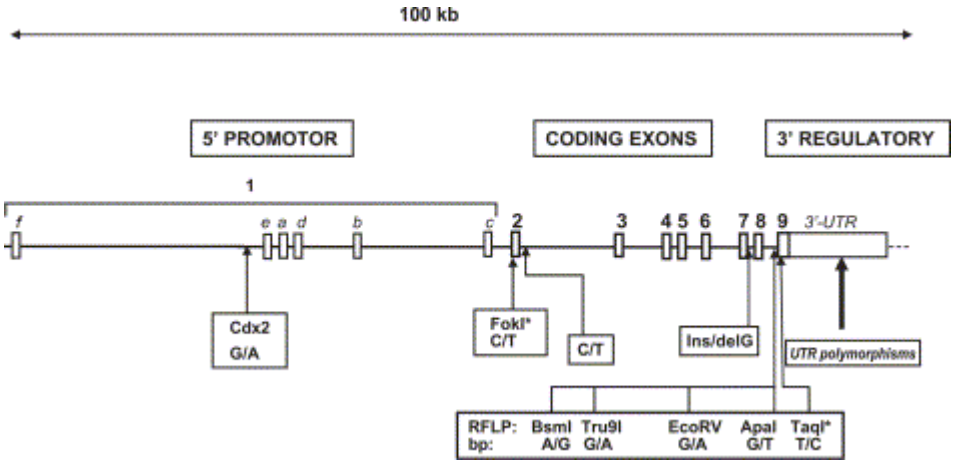


Fig. 1.12. Position of known polymorphisms in the exon–intron structure of the VDR gene. (*) indicates that these polymorphisms are in the coding sequence. (Uitterlinden etal,2004)

Table 1.2: Major VDR gene Polymorphisms

Polymorphism	Location	Codon	Amino acid change	Nucleotide Varient	References
CdX2	1e promoter: binding site for an intestinal-specific transcription factor	_	_	G/A	*Arai et al. 2001 *Uitterlin-den 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

As noticed , the only known protein polymorphism in the VDR gene is the FokI polymorphism (Utterlinden,2004): It is a thymine/cytosine (T/C) transition (changing ATG to ACG) at the first initiation codon (ATG) located in exon 2 of the VDR gene. Normally, the VDR gene has two potential translation initiation or ATG start sites, three codons apart. A start codon polymorphism (SCP) has been noted in the first ATG. Thus, the VDR gene from individuals with the T variant (containing the restriction site of FokI restriction enzyme, and thus denoted f allele), possesses 2 potential translation initiation codons. Therefore, while the translation of the VDR mRNA from the T (f) allele can initiate from the first start site, translation of mRNA from the C (F) allele must initiate from the second start site as shown in the following illustration:

Normal: (T or f allele)
(translated to MI (427aa) VDR)

ATG ——— **3 codons apart** ——— **ATG**
(1st Start codon) **(2nd potential start codon)**

↓

Polymorphic varient: (C or F allele)
(translated to M4 (424aa) VDR)

ACG ——————————— **ATG**
(only one start codon)

Such a difference, may provide structural change in the VDR protein that could potentially alter its function and thereby influence bone remodeling and bone mineral density (BMD). Interestingly, this T-C transition results in a three amino

acid shorter protein (424 aa residue(M4 isoform)instead of 427(M1 isoform))with increased biological activity (Harris etal.1997,Arai etal. 1997, Haussler etal,1998, Uitterlinden,2004b). In a more recent study on VDR gene polymorphism and osteoporosis, it was reported that the FokI SCP is strongly associated with reduced bone mineral density and predisposes women to Osteoporosis at the lumber spine (as seen in the next section).

In Sydney, Morrison et al carried out a study on MZ and DZ twins, where bone density was measured at different anatomical positions and correlated with VDR genotypes. From the results obtained, the **b allele (containing the BsmI restriction site) was associated with **higher bone density** and greater difference in bone densities at the lumbar spine was observed in DZ twins. Morrison et al described that allelic variations of the VDR gene accounts for 75% of the genetic effects on bone density and that VDR polymorphisms can be used to predict an increased risk of osteoporosis(Morrison etal,1994). This was the first study that showed such a correlation and since then, various studies have been carried out on different ethnic groups and different age groups to try to determine whether a correlation exists between these polymorphisms and BMD. However, among the most polymorphisms that seem to be associated with BMD in many studies are the BsmI and the FokI polymorphisms on which the following section will concentrate.

1.3.5.Overview About Previous Studies On BsmI & FokI Polymorphisms:

Of particular interest in this respect is the fact that not always the same risk allele is being found associated with bone parameters, preventing the straight forward interpretation of these associations: Surprisingly, while the initial studies by Morrison et al (1992,1994,1997) suggested the "B"allele of the **BsmI**-RFLP site to be the risk allele associated with low BMD, other studies either confirmed this, didn't find any effect, or found the opposite (i.e.the"b"allele to be the risk allele associated with low BMD) (Uitterlinden etal.2002). For example, contrasting the results obtained by Morrison et al are those of Hustmyer et al,1994, where no correlation was found between different VDR genotypes and BMD. This study was carried out on MZ and DZ twins of Caucasian origin at the Indiana University where ApaI, TaqI and BsmI were used as restriction enzymes. Hustmyer et al argued that the different results obtained from Morrison`s work may be due to the fact that VDR polymorphisms may be linked to another gene locus involved in bone density regulation. They also emphasized that this genetic linkage may be obscured by environmental factors that are particular to different populations (Hustmyer etal,1994).

Moreover, contrasting results were obtained in a study carried out on southern Chinese females (Kung etal.1998). The frequency of the B allele in this population was found to be lower when compared to that of western countries but is similar to that found in Koreans (Lim etal.1995). Kung et al , 1998 tried to find an

explanation for the various conflicting results obtained from the various studies. The role of environmental factors in the genetic effects of the VDR on BMD, especially dietary calcium intake, was highly emphasized. It was also stated that since the BsmI cleavage site is found in an intronic region, there is no actual difference in VDR expression between BB and bb genotypes. So it was concluded that VDR polymorphisms do not predict an increased risk of osteoporosis.

In another study carried out in the UK , an inverse association was found between VDR genotypes and BMD at the hip (Houston etal.1996). Unlike most of the studies, Houston et al found a lower BMD in individuals having the bb genotype when compared to the BB genotype (-0.79 sd). From this study, it was concluded that the VDR genotypes are associated with BMD in the population studied, explaining that the inverse findings found may reflect that reduced BMD may not be a direct cause of the VDR genotype but may be linked to another disease causing locus nearby. While Various studies were carried out on the BsmI restriction site showing its association with BMD, Lim et al concluded that VDR polymorphisms are not helpful in predicting osteoporosis (Lim etal.1995).

Considering the **FokI polymorphism**, A promising association between ff genotype and low BMD at the lumbar spine in a cohort of postmenopausal women has been published by Gross et al (1996) (Zajickova,2003). Moreover, In a study carried out in Boston in 1997, Harris and co-workers showed that postmenopausal women with the ff genotype (containing the FokI restriction site) had lower

femoral BMD (7.4%) when compared to the FF genotype. They also observed that a small difference in the distribution of these SCPs between black and white populations might explain part of the racial differences in BMD at the femoral neck (Harris etal.1997). Surprisingly, conflicting results were obtained by Ferrari et al, when they did not find correlation between VDR SCP and BMD in European-Caucasian women (Ferrari etal,1998). Interestingly, Kubota et al concluded that the FokI polymorphism is a stronger genetic indicator of osteoporosis than the BsmI polymorphism in pre-menopausal Japanese women (Kubota etal.2001), while van der Sluis et al predicted that VDR polymorphism predict height and bone size rather than bone density in children and young adults (van der Sluis etal.2003).

***Table 1.3,and table 1.4 below summarise various association studies (concerning the BsmI & FokI polymorphisms) with positive and negative findings respectively. Such conflicting findings – which aren't exclusive for the field of genetic association analysis – could have several reasons: The most likely explanations are that given the small effect on BMD, very often the statistical power is much too low and no conclusions on the presence or absence of an effect can in fact be drawn. Moreover, interaction between different genes and /or environmenta1 factors play a role in the action of this important steroid hormone receptor transcription factor. For example, dietary Ca-intake is known to differ substantially between countries while circulating serum vitamin D levels, which are determined by several metabolizing enzymes, also differ between populations.

Consequently, such gene-gene and gene-environment interactions can of course differ between different populations (Uitterlinden etal.2002).This is shown in table 1.5, where a summary of studies on this topic are shown.

Table 1.3 : Association studies with positive findings in the VDR gene (Bsm-I & Fok-I RFLPs).

Marker locus	n	Population characteristics	Phenotype and effect	P -value	Reference
BsmI	250	Normal healthy white twins	BMD of LS, FN	LS (0.000054, FN (0.038)	Morrison et al. 1994
BsmI	91	White individuals	Osteocalcin concentration (BB>bb)	BB vs bb: 0.0001	Morrison et al. 1992
BsmI	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
BsmI	470	Healthy premenopausal women, aged 44-50 yr	Spinal and trochanter BMD (BB>Bb, bb)		Salamone et al. 1996
BsmI	171	Pre-and postmenopausal women	FN bone density (bb<BB)	<0.02	Houston et al. 1996
BsmI	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
FokI	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
FokI	100	Postmenopausal Mexican-American white women	LS BMD (ff<Ff<FF) Decrease in BMD at FN in 2 yr (ff>FF)	0.01 0.005	Gross et al. 1996
FokI	110	Premenopausal Japanese women	LS BMD (mm>MM)	<0.05	Arai et al. 1997
BsmI	229	Healthy postmenopausal women	FN BMD (BB lowest among women >10 yr menopausal), rate of bone loss over 2 yr at spine, FN, radius (BB>other)	0.01	Krall et al. 1995
BsmI	72	Elderly individuals	LS BMD loss over 18 months (BB, loss; bb, no loss; Bb, change correlated with calcium intake) Change in LS BMD in reaction to calcium intake (Bb)	<0.05 for BB, <0.03 for Bb <0.03 (for Bb)	Ferrari et al. 1995
BsmI	60	Healthy late postmenopausal women	Fractional calcium absorption at low calcium intake (BB<bb)	0.044	Dawson-Hughes et al. 1995
BsmI	328 94	White aged 69-90 yr White aged 18-68 yr	Effect of calcium intake on BMD increase only in bb subjects FN BMD (bb>BB)	<0.05	Kiel et al. 1997
BsmI	84	Thai postmenopausal women	24 h urinary calcium excretion (bb>BB)	0.05	Ongphiph-adhanakul et al. 1997
FokI	72	Healthy children aged 7-12 yr	BMD (FF>Ff>ff) Calcium absorption (FF>Ff>ff)	0.02 0.04	Ames et al. 1999
BsmI	32	Healthy postmenopausal women	BMC (bone mineral content), weight (bb>Bb>BB)		Barger-Lux et al. 1995
BsmI	558	Non-obese postmenopausal women (BMI<30 kg/m)	FN BMD (bb>BB)	0.04	Vandevyv-er et al. 1997
BsmI	75	Yoyng Finns, 20-29 yr	Peak bone mass BMD in LS, FN (bb>BB)	LS, 0.030 FN, 0.049	Viitanen et al. 1996

Continued

Marker locus	n	Population characteristics	Phenotype and effect	P -value	Reference
BsmI	83 72	(white) (black)	FN BMD (whole group) (bb,Bb>BB)	FN BMD (0.034) LS BMD (0.036)	Fleet et al. 1995
BsmI	118 94 50	Old people (60-90 yr) with calcium intake >800 mg/day 18-68 yr Men from the above population	Trochanter BMD (bb>Bb, BB) FN BMD (bb>BB) FN BMD (bb>BB)	<0.05 <0.05 <0.05	Kiel et al. 1997
BsmI	380	Healthy women older than 70 yr, non-obese (BMI<30 kg/m)	FN BMD (bb>BB) Quadriceps strength (bb>BB) Grip strength (bb>BB)	<0.05 <0.01 <0.05	Geusens et al. 1997
BsmI	127	Brazilian women, 20-47 yr, premenopausal	LS BMD (bb>BB), FN BMD (bb>Bb>BB)	<0.05	Lazaretti-Castro et al. 1997
BsmI	92	Japanese women, healthy, 43 17 yr	LS BMD (bb>Bb)	<0.035	Tamai et al. 1997
BsmI	90	Japanese women, osteoporosis, 71 10 yr	LS BMD (BB>bb)	<0.025	Tamai et al. 1997
BsmI	78	Brazilian patients with IDDM	LS, FN BMD (BB<Bb,bb)		Hauache et al. 1998
BsmI	81	Old women, >70 yr	Increase of BMD in response to vitamin D supplementation (BB, Bb>bb)	0.03	Graafmans et al. 1997
BsmI	21	Premenopausal	Increase in serum osteocalcin after 7-day oral 1,25(OH) stimulation (BB<bb)	0.01	Howard et al. 1995
BsmI	589	Healthy infants	Body length, weight, surface area (BB girls>bb girls, BB boys<bb boys)		Suarez et al. 1997
BsmI	146	Normal men aged 20-83 yr	Bone density in the forearm (BB<Bb,bb) Bone area in the forearm (BB>Bb,bb)	0.030 (bone density) 0.026 (bone area)	Need et al. 1996
BsmI	170	Middle-aged white women	Femoral shaft expansion, increase in cortical area (bb>other)		Heaney et al. 1997
BsmI	66	Japanese patients with primary hyperparathyroid-ism	BMD in radius (bb<BB, Bb)		Kobayashi et al. 1998
BsmI	88	Japanese hemodialysed patients	Decrease of whole-body and FN BMD during 18 months (BB>Bb, bb)	<0.02	Karkoszka et al. 1998
BsmI	120	White postmenopausal women aged 61 0.6 yr	Intestinal Ca absorption (Bb<bb)	0.0015	Gennari et al. 1997
FokI	98	Postmenopausal osteoporotic French women aged 45-75 yr	FN BMD (ff<FF, Ff)	Significant	Lucotte et al. 1999
BsmI	191	Postmenopausal Japanese women	BMD loss in both early and late postmenopausal women (Bb>bb)	0.001	Kikuchi et al. 1999
BsmI	197	Prepubertal girls and peri- and postpubertal adolescents of white origen	LS BMD, height (BB<Bb, bb)	<0.02	Ferrari et al. 1998a

Continued

Marker locus	n	Population characteristics	Phenotype and effect	P value	Reference
BsmI	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
BsmI	677	Healthy white women aged 18-35 yr	Peak BMD at femoral neck (B: 0<1<2)	0.006	Rubin et al. 1999
BsmI	104	Healthy young men, aged 24.3 3.1 yr	BMD Z scores at LS and femoral trochanter (BB<Bb<bb)	0.03 (LS), 0.05 (femoral trochanter)	Ferrari et al.1999
BsmI	99	Healthy white girls, aged 16.9 1.2 yr	LS BMD (Bb>bb)	0.02	Lorentzon et al. 2001
FokI	118	Healthy Irish adults aged 16-67 yr	Serum osteocalcin (ff>Ff>FF), urinary pyridinoline and deoxypyridinoline (ff>Ff>FF		Sheehan et al. 2001
BsmI	102	Late postmenopausal women aged 47-77 yr	LS BMD (bb>BB)		Marc et al. 2000
BsmI	399	192 osteoporotic patients, 207 normal controls	BMD of intertrochantic region (bb>other) and total hip (bb>other)	<0.001 (BsmI, intertrochanter), <0.01 (BsmI, total hip)	Langdahl et al. 2000a
FokI	400	Postmenopausal women of Italian descent, 164 osteoporotic, 117 osteopenia, 119 normal	Lumbar BMD	0.06	Gennari et al. 1999
BsmI	326	Individuals of both sexes	Lumbar and FN BMD of women (bb>Bb, BB)		Gomez et al.1999
BsmI	24	Late postmenopausal women	Response to cyclic etidronate therapy with calcium supplementation: LS BMD increase (BB, Bb>bb) osteocalcin decrease (bb>BB)		Marc et al. 1999
FokI	163	Postmenopausal Chinese women in Taiwan	LS BMD (Ff>ff)	0.29	Chen et al. 2002
BsmI	171	Postmenopausal Chinese women in Taiwan	Lumbar and FN BMD	<0.001	Chen et al. 2001a
BsmI, TaqI	303	Postmenopausal Korean women who received HRT for 1 yr	LS BMD annual percentage change (bb, TT<Bb, Tt)		Kim et al. 2002a
BsmI	108	US mid-western postmenopausal white women	~5.8% spine BMD variation	0.004	Deng et al. 1999
BsmI	126	Hellenic postmenopausal women	BMD		Douroudis et al. 2003
BsmI		Postmenopausal Korean women	BMD		Kim et al. 2003

Table 1.4: Association studies with negative findings:

Marker locus	n	Population characteristics	Phenotype	Reference
BsmI	86MZ /39DZ	White	BMD	Hustmyer et al. 1994
BsmI	189	Premenopausal women, white	BMD, bone turnover	Garnero et al. 1995
BsmI	268 189	Postmenopausal women, 1-26 yr postmenopausal Female dizygous twins	Bone turnover, rate of bone loss, BMD Quantitative ultrasound of calcaneus or hip axis length	Garnero et al. 1996 Arden et al. 1996
BsmI	101	African-American women >65 yr	BMD, rate of postmenopausal bone loss, bone turnover	Zmuda et al. 1997
BsmI	84	Type I osteoporotic women (66.6 8.4 yr)	BMD	Vandevyver et al. 1997
BsmI	103	White women of Mexican descent, postmenopausal	BMD, bone metabolism	McClure et al. 1997
FokI	174	Premenopausal French women	BMD, calcium, parathyroid hormone, vitamin D, osteocalcin, alkaline phosphatase	Eccleshall et al. 1998
BsmI	163	Postmenopausal women	BMD	Boschitsch et al. 1996
BsmI	84	Thai, postmenopausal women	BMD, osteocalcin	Ongphiphadh-anakul et al. 1997
BsmI	81	Old women, >70 yr	FN BMD	Graafmans et al. 1997
BsmI	50	14 premenopausal 36 postmenopausal 43.3-62.8 yr German women	Markers of bone turnover BMD Ultrasound transmission velocity through bone	Rauch et al. 1997
BsmI	268 69	Chinese, 155 men aged 22-88, 113 premenopausal women aged 40-53 yr Premenopausal white women	BMD, BMC, markers of bone turnover BMD	Tsai et al. 1996 Alahari et al. 1997
BsmI	48 56 80	Men from southern Europe Premenopausal women from eastern Europe Postmenopausal women from western Europe	BMD	Spotila et al. 1996
BsmI	273	Young boys and girls, aged 8.2-16.5 yr	Forearm BMD gain	Gunnes et al. 1997
BsmI	92	49 young women aged 25-35 yr 43 elderly women aged 65-83 yr	Intestinal VDR protein concentration, serum 1,25(OH) D and radioactive calcium absorption	Kinyamu et al. 1997
BsmI	38		Abundance of VDR mRNA in peripheral blood mononuclear cells	Mocharla et al. 1997
BsmI	9		Levels of VDR expression, cellular responsiveness to 1,25(OH) D in cultured skin fibroblasts	Gross et al. 1998a
BsmI	723 110 108 109	Danish women	BMD at LS, hip, and forearm Early postmenopausal bone loss (age 51-53 yr) Late postmenopausal bone loss (age 63-69 yr) Long-term postmenopausal bone loss (age 51-69 yr)	Jorgensen et al. 1996
FokI	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

Continued

Marker locus	n	Population characteristics	Phenotype	Reference
FokI	124	Postmenopausal osteoporotic French women aged 45-90 yr	Age, yr since menopause, hight, weight, BMD at LS and FN	Lucotte et al. 1999
FokI	104	Community-dwelling African-American women aged >65 yr	Hip and calcaneal BMD, calcaneal ultrasound attenuation, hip geometry, biochemical markers of bone turnover, fractional calcium absorption	Zmuda et al. 1999a
BsmI	191 393	Postmenopausal Japanese women Women aged 45-53 yr	LS baseline BMD BBA, SOS	Kikuchi et al. 1999 Gregg et al. 1999
FokI	332	177 healthy premenopausal women aged 18.7-56.0 yr, 155 prepubertal girls aged 6.6-11.4 yr	BMD	Ferrari et al. 1998b
BsmI	172	Premenopausal women of white origin	BMD	Ferrari et al.1998a
BsmI	372	Pre- and perimenopausal women	Baseline BMD, change in BMD over 3 yr, bone-related serum markers	Willing et al. 1998
BsmI	509	272 Chinese women (mean age 75), 237 Chinese men (mean age 73 yr)	BMD	Lau et al. 1999
BsmI	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
BsmI	114	Postmenopausal Czech women aged 62.5 8.9 yr	BMD	Zajickova et al. 2002

Table 1.5: Gene x gene and gene x non-genetic factor interaction (related to the VDR gene)

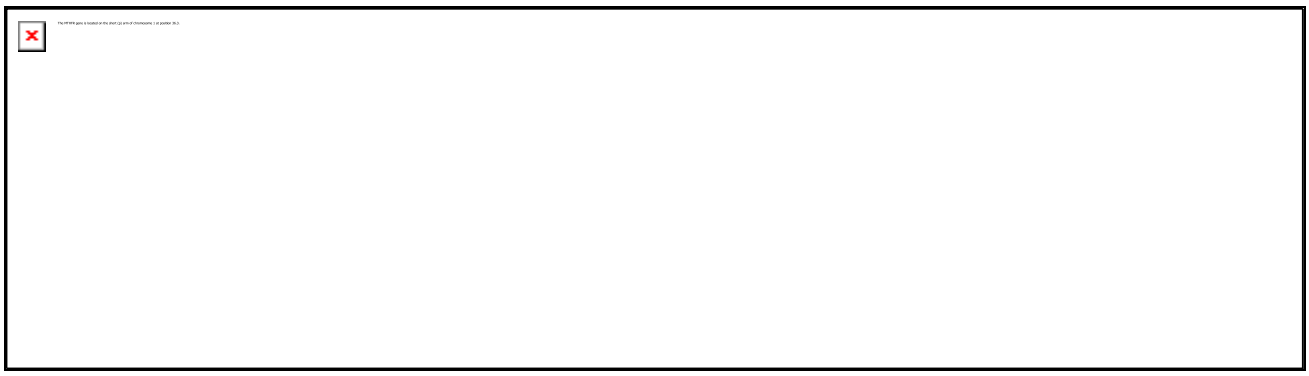
Factors	Traits	Population	Effect	P value	Reference
VDR (FokI) x VDR (BsmI and ApaI)	LS BMD	Prepubertal whites	ff was associated with a significant lower LS BMD in bb and aa individuals		Ferrari et al. 1998b
VDR BsmI x FokI	BMD	104 healthy men, aged 20.7-38.6 yr	Among BB, BMD were significantly lower in individuals carrying f allele		Ferrari et al. 1999
VDR x age, dietary calcium intake	BMD	369 healthy white females, aged 7-56 yr	BMD associated with VDR gene polymorphism only before puberty (BB<other); BMD accrual was increased in Bb and possibly BB prepubertal girls after increasing dietary calcium intake, bb individuals had the greatest spontaneous BMD accrual and remained unaffected by calcium supplements		Ferrari et al. 1998a
VDR x VitD supplementat-ion	BMD increase	81 women, aged 70 yr and over	BMD increase in the VitD supplementation group was greater in the BB and Bb genotype, compared with bb genotype	0.03	Graafmans et al. 1997
VDR BsmI x calcium intake	BMD	328 individuals aged 69-90 yr, 94 individuals aged 18-68 yr	In bb, not Bb or BB individuals, BMD was associated with calcium intake; BMD greater in persons with bb genotype only in the group with calcium intakes greater than 800 mg/day		Kiel et al. 1997
VDR x age	FN BMD	139 normal healthy women (53.2 14.5 yr) and 43 severely osteoporotic postmenopausal women (65.8 5.9 yr)	Age modulates the effect of VDR genotypes on FN BMD such that the effect of genotype was greatest among younger (premenopausal) women and declined with age so that there was no discernible difference by age 70 yr		Riggs et al. 1995
VDR x weight	BMD	807elderly women (>70 yr) ; 84 women with osteoporosis	Difference in FN BMD by BsmI genotype exists only in non-obese women (BMI <30 kg/m)		Vandevyv-er et al. 1997
VDR x years since menopause	BMD	400 postmenopausal women of Italian origin	Association of FokI genotypes with lumbar BMD more significant amont women in the first 5 yr of menopause	0.04	Gennari et al. 1999
VDR x caffeine	BMD	489 elderly women (aged 65 -77, 96 controls	When caffeine intake >300 mg/day, spine bone loss of tt>TT	0.05	Rapuri et al. 2001
VDR genotype x birthweight	BMD	165 men, 126 women aged 61-73 yr	Individuals in the lowest third of birthweight, spine BMD BB>other	0.02	Dennison et al. 2001
VDR x age	Bone loss	134 postmenopausal Irish women (age 58.19 - 7.69 yr)	Older than 60 yr FF>Ff, ff Less than 60 yr: no association	0.03	Drummo-nd et al. 2002
VDR (FokI, BsmI) x BMI	BMD	332 healthy early postmenopausal Danish women	BMI <25 kg/m : f allele associated with lower BMD of hip and forearm, b allele associated with lower spine BMD. FF, BB women: no difference in BMD between obese and thin women		Tofteng et al. 2002

1.4.Methylene Tetrahydrofolate Reductase (MTHFR):

1.4.1.MTHFR Gene and Gene Product:

The 5,10-methylenetetrahydrofolate reductase (*MTHFR)* gene is located on chromosome 1 at 1p36.3 (figure 1.13). The complementary DNA sequence is 2.2 kilobases long and appears to consist of 11 exons. Alternative splicing of the gene has been observed . (Goyette etal.1998, Botto&Yang, 2000).

Figure 1.13:MTHFR gene locus on chromosome 1 (U.S.National Library of Medicine)

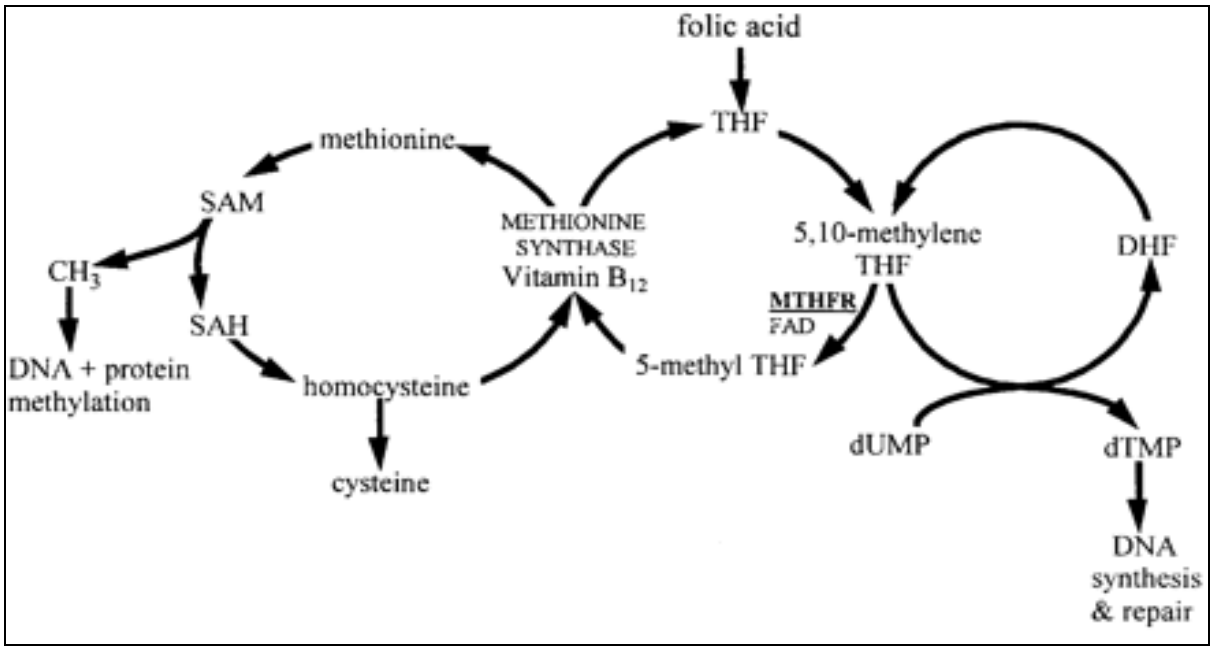


The major product of the *MTHFR* gene in humans is a catalytically active 77-kDa protein (although a smaller isoform of approximately 70kDa has been observed in some tissues(Rozen,1997)). *MTHFR* enzyme (EC 1.5.1.20) catalyzes the conversion of 5,10 methylenetetrahydrofolate into 5-methyltetrahydrofolate (5-MTHF) which is the major circulating form of folate (Figure 1.14).

The biochemical pathways involving folic acid and *MTHFR* are complex . Briefly, 5-MTHF(the methylated form of folate) provides the carbon moiety(participating in a single carbon transfer)that is used to convert homocysteine into methionine, a

reaction catalyzed by methionine synthase(vitamin B12 dependent). The remethylation of homocysteine to methionine is an important step in the metabolic network that regulates the biosynthesis of nucleosides, the methylation of DNA, proteins and lipids, and the levels of homocysteine and methionine . The metabolic network is complex and relies on multiple activators and inhibitors. For instance, a derivate of methionine, S-adenosyl methionine (SAM), is an allosteric inhibitor of *MTHFR* and an activator of cystathionine ß-synthase and regulates two main outflow paths of homocysteine. Although the complete effects of normal and abnormal folate metabolism are still incompletely understood, there is growing evidence that normal *MTHFR* activity may contribute to maintaining the pool of available circulating folate and methionine and prevent a buildup of homocysteine; conversely,abnormally low MTHFR activity may lead to lower levels of circulating folate,lower availability of methionine,and higher levels of homocysteine, since homocysteine cannot be converted to methionine (Botto & Yang,2000).

Figure 1.14:Simplified metabolic pathways involving MTHFR



***MTHFR catalysis the reduction of 5,10-methylene THF to 5-methyl THF(the predominant circulatory form of folate, and the methyl group donor required for the remethyation of homocysteine to methionine) (Frosst etal,1995)

1.4.2.MTHFR Gene (C677T)Polymorphism: *How is it Related to Osteoporosis?*

The MTHFR gene has a common polymorphism located to nucleotide 677, and is caused by a single base change (or point mutation) that converts a cytosine(C) into a thymine (T) leading to an amino acid substitution (alanine to valine) at position 222 in the MTHFR enzyme (Cashman,2005). The *C677T* allele is commonly called "thermolabile," because the activity of the encoded enzyme (thermobile MTHFR)is reduced at *37ºC* or more (Kang,1991). Thus, MTHFR activity among *C677T* homozygotes (denoted TT or VV)is 50-60% lower at *37ºC* ,and approximately 65% lower at *46°C* than in similarly treated controls. Hetero-zygotes (denoted CT or AV)are in the intermediate range. People who are

homozygous for the *C677T* allele tend to have mildly increased blood homocysteine levels if their folate intake is insufficient, but normal blood levels if their folate intake is adequate (Rozen,1997).

In the general population, a mildly elevated plasma homocysteine is a common condition. However,high circulating levels of homocysteine (also referred to as hyperhomocysteinemia) is associated with an increased risk of neural tube defects, Alzheimer's dementia, pregnancy complications, and inflammatory bowel disease.Recently, studies reported that high homocysteine levels significantly increase the risk of osteoporotic fracture. Moreover, association studies linked the common allelic polymorphism in the MTHFR gene with low bone mineral density (BMD) and/or fracture risk, together with evidence of generalized osteoporosis in patients with homocystinuria, a condition in which there is dramatically elevated plasma homocysteine levels.

The underlying pathophysiological mechanism for the occurrence of early osteoporosis in patients with elevated homocysteine levels is not completely understood. However, this finding has been attributed to a competitive inhibition of lysyl oxidase, an enzyme that is involved in the synthesis of cross-links that stabilize the collagen fibrils in bone, by high homocysteine levels. However, while this has been shown clearly in vitro, in vivo evidence of the link between homocysteine and generalized osteoporosis is limited (Cashman,2005, Morita,1998).

1.4.3. Association Studies on MTHFR(C677T) and Osteoporosis:

Recently, a number of studies have investigated the association between the MTHFR (C677T) genotype and osteoporosis (although some are contradictory). For example, based on the fact that early-onset osteoporosis is associated with homocystinurea (Morita etal,1998), and that plasma homocystein concentration is higher in postmenoposal than in premenoposal women (Kang&Trelstad, 1973): Miyao et al. formulated the hypothesis that homocysteine might participate in the pathophysiological process of osteoporosis, and they investigated the influence of MTHFR(C677T) polymorphism on BMD. In their study on 307 post- menopausal healthy women, there was a significant effect of the MTHFR (C677T) mutation on lumbar spine BMD (with the *TT* genotype having the lowest BMD) and a trend in the same direction for total body BMD. These investigators demonstrated that this allelic polymorphism in the MTHFR gene was associated with reduced BMD in postmenopausal Japanese women.Actually, this was the first report suggesting that the *TT* genotype is one of the genetic risk factors for low BMD (Miyao etal, 2000). Furthermore, Abrahamsen et al. reported that early postmenopausal Danish women with the *TT* MTHFR genotype had significantly lower BMD at the femoral neck, hip and spine and increased fracture incidence (Abrahamsen,2003). This was further confirmed in two other Danish cohorts (Bathum etal.2004,Villadsen etal.2004). In contrast, Jorgensen et al. reported an association between the C677T polymorphism (TT) in the MTHFR gene with a reduced risk of osteoporotic fracture of the forearm and hip in a case-control study in Danish postmenopausal

women relative to those with the wild-type CC genotype, even though BMD at the forearm and ultrasound parameters measured at the calcaneus were similar in both genotype groups (Jorgensen etal.2002).

*The reason for the discordant findings of studies investigating the relationship between MTHFR genotype and BMD/fracture risk is unclear (Cashman,2005). Dietary factors could be an explanation for this cotraversy, in particular B vitamins, which play a role in lowering circulating homocysteine (Bronstrup etal.1999 , McKay etal.2000). Results from the Framingham study suggested that the discrepancies could also be due to differences in folate status, as the MTHFR *TT* genotype may require -or perhaps induce- low plasma folate for development of low BMD (McLean et al.2004). Recently, a key role for riboflavin in preventing low BMD in the TT genotype was suggested by analysis of food records in the Aberdeen Osteoporosis Screening Study (Macdonald et al.2004) . Analyzing food records collected at inclusion to the Danish Osteoporosis Prevention Study, Abrahamsen et al. confirmed reports that BMD in the MTHFR TT genotype is only significantly reduced in the lowest quartile of riboflavin, B 12, B6, and folate intake, at least at the time of menopause. Vitamin B supplementation would only be expected to benefit BMD in about 2% of the population, i.e., those with the TT genotype and low vitamin B intake (Abrahamsen etal.2005).

1.5. Aim & Significance of this Study:

Osteoporosis represents one of the leading health challenges in most societies around the world. The impact of this disease on individual populations depends in part on the role of each one of the spectrum of risk factors, the rate of its occurrence in the population, and from the relatively high cost of health care of inflicted patients. Definitely, prevention programs against osteoporosis are very important in the fight against the development and spread of this disease in the population. This strategy is even of higher significance especially in under developed societies and weak economies like our own society.

This study is the first of its kind in Palestine. At present, there is no data or knowledge concerning the contribution of the various genetic risk factors to the occurrence of osteoporosis in our community.

In this study, various polymorphic genetic markers that are associated with the occurrence of osteoporosis (Bsm-I &Fok-I RFLP of the VDR gene , and the C677T polymorphism of the MTHFR gene) were investigated among women and their normal counterparts in Bethlehem district.

Specific aims of this study:

1. To investigate the genetic distribution of MTHFR and VDR (Bsm-I & Fok-I) polymorphisms among osteoporotic, osteopenic, and normal women (aged 49 years or more) in our population.

2. To study the association between these polymorphisms and BMD at the spinal, hip, or femoral neck skeletal sites.

3. To observe the association of gene – gene interactions with BMD at each of the mentioned skeletal sites.

4. To study the association of other non-genetic environmental factors including years since menopause, weight, and height with BMD in the different indicated genes polymorphisms.

*__Significance of the study :__

1. This will be the first study of its kind among osteoporotic patients in Palestine and the whole Arab population. At the same time, it's the first study worldwide in cross-genotyping the MTHFR gene polymorphisms with the VDR gene polymorphisms, and their association with BMD.

2. It will provide us with better understanding of the genetic markers background that is characteristic of our patients in comparison to similar patients from other ethnic groups.

3. Due to the fact that genetic markers are part of the individual's identity through life, the results may provide a very important and powerful tool to test individuals at an early age. This will help to identify individuals with high risk of osteoporosis based on their genetic background, and alert them to modify their life style to minimize the risk of acquiring the disease or decrease its severity.

4. The results can potentially be used in genetic counseling to advise against marriages between individuals who are heterozygous for the indicated markers especially among families with established history of Osteoporosis.

5. The results of this study will enhance other similar studies to be done on the whole west bank districts other than Bethlehem.

Chapter 2
Materials & Methods

2.1. Sample Collection:

In a previous study done by our group, showing the awareness and prevalence of osteoporosis among postmenopausal women in Bethlehem district , about 500 women were selected according to a personal interview , and then recalled to visit the Palestinian Osteoporosis Prevention Society in Beit Jala to perform BMD testing using Dual Energy X-ray Absorptiometry (DEXA).The target population constituted of mixed urban , rural , and camp sub-populations, including women over 49 years of age, from which representative subjects residing in cities, villages, and camp locations were selected using a multistage random sampling technique (Intissar Issa, 2004).

In this present study, all women from this group who performed BMD testing , including osteoporotic , osteopenic , and normal women , were phoned and asked if they agree to participate in this study (by allowing us to collect 5ml blood sample from each , for DNA extraction).

Actually, from 502 women, 344 (165 osteoporotic , 93 osteopenic , and 86 normal cases) agreed to participate . Everyone of these women has known bone scanning results, BMDs, disease severity, family history, and other information including age since menopause, height, and weight.

* Table 2.1 describes the sample distribution between the cities, villages, and camp locations in Bethlehem district:

Table 2.1: Sample distribution

Location	Osteoporotic Patients	Osteopenia cases	Normal cases	Total
Bethlehem	29	18	14	61
Beit-Jala	24	21	11	56
Al-Duhaisha Camp	19	10	5	34
Al-Obeidia	32	17	11	60
Battier	16	10	13	39
Al-Khader	19	9	7	35
Beit-Fajjar	27	8	25	60
Total	166	93	86	345
		179		

***** *Blood samples from Bethlehem, Beit Jala, Al-Duhaisha Camp, Al-Obeidia, Batteer, and Al-Khader were collected in collaboration with Aknaf Beit-AlMaqdes Charitable Society Medical center at Al-Obeidia, while the Beit Fajjar blood samples were collected in Al-Haitham Medical lab in Beit-Fajjar.*

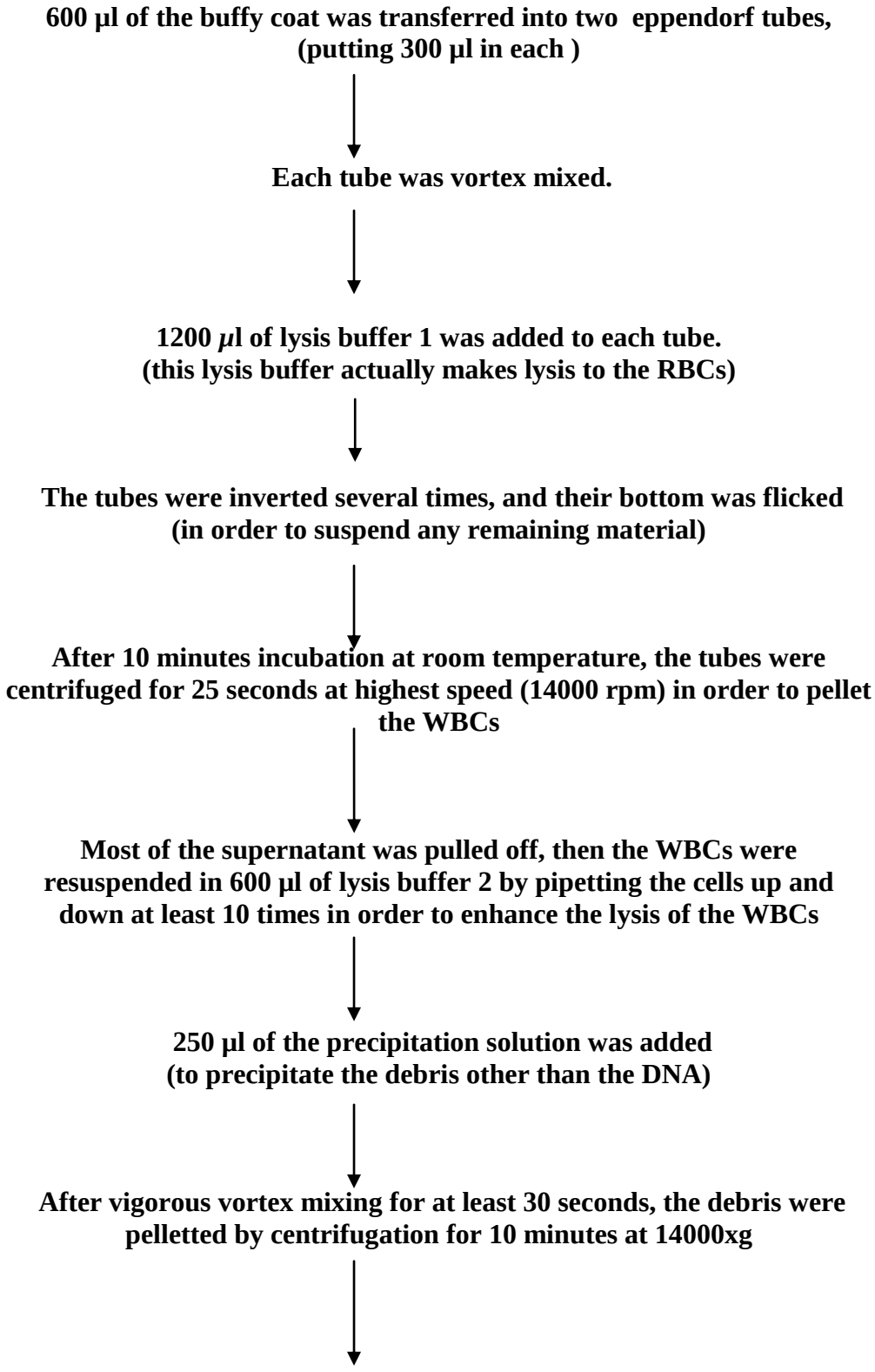
2.2. DNA preparation:

2.2.1. DNA Extraction:

EDTA blood samples were collected (5 ml when possible), and centrifuged for 10 minutes at 3000 rpm . Hemolysed samples were asked to repeated.

Then , DNA was extracted from the buffy coat using a commertially available kit(Master Pure™ Genomic DNA Purification Kit, Epicentre Technology Co.

Cat.No.MG71100). According to this kit, the procedure of DNA extraction included the following steps:



The supernatant was poured into new clean eppendorf tubes and 700µl of cold isopropanol was added

↓

After inverting the tubes gently for 30-40 times, a stringy precipitate of DNA was visible

↓

The DNA was pelletted by centrifugation for 10 minutes at 14000 rpm, and the supernatant was carefully poured off without dislodging the pellet

↓

The DNA was rinsed twice with cold ethanol in order to wash the isopropanol out (by adding 200µl of 75% cold ethanol,centrifuging for 1min,and pouring off the ethanol being careful not to dislodge the pellet)

↓

All of the residual ethanol was then removed with a pipette, and the pellet was allowed to air dry at room temperature for 2-3 minutes

↓

Finally, the DNA was dissolved in TE buffer (10mM Tris-HCl and 1mM EDTA, pH 8.0), and frosen at -30C.

***** Note: this 2-tube procedure for each sample was done for sample 1 until sample number 283(In which finally, the contents of each two tubes were mixed togetherin one tube). However, samples numbered 284 to 352 were done in a 1-tube procedure.

2.2.2. DNA Quantification:

DNA samples were quantified spectrophotometrically (Gene Quant II-Pharmacia Biotech.) at Al-Makassed hospital, after diluting the extracted DNA

1:50 by adding 10 µl of DNA to 490 µl TE buffer (10mM Tris-HCL & 1MmNa2EDTA,pH8) in which the following parameters were assessed:

-Absorbance at wavelength 230nm: indicating salt concentration.

-Absorbance at wavelength 260nm: indicating DNA concentration.

-Absorbance at wavelength 280nm: indicating protein concentration.

-The ratio 260/280: to show the amount of DNA to proteins (and thus the purity)in each extracted sample.

-DNA concentration in µg/µl for each sample.

-Protein concentration in mg/ml for each sample.

2.3. MTHFR gene (C677T) Polymorphism Screening:

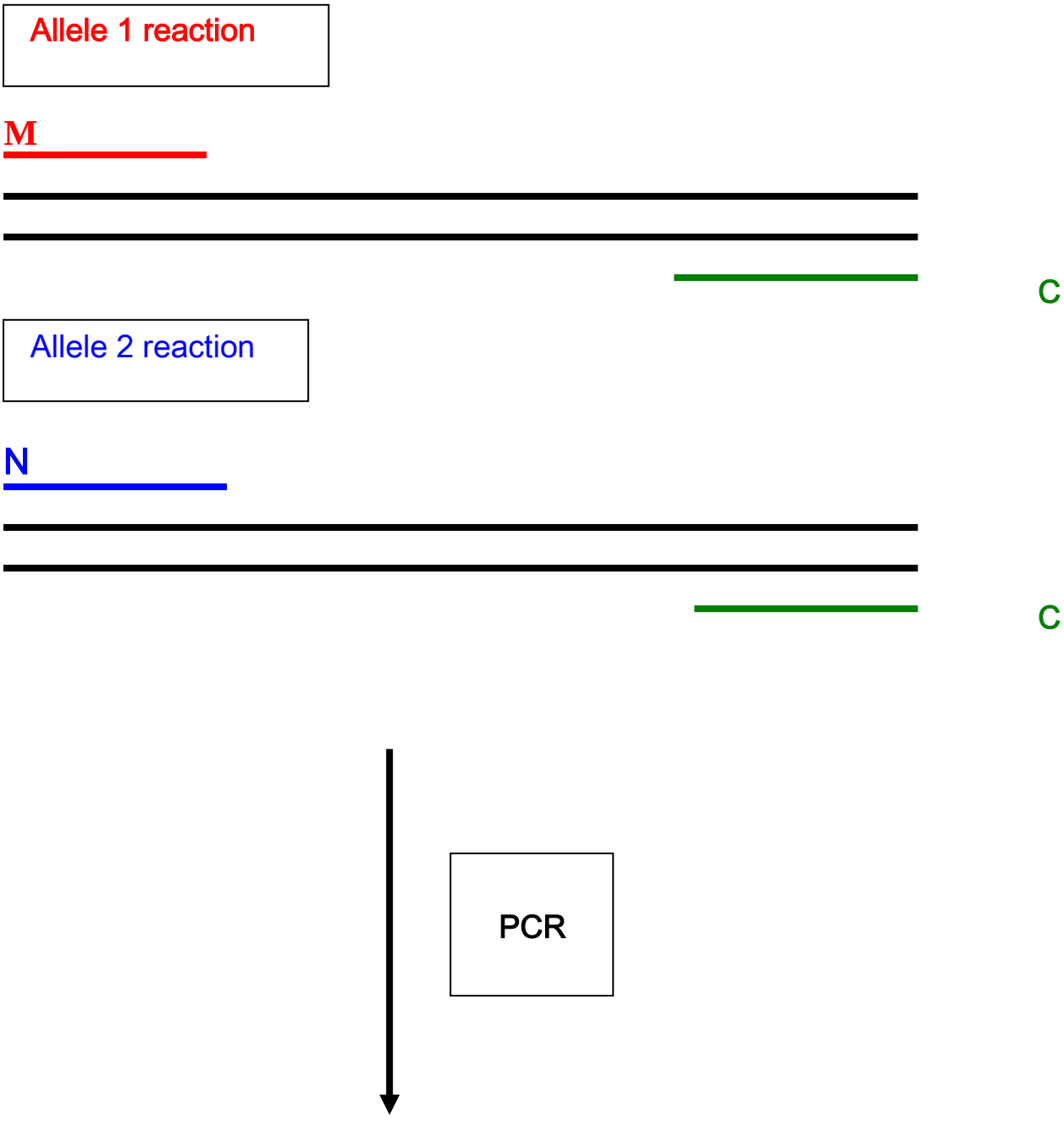
The C677T polymorphism of MTHFR of all samples was analyzed using the Amplification Refractory Mutation System (ARMS)-PCR.

ARMS is a PCR-based method, which uses allele-specific priming. Forward primers are designed so that the 3' end hybridizes to a known polymorphism (usually single nucleotide polymorphism (SNP), which can be named as a normal sequence or a mutated one), and reverse primers are designed normally. Therefore, allele determination requires two PCR reactions (one for each allele) for the same genomic DNA, combining both the amplification and diagnostic steps accurately, simply, and rapidly.

As illustrated in figure2.1 , three primers are utilized in ARMS: a common primer (C), a normal specific primer (N), and a mutation specific primer (M). The mutant

primer, coupled with a common primer, is used in one PCR reaction. In parallel, the corresponding normal primer coupled with a common primer is used in another PCR reaction. Exponential amplification of PCR product requires DNA synthesis to be initiated from both the forward and reverse primers. And extension of the forward primer cannot occur unless the 3' end is correctly hybridized to the target sequence. Which means that each primer should be very specific to amplify a particular allele. The presence of an amplification product in the first reaction indicates the presence of the mutation, while its absence suggests presence of the normal DNA sequence at that specific site. In the second reaction, however, the presence of an amplified product suggests presence of a normal DNA sequence at that specific site, while its absence suggests presence of the mutation (HUGO Lab notes, 2004; Najmabadi etal, 2001; Li etal,1998).

Figure2.1 : ARMS-PCR



2.3.1. ARMS-PCR Procedure:

As detailed in table 2.3, genomic DNA was amplified in a total volume of 25µl reaction mixture containing the following:

200-500ng of template DNA, (for the first 58 samples, 400ng DNA was used. Then, it was found that 200ng DNA was enough, so this amount was used for all the remaining samples), with 0.15µg of each primer (Invitrogen Life Technologies, 093018) (* All the commercially synthesized primers used here, and for all the other polymorphisms are listed in table 2.2), 2.5µl of 10X PCR buffer (TaKaRa Bio Inc., Code No. R001A. Components: 100mM Tris HCl (pH 8.3), 500mM KCl, and 15mM MgCl2), 2µl dNTPs (TaKaRa Bio Inc: 2.5mM of each dNTP, pH 7-9), and 0.625U of Taq polymerase (TaKaRa Taq TM, Code No. R001a) after completing the total volume up to 25µl with sterile distilled water (Birzeit-Palestine Pharm.Co.).

The mixture was then overlaid with one drop of mineral oil (Promega, Catalog # DY1151) to prevent vaporization.

Finally, the tubes were placed in an automatic thermal cycler (Gene Amp® PCR system 9700), so that the PCR amplification was performed according to the following conditions: One cycle of initial denaturation at 98º C for 2 minutes, followed by 32 cycles of second denaturation at 98º C for 20 seconds, annealing at 56º C for 30 seconds, and extension at 72º C for 30 seconds, followed by a final extension cycle at (72º C) for 10 minutes.

* The number of cycles and annealing temperatures and all conditions of all the PCRs for all the polymorphism tested, are outlined in table 2.4.

* Negative control was used by putting TE buffer (10Mm Tris-HCL,pH 8.0 and 1 mM EDTA) , instead of DNA, in the mix.

* The PCR products (207 bp) of the so called " Normal " primer were denoted "C", indicating the C677 polymorphism , while the PCR products (197bp) of the so called "Mutant" primer were denoted "T", indicating the T677 polymorphism.

* In order to be accurate in reading the results, the PCR of every sample giving a negative or questionable result in any allele was repeated and read another time.

Table 2.2: Primers used in all PCR Protocols:

Gene	Polymorphism	Primer type	5'-3' Primer Sequence	Product size (bp)	Reference
MTHFR	C677T polymorphism	Normal	CTCCCTCTCTGA AGGAGAAGGTGT CTGCGG	207	George Endler
		Mutated	GAAGGAGAAGG TGTCTGCGGAAG T	197	
		Common	AGGACGGTGCGG TGAGAGTG		
VDR	Fok-1 polymorphism	Sense (Forward)	AGCTGGCCCTGG CACTGACTCTG	250	Tofteng etal, 2002
		Antisense (Reverse)	TCTTCTCCCTCCC TTTCCACTG		
	Bsm-1 polymorphism	Forward	AACCAAGACTAC AAGTACCGCGTC AGTGA	820	Tofteng etal, 2002
		Reverse	AACCAGCGGGAA GAGGTCAAGGG		

Table 2.3: ARMS-PCR procedure

Reagents			PCR mix/1 sample	
			Tube 1 (N)	Tube 2 (M)
1.	Template DNA		200-400 ng	200-400 ng
2.	Primers 0.15 Mg/Ml	Normal (N)	1 µl	_
		Mutated (M)	_	1 µl
		Common (C)	1 µl	1 µl
3.	10x PCR buffer		2.5 µl	2.5 µl
4.	dNTP mixture (2.5 mM each)		2 µl	2 µl
5.	Taq polymerase (5u/Ml)		0.125 µl (0.625U)	0.125 µl (0.625U)
6.	Sterile water		up to 25 µl	up to 25 µl
Total volume			25 µl	25µl

Table 2.4: PCR Conditions for All polymorphisms

PCR Conditions	MTHFR-C677T		VDR (For Bsm-I)		VDR (for Fok-I)	
Initial denaturation	98º C, 2min		94 C, 2min		94 C, 5min	
2nd denaturation	98º C,20sec		94º C,30sec		95º C, 1min	
annealing	56º C,30sec	32 cycles	60º C,45sec	30 cycles	53º C,1min	30 cycle
extension	72º C,30sec		72 ºC,1min		72º C,2min	
Final extension	72º C, 5min		72º C, 10min		72º C, 5min	

2.3.2. Agarose Gel Electrophoresis:

ARMS products were separated on horizontal 2% (w/v) agarose gels, prepared consisting of 2g agarose (GIBCO, Invitrogen Corporation, Cat No. 15510-019) in 100L 1x Tris Acetate EDTA (TAE) working buffer (10X: 48.4g Tris base (Applichem, M=121.14g/mol), 11.42ml glacial acetic acid (CAT No. 5550020, m.w.=60.05), 20ml of 0.5M EDTA (pH8) up to 1L D.W.).

After boiling the mixture, and allowing it to cool (~60ºC), 10µl ethedium bromide(10mg/ml) was added to the agarose solution, and mixed thoroughly. Then, the warm agarose was poured evenly into a tray containing 2 combs to produce wells when the gel was set. After approximately 15 min, the tray was placed into an electrophoresis tank (Bio-Rad, sub-cell®GT) filled with 1X TAE gel loading buffer. The combs were lifted up carefully, and PCR products were loaded into the gel as follows:

-3µl of loading dye(Blue/Orange Loading dye,6X,Promega,Cat No.G1881: 0.03%bromophenol blue,0.03%Xylene cyanol FF,0.4%orange G,15%Ficoll 400,10mM Tris-HCl (pH8)) was mixed with 10µl PCR product, and loaded into each well.

-For size determination, a ladder marker was loaded by mixing 1µl of a 100 bp DNA ladder(Invitrogen Life Tech., Cat.No.15628 – 019) with 3µl loading dye, and 9 µl TE buffer, giving bands at 1000, 900, 800, 70, 600, 500, 400, 300, 200,& 100 bp.

-Gels were allowed to run at 120 volt for approximately 20-45 minutes.

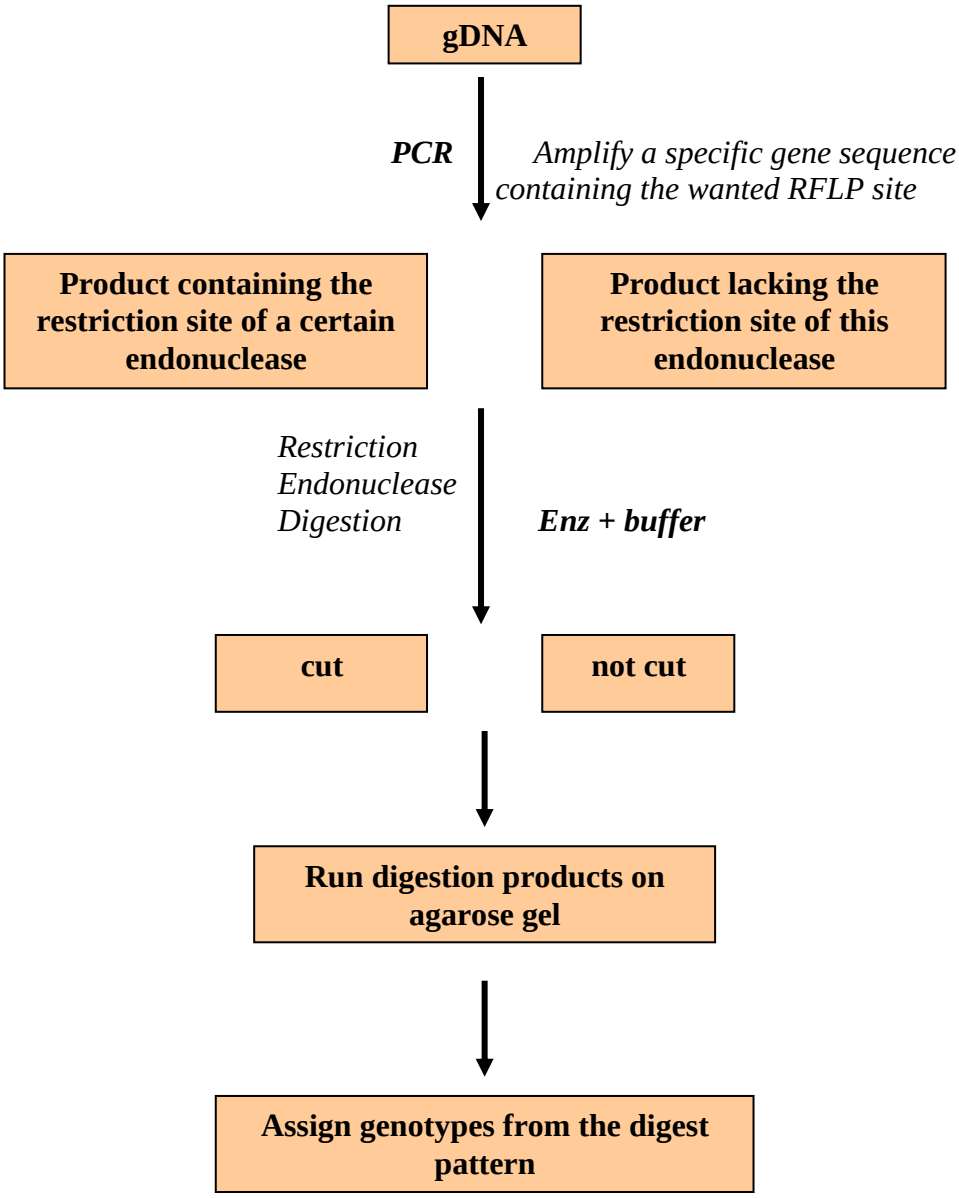
-After electrophoresis, gels were examined and photographed with a Polaroid camera (Pharmacia Biotech, Image Master VDS).

2.4. VDR gene Polymorphisms Screening

- PCR-Restriction Fragment Length Polymorphism (PCR-RFLP) was used for the screening of the VDR gene polymorphisms (Bsm-1 and Fok-1-RFLPs).

- This method is based on the principle that treatment of DNA fragments by a restriction enzyme will show an aberrant pattern if polymorphisms exist in restriction sites. This aberrant pattern can be detected by ethedium bromide staining during agarose gel electrophoresis (Tauata etal, 2000). Thus, it is a useful diagnostic tool to distinguish between homozygous and heterozygous alleles, as shown in the schematic representation below:

Fig 2.2 : Schematic representation of RFLP-PCR



2.4.1. Bsm-I RFLP Screening

2.4.1.1. PCR Procedure:

Amplification of intron 8 of the VDR gene (containing the Bsm-1 restriction enzyme site) was performed in 30 µl of reaction mixtures, containing: 400 ng of

genomic DNA, with 150 ng of each reverse and forward primers (see table 2.2) , 3µl of 10X PCR buffer (TaKaRa Bio Inc,Code No.R001A), 2µl dNTPs (TaKaRa Bio Inc, containing 2.5mM of each dNTP), and 0.625U of Taq polymerase (TaKaRa Taq™,Code No.R001A) ,after completing the total volume up to 30 µl with sterile distilled water (Birzeit-Palestine Pharm. Co.). To prevent vaporization, the tubes were overlaid with 1drop mineral oil (Promega, Cat.No.DY1151),and finally they were put in an automatic thermal cycler (Gene Amp® PCR system 9700) according to the conditions described in table 2.4: One cycle of initial denaturation at 94ºC for 2 minutes, 32 cycles of second denaturation at 94ºC for 30 seconds, annealing at 60ºC for 45 seconds, and extension at 72ºC for 1 minute , followed by a final extension cycle at 72ºC for 10 minutes.

*In each run, a negative control was used by putting TE buffer (10Mm Tris-HCL, pH 8 and 1mM EDTA) in the mix instead of genomic DNA.

2.4.1.2. Agarose Gel Electrophoresis for PCR Products:

All PCR products (820 bp) were viewed on ~2%(w/v) agarose gels (prepared, loaded, and photographed as described in section II.3.2.) in order to determine successful amplification.

2.4.1.3. Bsm-I Enzyme Digestion Procedure:

The PCR products were digested with Bsm-I as follows:

*For the first 152 samples, 10 µl of PCR products were cleaved by adding a 5µl mix containing 1.5 µl of 10X Bsm-I buffer, 5U (0.5µl) Bsm-I enzyme (Roche, Cat.

No.1292307, containing 200 units(10U/µl)), and 3 µl of sterile distilled water (Birzeit-Palestine Pharm.Co.).

*For all samples from 153 up to 345, the volume of the digestion reaction was minimized (after testing the new volume on previously tested samples, and obtaining the same results), so that 8µl of PCR products were cleaved by adding a mix of 1µl 10X Bsm-I enzyme buffer, 0.3 µl of Bsm-I enzyme(Roche, Cat. No. 1292307), and 2 µl sterile distilled water.

*The digestion reaction, for all the samples, took place in a water bath (Unitronic-OR) at 65ºC for 1 hour.

2.4.1.4. Agarose Gel Electrophoresis for the Digested PCR Products:

* Restriction fragments were visualized by electrophoresis through 1.6%(w/v) agarose gels stained with 10µl Ethedium Bromide(10mg/ml), and loaded as follows:

10 µl of the digested products was mixed with 3 µl of Blue/Orange loading dye (6X,Promega,Cat.No. G1881),and loaded into each well.

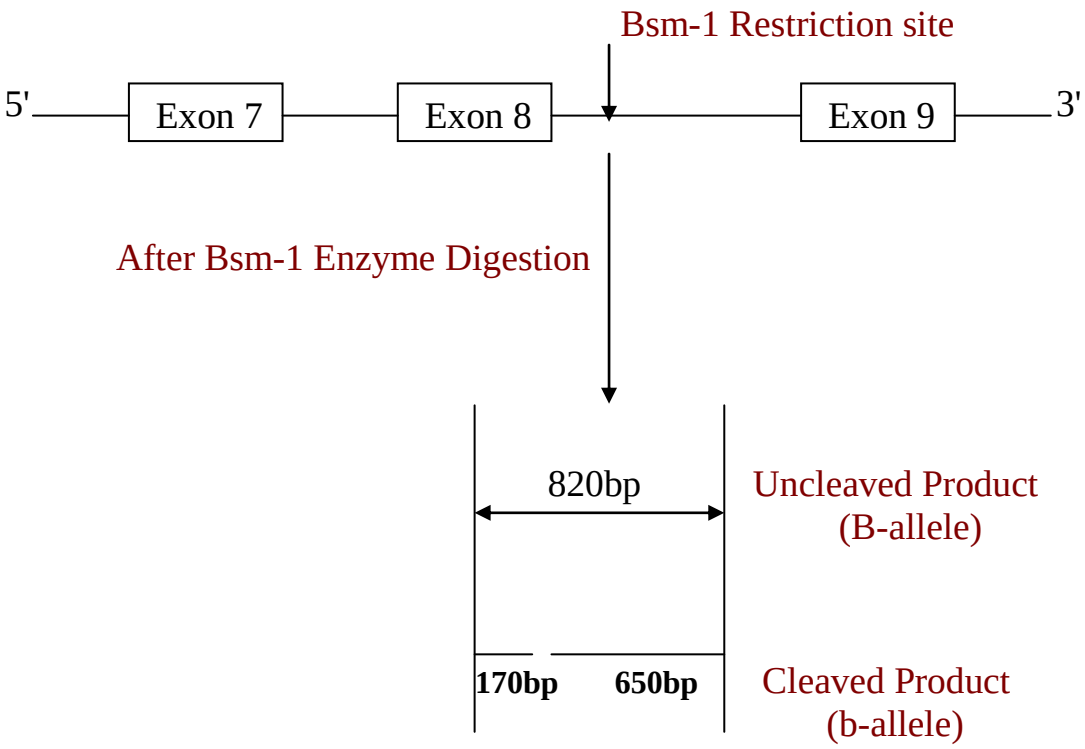
For size determination , a ladder marker was loaded by mixing 1µl of a 100 bp DNA ladder (Invitrogen Life Technologies,Cat. No.15628 - 019) with 3µl loading dye and 9 µl TE buffer.

* All gels were run at 120 volt , then photographed with a Polaroid camera (Pharmacia Biotech., Image Master VDS).

* The uncleaved PCR products of ~820 bp were denoted "B" , and the presence of the Bsm-I restriction site (cleaved to 650 bp & 170 bp) was denoted "b", as illustrated in figure 2.3.

Figure 2.3

Schematic Diagram Demonstrating the Bsm-I restriction site (Restriction Pattern for PCR products)



2.4.2. Fok-I RFLP SCREENING

2.4.2.1. PCR Procedure:

Exon 2 of the VDR gene (containing the restriction site of the Fok-I endonuclease) was amplified in 30 µl of reaction mixtures, containing 400 ng of genomic DNA, with 150 ng of each reverse and forward primers (see table 2.2), 3µl of 10X PCR buffer (TaKaRa Bio Inc.,Code No. R001A), 2µl dNTPs (TaKaRa Bio Inc.), 0.625 U of Taq Polymerase (TaKaRa Taq™,Code No.R001A) ,and the final volume of 30µl was completed with sterile distilled water (Birzeit-Palestine Pharm.Co.) . After overlaying with one drop of mineral oil (Promega, Cat.No. DY1151), each tube was placed in the automatic thermal cycler (Gene Amp ® PCR system 9700) according to the conditions described in table 2.4 :

One cycle of initial denaturation at 94ºC for 5 minutes , followed by 30 cycles of second denaturation at 94ºC for one minute , annealing at 53ºC for 1 minute , and extension at 72ºC for 2 minutes , then followed by a final extension cycle at 72ºC for 5 minutes.

* In each run , a negative control was used by putting TE buffer in the mix instead of genomic DNA.

2.4.2.2. Agarose Gel Electrophoresis for the PCR Products:

In order to determine successful amplification, all PCR products (250 bp) were viewed on ~ 2% (w/v) agarose gels which were prepared,loaded, and photographed as described in section II.3.2.

2.4.2.3. Fok-I Enzyme Digestion Procedure:

All PCR products were digested with Fok-I as follows:

10 µl of PCR products were cleaved with 5U Fok-I (Promega, Cat.No. R6781, 10u/µl) , after 3 hours incubation in a water bath (memmert) at 37ºC.

2.4.2.4. Agarose Gel Electrophoresis for Digested PCR Products:

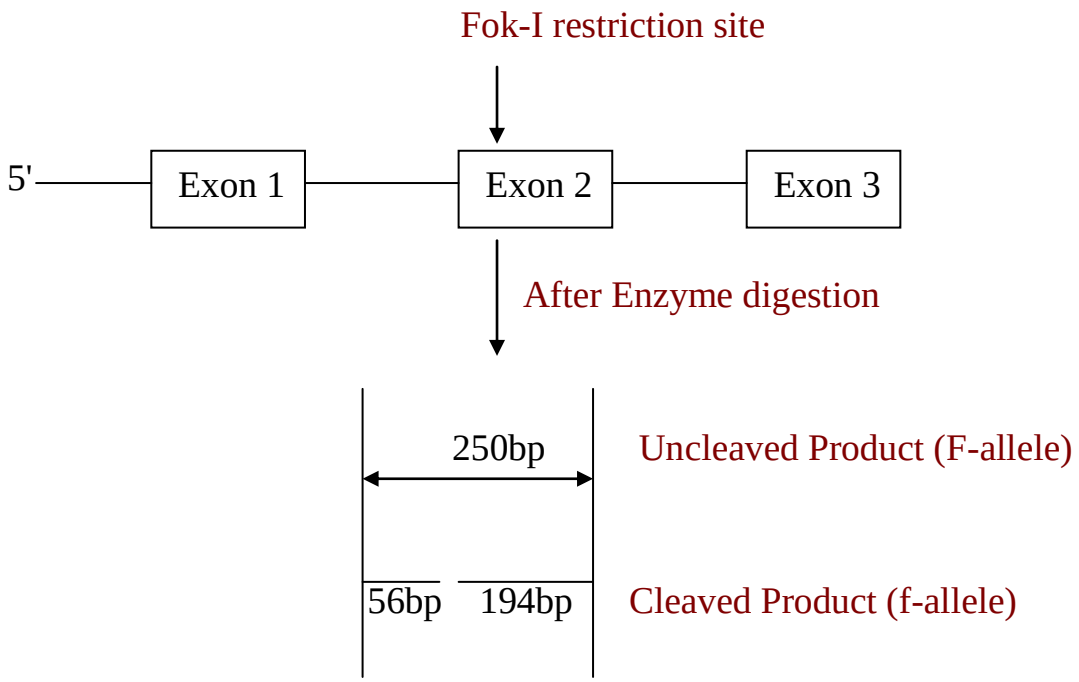
* Size separation of the digested PCR products was performed on 3%(w/v) agarose gels stained with 10 µl Ethedium Bromide(10 mg/ml), by mixing the digested products with 3µl of Blue/orange loading dye (6X, Promega, Cat.No. G1881) , and loading them in each well. Size determination was achieved by using a 100 bp DNA ladder (Invitrogen Life Technologies, Cat.No.15628 – 019) , loaded after mixing 1µl of it with 3 µl loading dye and 9 µl TE buffer.

* Gels were run at 120 - 150 volt , then photographed with a Polaroid camera (Pharmacia Biotech., Image Master VDS).

* The uncleaved products of 250 bp were denoted "F", while alleles having the Fok-I restriction site (and thus cleaved to 56 bp & 194 bp) were denoted "f". (Figure 2.4)

Figure 2.4

Schematic Diagram Demonstrating the Fok-I restriction site (Restriction Pattern for PCR products)



2.5. Statstical Analysis:

Using the standard SPSS package for windows, the frequency distribution of all genotypes and other patients' parameters was determined. Chi square tests were performed for testing the various polymorphic distributions of MTHFR and VDR genes.

The subjects were classified into three groups according to their genotype. Comparison of BMD and other factors among these three groups were performed using analysis of variance (ANOVA), followed by Scheffe's post hoc test. When the differences in the BMD and other factors were tested between two groups, statistical analysis was performed using independent samples' T- test. A p-value less than 0.05 was accepted as the value of significance.

Chapter (3)

Results

3.1. DNA Quantification :

Spectrophotometrically , the following readings were observed:

In all samples, protein concentration was between 0.1 and 1.3mg/ml, the 260/280 ratio was between 1.730 and 1.985, and the DNA concentration was approximately between 0.1 and 1.3 µg/µl, except for one sample, whose ratio and DNA concentration were 1.3 and 0.022 µg/µl respectively.

3.2. MTHFR ARMS-PCR Results:

According to the ARMS-PCR results, MTHFR genotypes in the individual samples were denoted: CC,CT,or TT as shown in a representative gel below (figure 3.1):

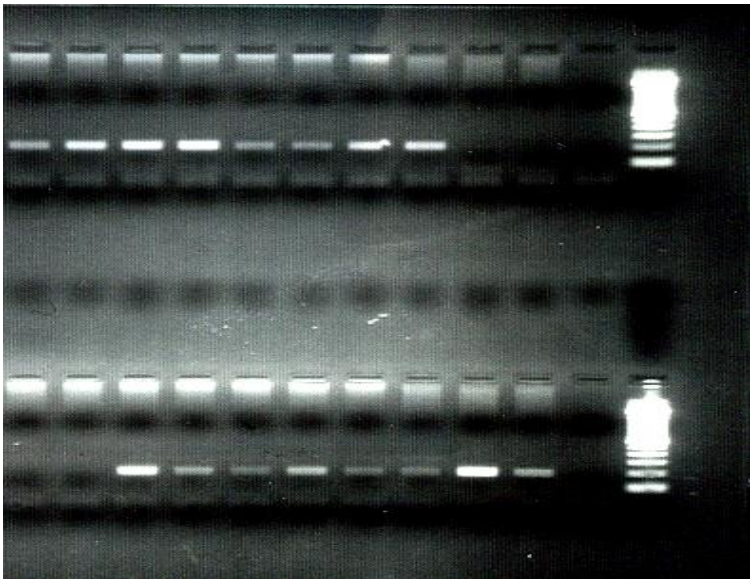


Figure (3.1):Agarose gel electrophoresis for the ARMS PCR of the MTHFR gene:

- lanes (1-11) upper : represent bands of PCR reactions with the normal primer (C allele polymorphism).

- lanes (1-11) lower : represent bands of PCR reactions with the mutated primer (T allele polymorphism).

-Lanes 11(upper and lower): are negative controls.

-Lanes 12(upper & lower): are DNA ladder markers.

-The genotype results for samples (1-10) are as follows:

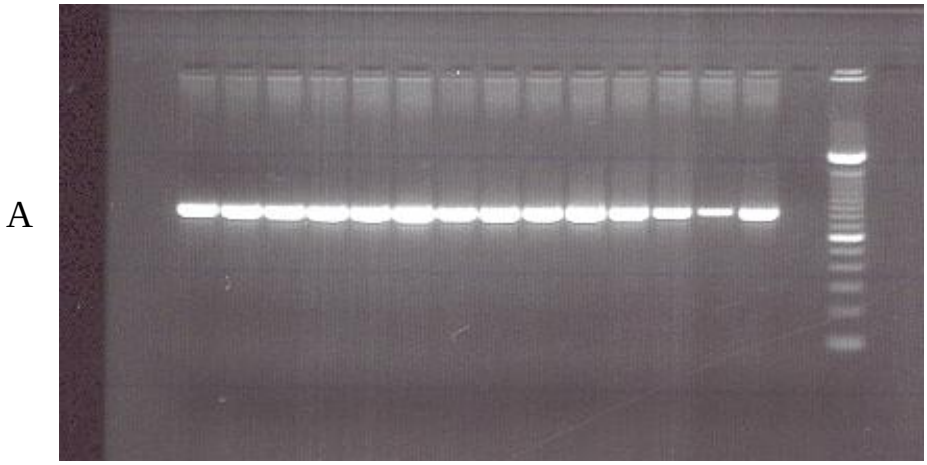
CC,CC,CT,CT,CT,CT,CT,CT,TT,TT.

3.3 VDR Gene RFLP Results :

3.3.1 Bsm-I RFLP Results:

3.3.1.1. Bsm-I PCR Gel Products' Results:

The two gels below (figure 3.2) show representative Bsm-I PCR products (820bp). These amplification bands were then followed by enzyme digestion:



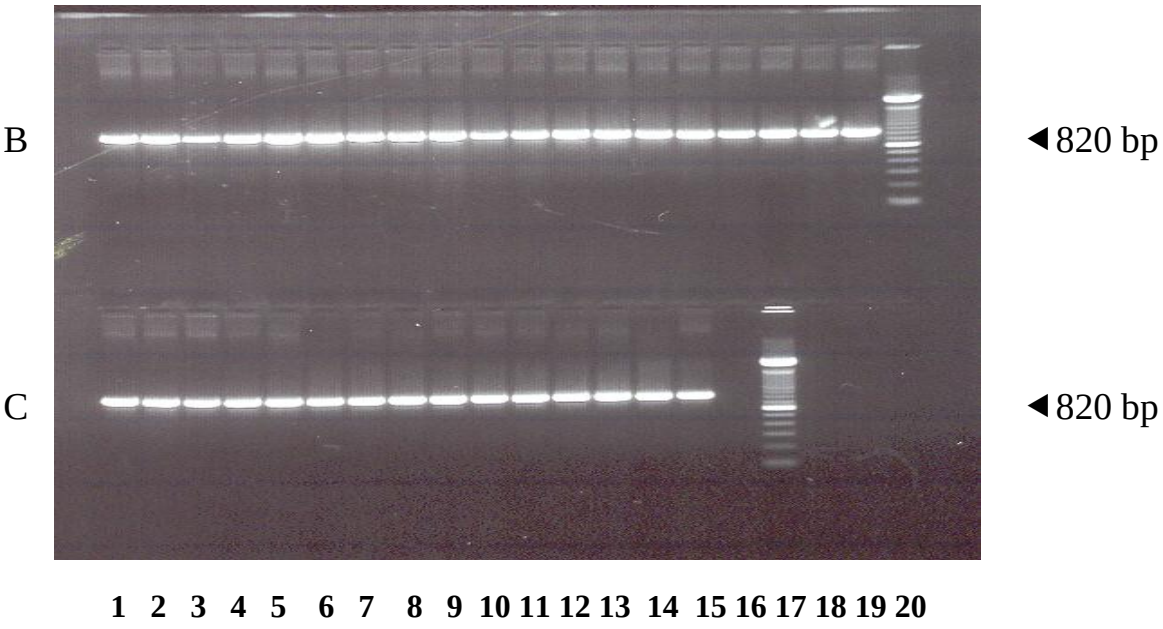


Figure 3.2:Some of the Bsm-I PCR Products:

* Lanes (1-14)A,(1-19)B,and (1-15)C are samples, giving the bands of 820bp size.

* Lanes 15A & 16C reprersent negative controls.

* Lanes 16A, 20B, & 17C are DNA ladder markers.

3.3.1.2. Results of Bsm-I - Digested PCR Products:

According to the gel results, the Bsm-I polymorphisms' genotypes were denoted: BB, Bb, or bb.

Figures (3.3) & (3.4) demonstrate the Bsm-I digestion products results for the amplified samples.

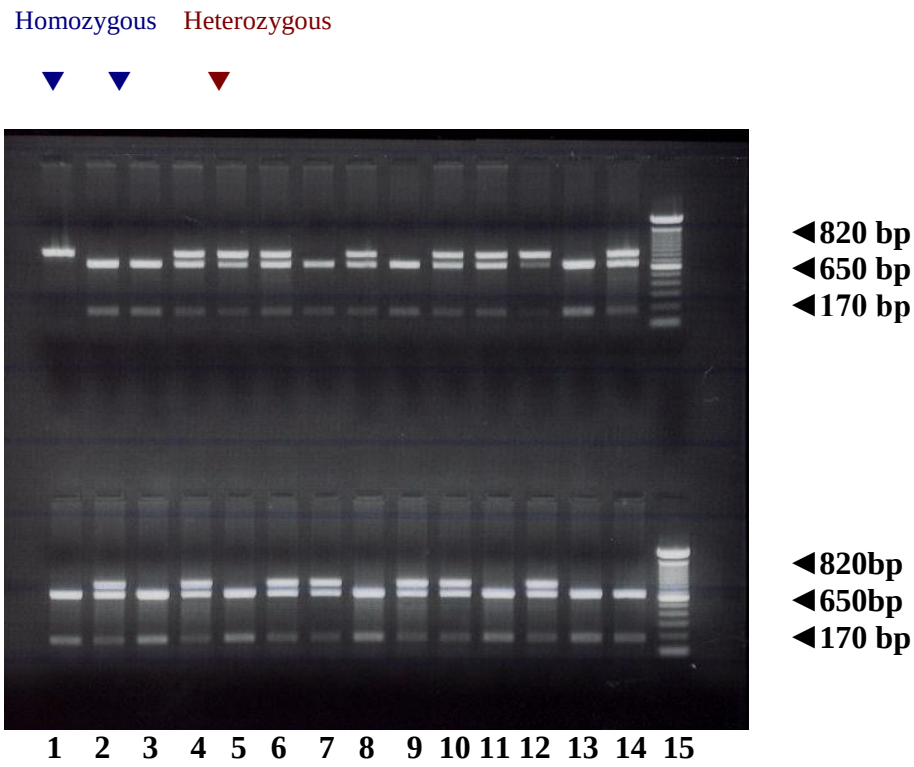


Figure 3.3: Results of Bsm-I-Digested PCR Products:

-The upper and lower (1-14) lanes :are samples.

-Lanes 15: are DNA ladders.

-If the DNA gives only one band (820 bp)-as in the upper lane 1 , this means that the two alleles don't contain the Bsm-1 restriction site, and thus denoted as"BB".

-If two bands appeared (650bp & 170bp)-as in the upper lanes 2+3, this means that the two alleles contain the Bsm-I reatriction site, and thus denoted as"bb".

-If three bands appeared (820bp, 650bp, &170bp) - as in the upper lanes 4+5+6, then this sample is heterozygous, containing one cut allele and one noncut allele, and thus denoted as"Bb".

-Accordingly, the genotype results of the samples shown above are:

BB, bb, bb, Bb, Bb, Bb, bb, Bb, bb, Bb, Bb, Bb, bb, Bb in the upper lanes.

bb, Bb, bb, Bb, bb, Bb, Bb, bb, Bb, Bb, bb, Bb, bb,bb in the lower lanes.

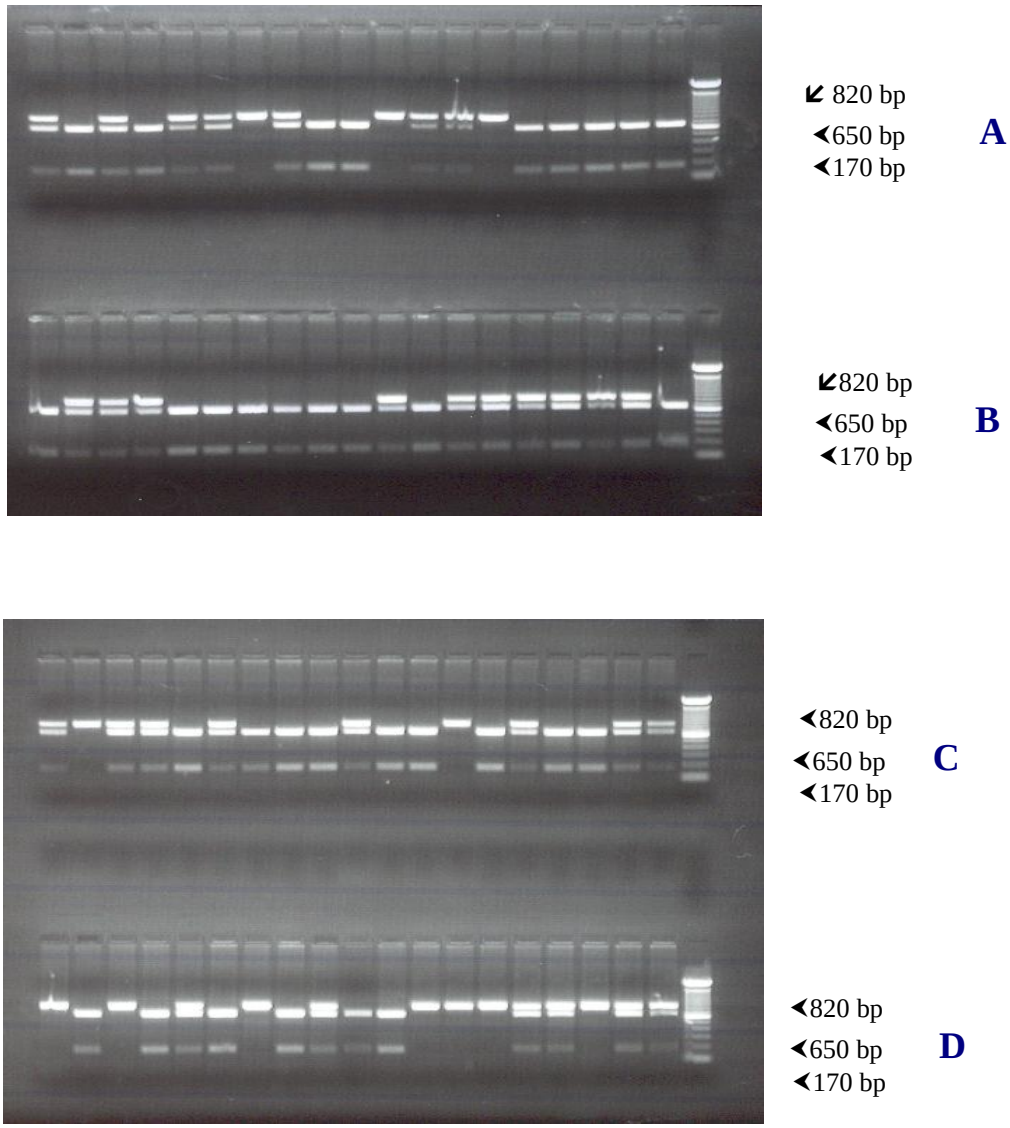


Figure 3.4: Results of Bsm-I digestion of amplified PCR fragments For 76 of the Samples:

* The results were read as followed:

Row A: Bb,bb,Bb,bb,Bb,Bb,BB,Bb,bb,bb,BB,Bb,Bb,BB,bb,bb,bb,bb,bb

Row B: bb,Bb,Bb,Bb,bb,bb,bb,bb,bb,bb,Bb,bb,Bb,Bb,Bb,Bb,Bb,Bb,bb

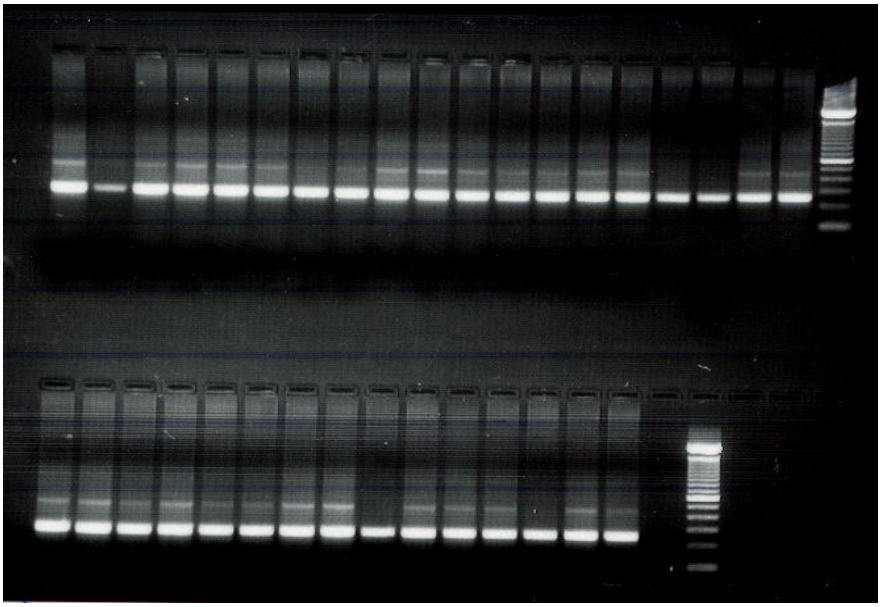
Row C: Bb,BB,Bb,Bb,bb,Bb,bb,bb,bb,Bb,bb,bb,BB,bb,Bb,bb,bb,Bb,Bb

Row D: BB,bb,BB,bb,Bb,bb,BB,bb,Bb,bb,bb,BB,BB,BB,Bb,Bb,BB,Bb

3.3.2. Fok-I RFLP Results:

3.3.2.1. Fok-I PCR Gel Products' Results:

The Fok-I PCR products are 250 bp. The amplified products were then digested by Fok-I enzyme. Two of the PCR gels are shown in figure (3.5). In addition to the samples PCR products, each gel contains a negative control and a DNA ladder marker . Nonspecific bands appeared,but these bands had no effect on interpreting the results:



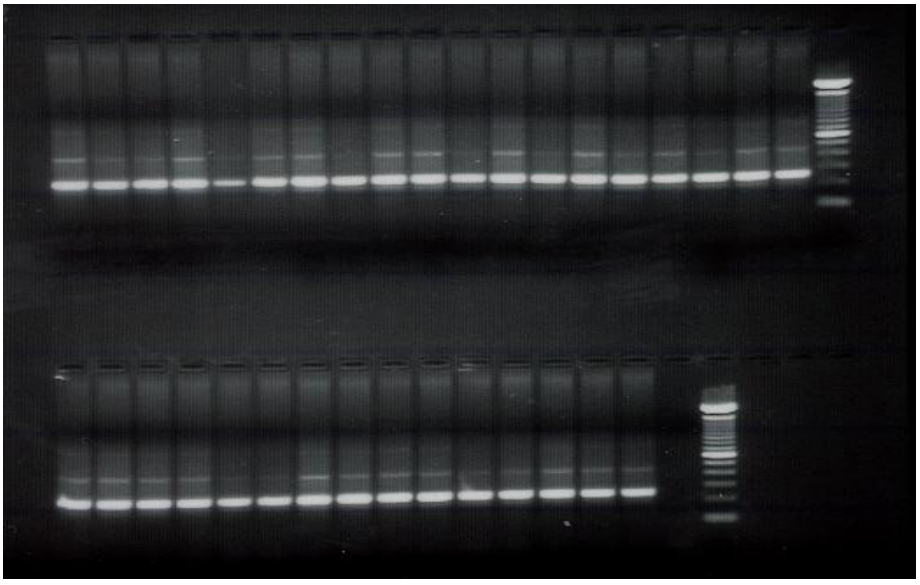


Figure 3.5: Products of Fok-I Digestion of amplified PCR fragments:

* The last lane of each row is a DNA ladder.

* The 34 samples of each gel gave a 250 bp band ,ending with a negative control.

3.3.2.2. Gel Results for Fok-I - Digested PCR Products:

The three genotypes of the Fok-I polymorphisms are denoted: FF, Ff, or ff, and demonstrated in figure (3.6) .

Figures 3.7 & 3.8 show the results of nine and fourteen of the samples respectively.

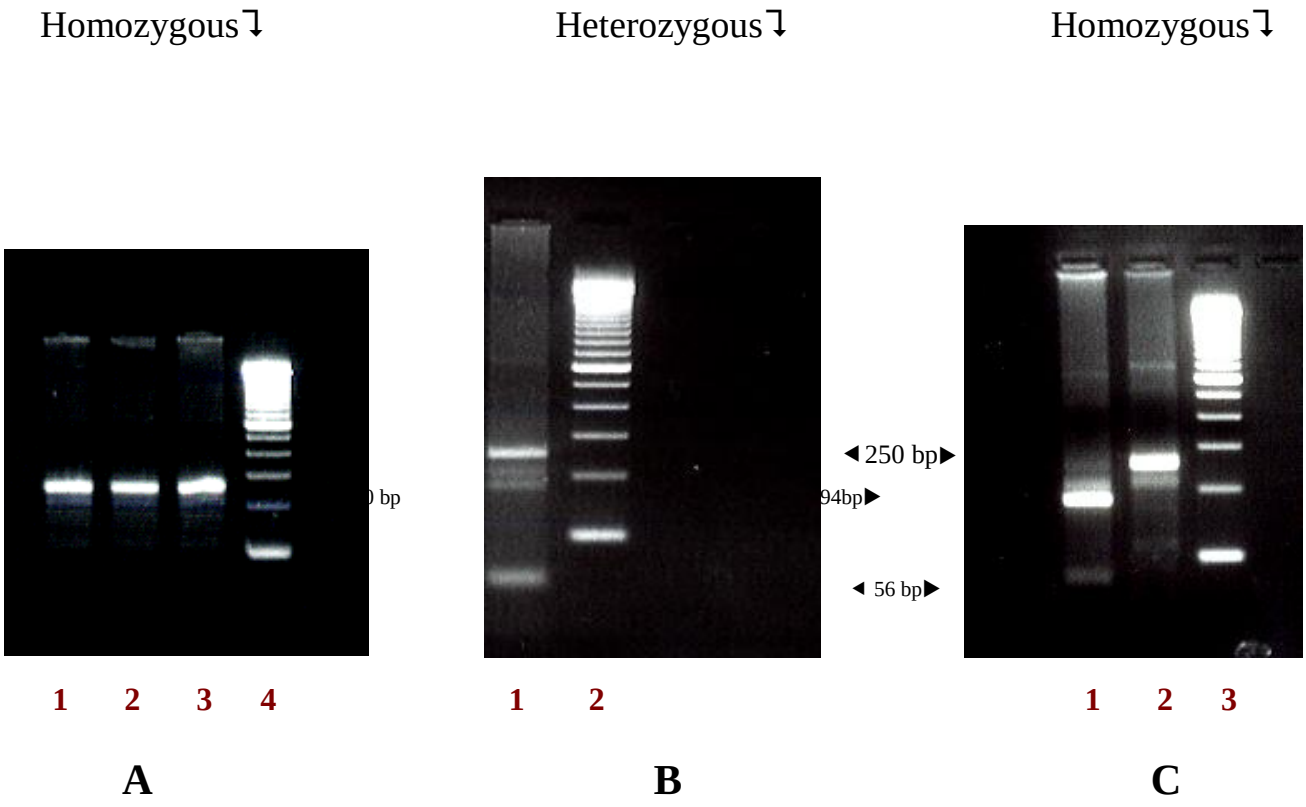


Figure 3.6: Reading the Fok-I Polymorphisms' Genotypes:

* The lanes 4A, 2B, and 3C are DNA ladders.
* The uncleaved products of 250 bp were denoted "FF", as in 1A, 2A, 3A, and 2C.
* The totally cleaved products_giving the two bands:194 bp & 56 bp were denoted "ff", as in 1C .
* The products giving three bands (as 1B) are heterozygous, denoted "Ff".
* The band appearing between 250 & 194 is a nonspecific band, which didn't affect the reading of the results.

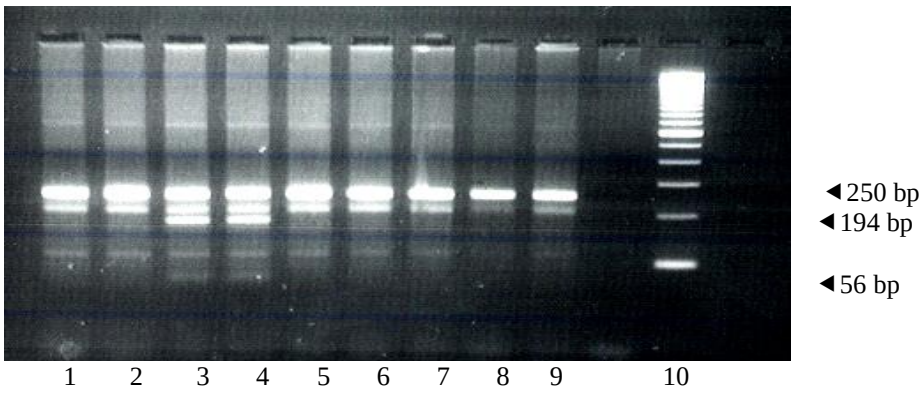


Figure 3.7:

Agarose Gel Electrophoresis Result For 9 Samples After Fok-I Digestion:

The samples' genotypes (1 to 9) were denoted: FF, FF, Ff, Ff, FF, FF, FF, FF, FF.

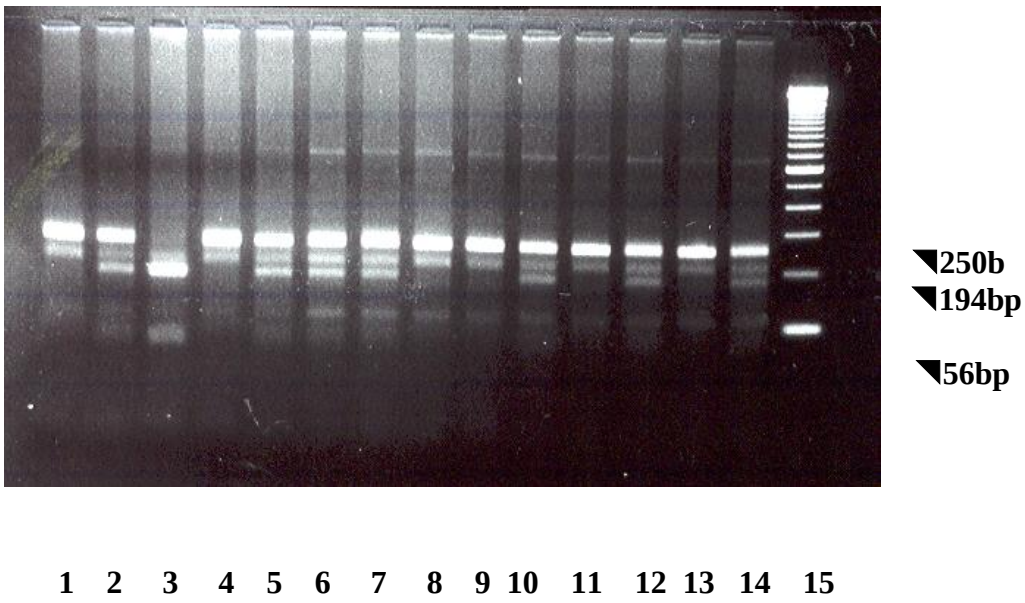


Figure 3.8:Agarose Gel Electrophoresis Result For 14 Fok-I Digested Samples:

According to the gel, the samples genotypes (from 1 to 14) were read as:

FF, Ff, ff, FF, Ff, Ff, Ff, FF, FF, Ff, FF, Ff, FF, Ff.

3.4. Genotype Distribution of MTHFR & VDR Genes' Polymorphisms:

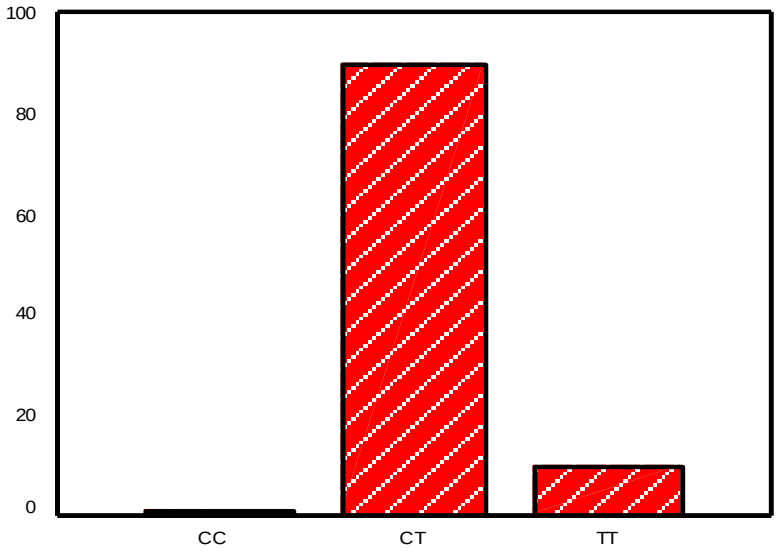
3.4.1. Among The Whole Population:

* Figures 3.9, 3.10, and 3.11 show three tables - each followed by a histogram - demonstrating the genotypic or allelic frequency of MTHFR, Bsm-I, and Fok-I polymorphisms respectively: In all the 345 tested samples, it is noticed that among the Bsm-I genotypes, Bb had the highest frequency (Bb=47%) in this population. On the other hand, the FF genotype had the highest frequency among the three Fok-I genotypes (FF= 56.5%). However, the most noticeable result is in MTHFR, where the majority of the population were heterozygous (CT= 89.3%) , some had the TT homozygous genotype (TT=9.9%), while there was a relative lack of the CC genotype (only 0.9%).

*Cross-genotyping was done between the MTHFR, the Bsm-I, and the Fok-I polymorphisms (figures 3.12, 3.13, and 3.14). However, no significant association in distribution was observed between any of these genotypes ($p > 0.05$, Chi square tests).

Figure 3.9: The frequency of MTHFR genotypes among all the entire, tested samples:

MTHFR Genotype	Frequency	Percent
CC	3	0.9%
CT	308	89.3%
TT	34	9.9%
Total	345	100%



MTHFR Polymorphisms

Figure 3.10: The Frequency of Bsm-1genotypes among the entire tested samples:

Bsm-I Genotype	Frequency	Percent
BB	58	16.8%
Bb	162	47.0%
bb	125	36.2%
Total	345	100%

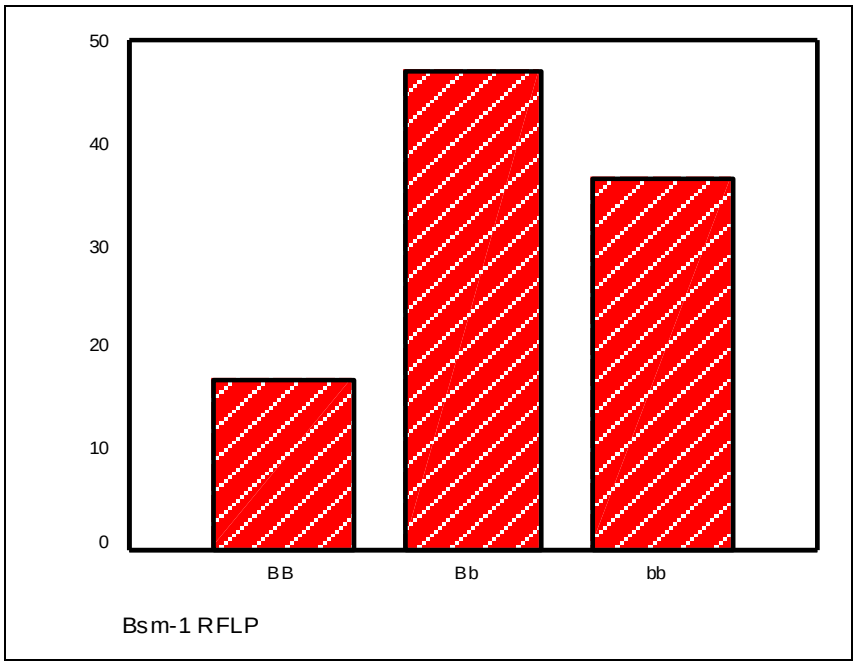


Figure 3.11: The Frequency of Fok-I genotypes among the entire tested samples

Fok-I Genotype	Frequency	Percent
FF	195	56.5%
Ff	125	36.2%
ff	25	7.2%
Total	345	100%

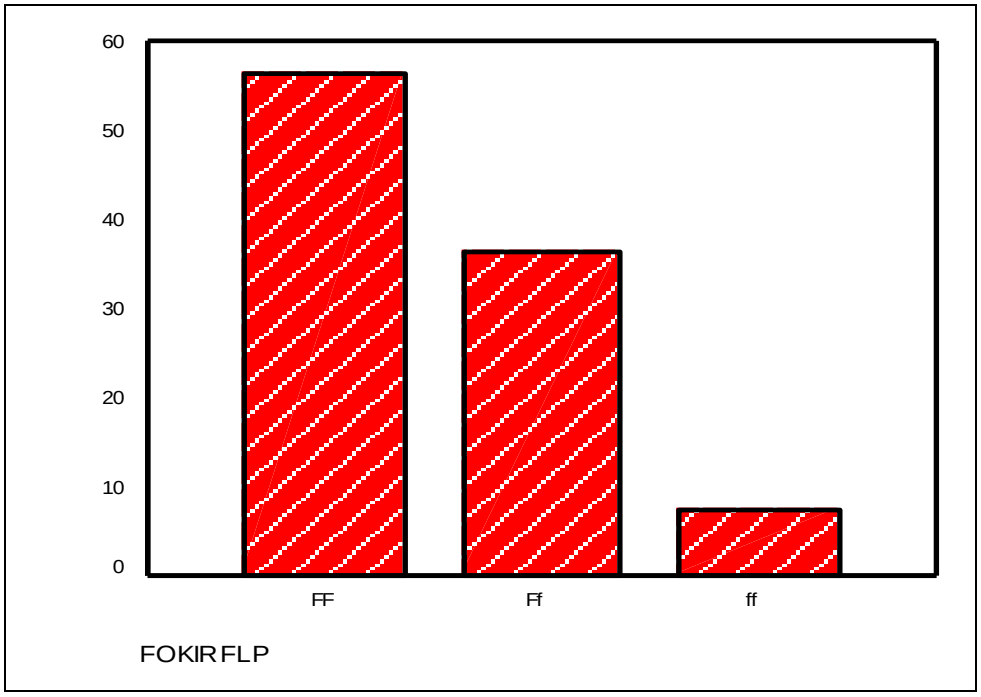
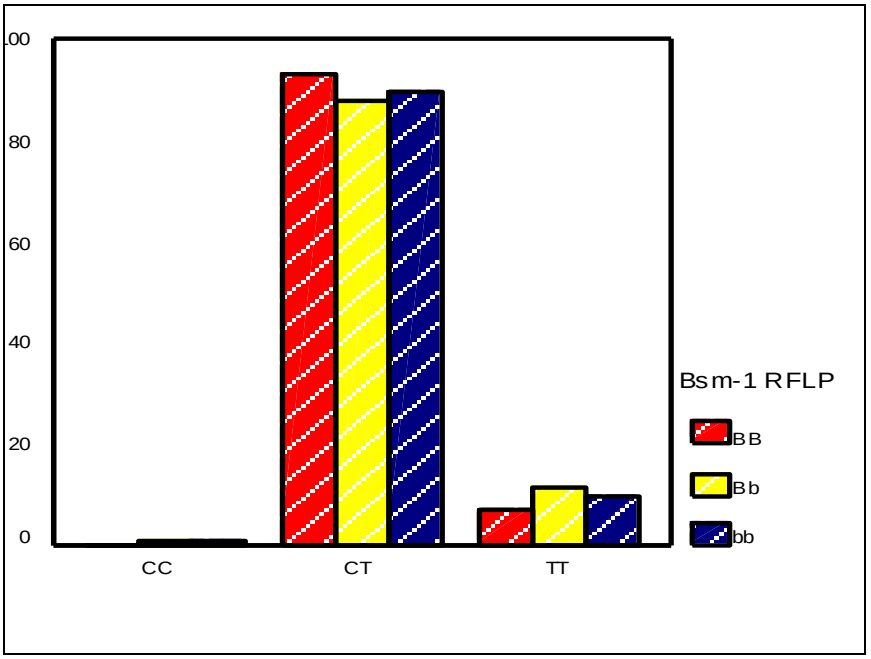


Figure 3.12:Cross- Genotyping of MTHFR with Bsm-I & Fok-I Polymorphisms



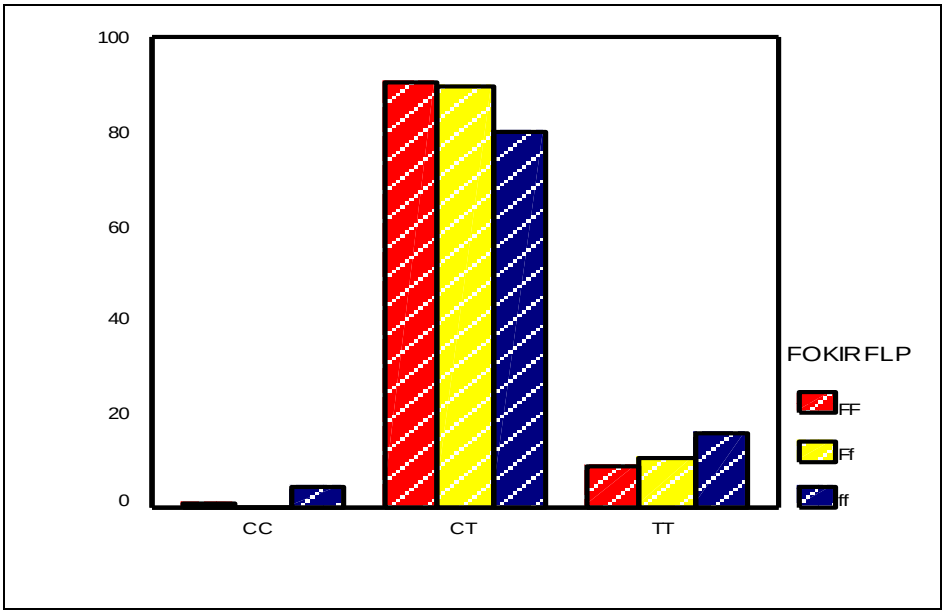
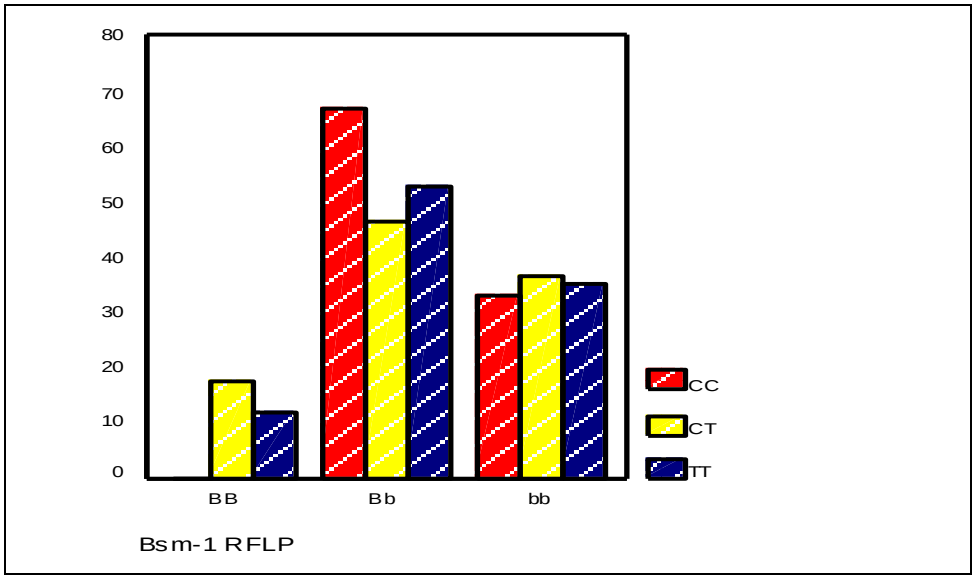


Figure 3.13: Cross-Genotyping of Bsm-I with MTHFR & Fok-I



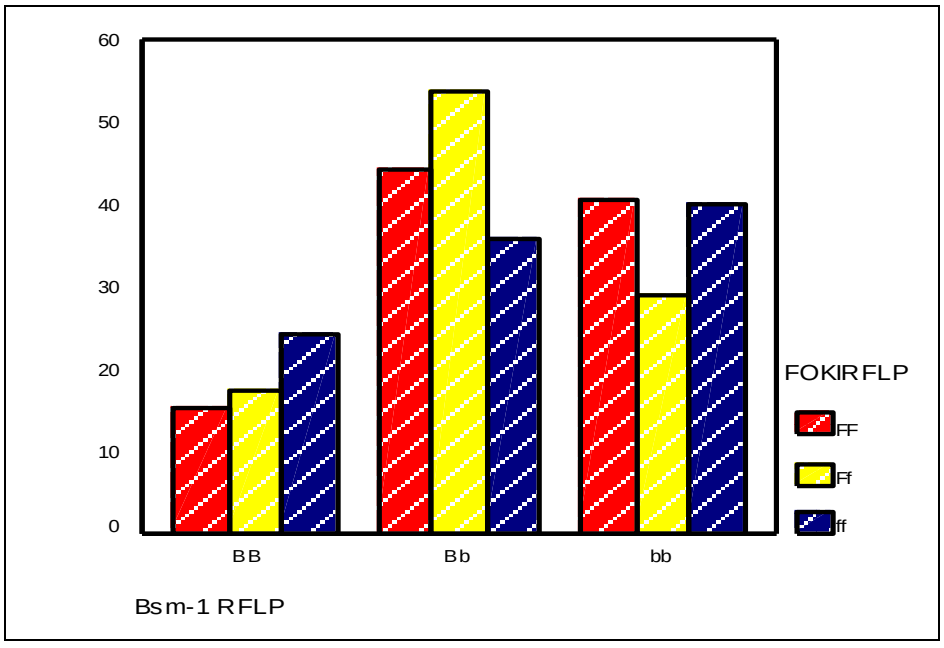
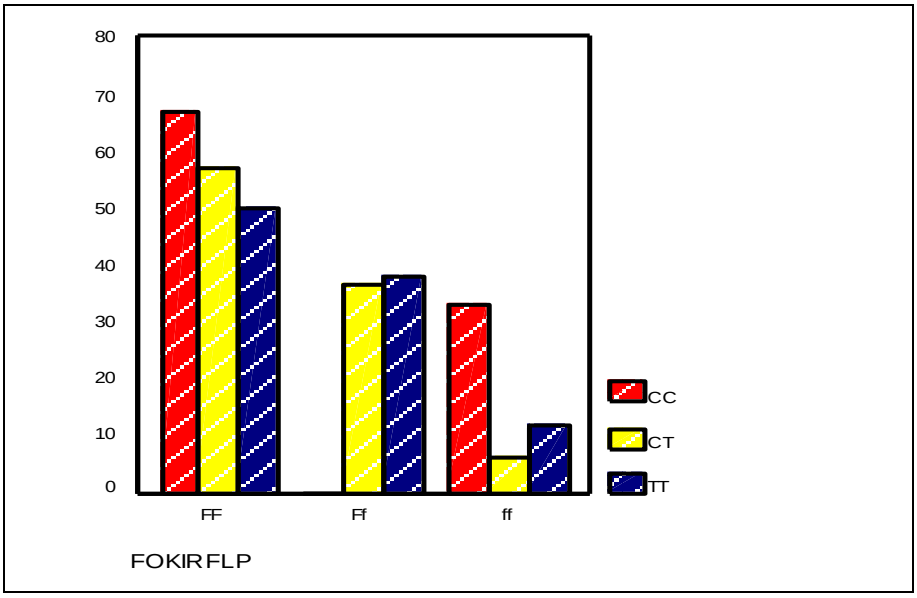
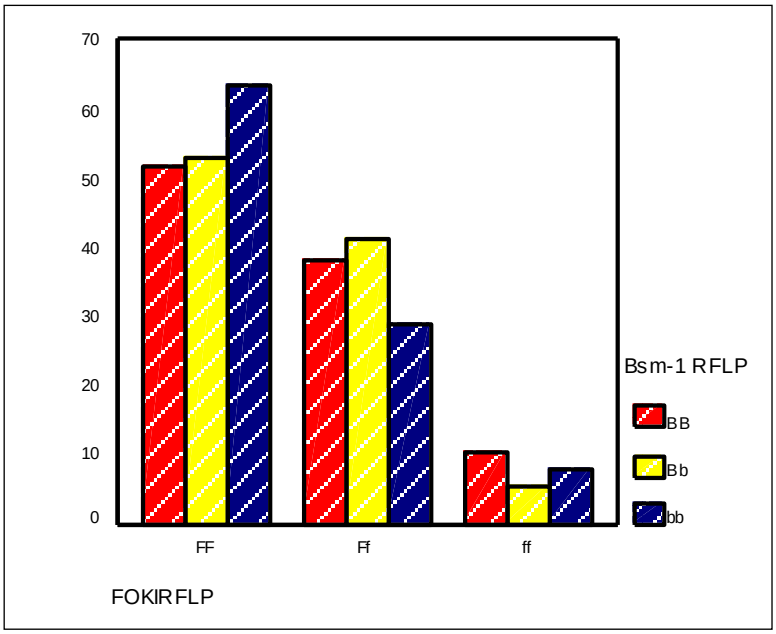


Figure 3.14: Cross Genotyping of Fok-I with MTHFR & Bsm-I





3.4.2. Among the Osteoporotic, Osteopenic, and Normal Cases:

The tables below (tables(3.1) to (3.9))describe the various genotypic distribution of the VDR and MTHFR genes' polymorphisms among the three cases – classified as osteoporosis, osteopenia, or normal according to their measured BMD in either the hip, spine, or femoral neck skeletal sites.

The numbers shown in these tables give us a descriptive image. However, significant associations with BMD, after statistical analysis to these data, are shown in the next sections .

Table 3.1: The Distribution of **MTHFR** Gene Polymorphism Among **Hip** Osteoporotic, Osteopenic, & normal Cases

Hip Fracture Risk		MTHFR genotype			Total
		CC	CT	TT	
osteoporosis	No.	-	52	5	57
	% within hip fracture risk	0%	91.2%	8.8%	100.0%
	% within MTHFR genotype		16.9%	14.7%	16.5%
osteopenia	No.	-	153	10	163
	% within hip fracture risk	0%	93.9%	6.1%	100.0%
	% within MTHFR genotype		49.7%	29.4%	47.2%
normal	No.	3	103	19	125
	% within hip fracture risk	2.4%	82.4%	15.2%	100.0%
	% within MTHFR genotype	100.0%	33.4%	55.9%	36.2%
Total	No.	3	308	34	345
	% within hip fracture risk	0.9%	89.3%	9.9%	1 00.0%
	% within MTHFR genotype	100.0%	100.0%	100.0%	100.0%

Table 3.2: **Bsm-I** Genotypic Distribution Among **Hip** Osteoporotic, Osteopenic, & Normal Cases

Hip Fracture Risk		Bsm-1 RFLP			Total
		BB	Bb	bb	
osteoporosis	No.	11	32	14	57
	% within hip fracture risk	19.3%	56.1%	24.6%	100.0%
	% within Bsm-1 RFLP	19.0%	19.8%	11.2%	16.5%
osteopenia	No.	3C	73	60	163
	% within hip fracture risk	18.4%	44.8%	36.8%	100.0%
	% within Bsm-1 RFLP	51.7%	45.1%	48.0%	47.2%
normal	No.	17	57	51	125
	% within hip fracture risk	13.6%	45.6%	40.8%	100.0%
	% within Bsm-1 RFLP	29.3%	35.2%	40.8%	36.2%
Total	No.	58	162	125	345
	%within hip fracture risk	16.8%	47.0%	36.2%	100.0%
	% within Bsm-1 RFLP	100.0%	100.0%	100.0%	100.0%

Table 3.3: **Fok-I** Genotypic Distribution Among **Hip** Osteoporotic, Osteopenic, & Normal Cases

Hip Fracture Risk		FOKI RFLP			Total
		FF	Ff	ff	
osteoporosis	No.	34	20	3	57
	% within hip fracture risk	59.6%	35.1%	5.3%	100.0%
	% within FOKI RFLP	17.4%	16.0%	12.0%	16.5%
osteopenia	No.	97	54	12	163
	% within hip fracture risk	59.5%	33.1%	7.4%	100.0%
	% within FOKI RFLP	49.7%	43.2%	48.0%	47.2%
normal	No.	64	51	10	125
	% within hip fracture risk	51.2%	40.8%	8.0%	100.0%
	% within FOKI RFLP	32.8%	40.8%	40.0%	36.2%
Total	No.	195	125	25	345
	% within hip fracture risk	56.5%	36.2%	7.2%	100.0%
	% within FOKI RFLP	100.0%	100.0%	100.0%	100.0%

Table 3.4: The Distribution of **MTHFR** Gene Polymorphism Among **Spinal** Osteoporotic, Osteopenic, & Normal Cases

Spinal Fracture Risk		MTHFR Genotypes			
		CC	CT	TT	Total
osteoporosis	No.		116	10	126
	% within spinal fracture risk		92.1%	7.9%	100.0%
	% within MTHFR Genotype		37.8%	29.4%	36.6%
osteopenia	No.		118	6	124
	% within spinal Fracture risk		95.2%	4.8%	100.0%
	% within MTHFR Genotype		38.4%	17.6%	36.0%
normal	No.	3	73	10	94
	% within spinal Fracture risk	3.2%	77.7%	19.1%	100.0%
	% within MTHFR Genotype	100.0%	23.8%	52.9%	27.3%
Total	No.	3	307	34	344
	% within spinal Fracture risk	0.9%	89.2%	9.9%	100.0%
	% within MTHFR Genotype	100.0%	100.0%	100.0%	100.0%

Table 3.5: **Bsm-I** Genotypic Distribution Among **Spinal** Osteoporotic, Osteopenic, & Normal Cases

Spinal Fracture Risk		Bsm-1 RFLP			Total
		BB	Bb	bb	
Osteoporosis	No.	28	55	43	126
	%within spinal fracture risk	22.2%	43.7%	34.1%	100.0%
	% within Bsm-1 RFLP	48.3%	34.0%	34.7%	36.6%
Osteopenia	No.	15	63	46	124
	% within spinal fracture risk	12.1 %	50.8%	37.1%	100.0%
	% within Bsm-1 RFLP	25.9%	38.9%	37.1 %	36.0%
Normal	No.	15	44	35	94
	% within spinal fracture risk	16.0%	46.8%	37.2%	100.0 %
	% within Bsm-1 RFLP	25.9%	27.2%	28.2%	27.3%
Total	No.	58	162	124	344
	% within spinal fracture risk	16.9%	47.1%	36.0%	100.0%
	% within Bsm-1 RFLP	100.0%	100.6%	100.0%	100.0%

Table 3.6: Fok-I Genotypic Distribution Among **Spinal** Osteoporotic, Osteopenic, & Normal Cases

Spinal Fracture Risk		FOKI RFLP			Total
		FF	Ft	ff	
osteoporosis	No.	72	42	12	126
	% within spinal fracture risk	57.1%	33.3%	9.5%	100.0%
	% within FOKI RFLP	36.9%	33.9%	48.0%	36.6%
osteopenia	No.	68	50	6	124
	% within spinal fracture risk	54.8%	40.3.%	4.8%	100.0%
	% within FOKI RFLP	34.9%	40.3%	24.0%	36.0%
normal	No.	55	32	7	94
	% within spinal Fracture risk	58.5%	34.0%	7.4%	100.0%
	% within FOKI RFLP	28.2%	25.8%	28.0%	27.3%
Total	No.	195	124	25	344
	% within spinal fracture risk	56.7%	36.0%	7.3%	100.0%
	% within FOKI RFLP	100.0%	100.0%	100.0%	100.0%

Table 3.7

The Distribution of **MTHFR** Gene Polymorphisms Among the **Femoral Neck** Osteoporotic, Osteopenic, & Normal Cases

Femoral Neck Risk		MTHFR Genotypes			Total
		CC	CT	TT	
osteoporosis	No.		94	4	98
	% within neck risk		95.9%	4.1%	100.0%
	% within MTHFR Genotype		30.5%	11.8%	28.4%
osteopenia	No.	1	146	16	163
	% within neck risk	0.6%	89.6%	9.8%	100.0%
	% within MTHFR genotype	33.3%	47.4%	47.1%	47.2%
normal	No.	2	68	14	84
	% within neck risk	2.4%	81.0%	16.7%	100.0%
	%within MTHFR genotype	66.7%	22.1%	41.2%	24.3%
Total	No.	3	308	34	345
	%within neck risk	0.9%	89.3%	9.9%	100.0%
	% within MTHFR genotype	100.0%	100.0%	100.0%	100.0%

Table 3.8: **Bsm-I** Genotypic Distribution Among the **Femoral Neck** Osteoporotic, Osteopenic, & Normal Cases

Femoral Neck Risk		Bsm-1 RFLP			Total
		BB	Bb	bb	
Osteoporosis	No.	18	47	33	98
	% within neck risk	18.4%	48.0%	33.7%	100.0%
	% within Bsm-1 RFLP	31.0%	29.0%	26.4%	28.4%
Osteopenia	No.	29	75	59	163
	% within neck risk	17.8%	46.0%	36.2%	100.0%
	% within Bsm-l RFLP	50.0%	46.3%	47.2%	47.2%
Normal	No.	11	40	33	84
	% within neck risk	13.1%	47.6%	39.3%	100.0%
	% within Bsm-l RFLP	19.0%	24.7%	26.4%	24.3%
Total	No.	58	162	125	345
	% within neck risk	16.8%	47.0%	36.2%	100.0%
	% within Bsm-1 RFLP	100.0%	100.0%	100.0%	100.0%

Table 3.9: **Fok-I** Genotypic Distribution Among the Femoral Neck Osteoporotic, Osteopenic, & Normal Cases

Femoral Neck Risk		FOK-I RFLP			Total
		FF	Ft	ff	
osteoporosis	No.	61	32	5	98
	% within neck risk	62.2%	32.7%	5.1%	100.0%
	% within FOKIRFLP	31.3%	25.6%	20.0%	28.4%
osteopenia	No.	94	57	12	163
	% within neck risk	57.7%	35.0%	7.4%	100.0%
	% within FOKIRFLP	48.2%	45.6%	48.0%	47.2%
normal	No.	40	36	8	84
	% within neck risk	47.6%	42.9%	9.5%	100.0%
	% within FOKIRFLP	20.5%	28.8%	32.0%	24.3%
Total	No.	195	125	25	345
	% within neck risk	56.5%	36.2%	7.2%	100.0%
	% within FOKIRFLP	100.0%	100.0%	100.0%	100.0%

3.5. Genetic Association Analysis Results:

3.5.1. MTHFR & VDR Genotypes' Association with BMD:

* Among the **MTHFR** genotypes, the homozygous TT and CC were associated with significantly higher spinal BMD compared to CT (**for TT: p=0.03, ANOVA**; **p=0.009, T-test. For CC: p=0.03, ANOVA**). In the hip or femoral neck, however, no significant BMD differences were observed . Among osteoporotic patients, no significant change in BMD was observed in any skeletal site.

* The BB genotype of the **Bsm-I** site was significantly associated with lower BMD at the total hip than the bb genotype **(p=0.026 , ANOVA)**, while no significant differences appeared at the other skeletal sites. Interestingly, among spinal osteoporotic women, a significant lower BMD was observed in Bb than in bb genotypes (**p=0.016, ANOVA**) (See table 3.10).

Table 3.10 : MTHFR & VDR Genotypes' Association with BMD:

Polymorphism	BMD site	Effect	P-value
MTHFR (C677T)	spine	TT,CC>CT	0.03
VDR (* Bsm-I)	hip	BB<bb	0.026
VDR (Fok-I)	_	_	>0.05

*Among spinal osteoporotic women, a significant lower BMD was observed in Bb than in bb genotypes (p=0.016)

* Considering the **Fok-I** genotypes, there was no significant difference in BMD at any of the three skeletal sites (**p>0.05, ANOVA**) both in all the 344 cases and among the osteoporotic cases.

3.5.2. Gene × Gene Interaction Effects on BMD:

When testing the Cross genotyping effects of the various polymorphisms on BMD, the following results were obtained:

1. Among the Bb individuals, those having the FF genotype had significantly lower femoral neck BMD than those having the Ff (**p=0.027, ANOVA**) or the ff (**p=0.002, ANOVA**) genotypes. Moreover, the BbFF individuals are significantly associated with low hip BMD when compared to BbFf individuals (**p=0.022, ANOVA**).

At the same time, among FF individuals, those having the Bb genotype are significantly associated with low hip (**p=0.011, ANOVA**) and neck (**p=0.007, ANOVA**) BMD than the bb ones .

2. Among ff individuals, there was a significant decrease in hip BMD for those having the BB genotype when compared to Bb genotype (**p=0.04, ANOVA**).

3. TT women having the BB genotype are significantly associated with lower hip BMD when compared to bb (**p=0.005, ANOVA**),or Bb (**p=0.006, ANOVA**) women. Moreover, these TTBB women had significantly lower femoral neck BMD when compared to TTbb (**p=0.02, ANOVA**),or TTBb (**p=0.01, ANOVA**) women.

4. Among Bb women, those having the CT genotype had significant lower hip BMD (**P=0.014**), and significant lower femoral neck BMD (**P=0.005**) than those having the TT genotype.

5. FF individuals having the CT genotype are significantly associated with lower BMD at both the hip (**p=0.033**) and femoral neck (**p=0.013**), than those having the TT genotype.

3.5.3. Gene × Nongenetic Interaction Effects on BMD:

To investigate whether genotype effects are influenced by other environmental & non-environmental factors, the various genotypes were analyzed by grouping women according to years since menopause, height, and weight .

Since only 3 women (from the total 345) had the CC genotype (table3.1), the statistical analysis for the differences in the BMD in the MTHFR gene with nongenetic interactions were tested only between the CT and TT individuals. The results of this analysis are described below :-

3.5.3.1. Gene × Years Since Menopause Effects on BMD:

***Bsm-I × years since menopause:**

After 15 years of menopause, women having the BB genotype had significant lower hip BMD than those having the bb genotype (**p=0.045, ANOVA**). This significant association isn't observed when the years since menopause are < or = 15 years (**p>0.05, ANOVA**).

***Fok-I × years since menopause:**

No significant difference in BMD was observed with years since menopause between the three Fok-I genotypes at any of the skeletal sites (**p>0.05, ANOVA**).

***MTHFR × years since menopause:**

During the first 20 years of menopause, CT women had significant lower BMD at the spine (**p=0.010**), hip (**p=0.023**), and femoral neck (**p=0.017**) than TT women. On the other hand, after more than 20 years since menopause, no significant differences in BMD are observed between these two genotypes (**p >0.05**).

3.5.3.2. Gene × Weight Effects on BMD:

***Bsm-I × weight :**

Among individuals weighing 80 Kg or more, those having the BB genotype had significant lower hip BMD than the bb genotype (**p=0.03, ANOVA**). This significant difference, however, isn't observed in individuals weighing < 80 Kg (**p>0.05, ANOVA**).

***Fok-I × weight :**

No significant difference in weight-adjusted BMD is observed among the Fok-I genotypes at any skeletal site.

***MTHFR × weight :**

CT women weighing 70 Kg or more are found to be associated with significant decrease in BMD at both hip (**p=0.027**) and spine (**p=0.041**), as compaired to TT women having the same weight. However, this decrease in BMD isn't found in CT

women weighing less than 70 Kg, and surprisingly in women weighing 90 Kg or more.

3.5.3.3. Gene × Height Effects on BMD :

Women were classified in five groups according to their height. However, significant associations appeared at 150 and 160 cm height as follows:

***Bsm-I × height :**

Women whose height was 150 cm or more and having the BB genotype, were significantly associated with lower hip BMD than did the bb women having the same height (**p=0.025, ANOVA**). Indeed,this significant association disappeared in women less than 150 cm height (**P>0.05, ANOVA**). Moreover, BB women with height of 160 cm or more, had significantly lower spinal BMD than did the bb ones (**p=0.009, ANOVA**).

***Fok-I × height :**

Short FF women (< 150 cm height), had significantly lower femoral neck BMD than ff women (**p=0.031, ANOVA**), while those with 150cm or more didn't show any effect on BMD **(P>0.05, ANOVA).**

***MTHFR × height :**

Significant differences appeared only in tall women, of 160cm height or more: If they had the CT genotype, their BMD was significantly lower at the spine (**p=0.028**),hip (**p=0.045**),and femoral neck (**p=0.011**) than those of TT genotype.

3.5.3.4. Gene × Height & Weight Effects on BMD :

After combining the effects of height and weight together, with the genotypic influence, the following interesting results are obtained (table 3.11):-

Table 3.11: Gene × Height & Weight Association with BMD:

Polymorphism	Height (cm)	Weight (kg)	BMD site	effect	P-value
Bsm-I	>=160	>=80	spine	BB , Bb<bb	0.00 for BB 0.008 for Bb
	<150	<80	hip, f.neck	Bb<bb	0.033 for hip 0.014 for f.neck
		>=80	hip	BB<Bb	0.034
Fok-I *					
MTHFR	<160	<60	f.neck	CT<TT	0.032
	>=160	>=70 <80	spine	CT<TT	0.000
		>=90	hip spine f.neck	CT<TT	0.002 for hip 0.044 for spine 0.002 for f.neck

* *No high significant findings were found, FF(<160 cm, >=70 Kg) < ff (f.neck,p=0.045)*

*Bsm-I × height & weight:

The interesting observation is with BB, or Bb tall women whose height is 160 cm or more . If women weigh 80 Kg or more, their spinal BMD is significantly lower than bb women with the same height and weight (**p=0.00 for BB, p=0.008 for Bb, ANOVA**). Nothing is observed on BB or Bb tall women (>= 160 cm) weighing less than 80 Kg , nor on BB or Bb women less than 160cm height. However, very short women (< 150 cm height), weighing less than 80 Kg, also had a significant lower BMD at the hip (**p=0.033, ANOVA)** and femoral neck

(**p=0.014, ANOVA**) if they had the Bb genotype, when compared to bb genotype at the same conditions. If they had the BB genotype, however, and their weight was 80 Kg or more, their hip BMD was significantly lower than those having the Bb genotype of the same height and weight (**p=0.034, ANOVA**).

***Fok-I × height & weight :**

No significant findings were found, except for FF women whose height was less than 160 cm: If these women had a weight of 70 Kg or more, they showed a significant lower femoral neck BMD than those having the ff genotype **(p=0.045, ANOVA).**Fortunately, FF women with the same height, showed nothing if their weight was < 70 Kg **(p > 0.05, ANOVA)**.

***MTHFR × height & weight :**

Women **less than 160 cm** height showed nothing, except when their weight was less than 60 Kg: If they had the CT genotype, their femoral neck BMD was significantly lower than those having the TT genotype (**p=0.032**).

Unfortunately, CT **tall women** with 160 cm or more, weighing 70 Kg or more, showed many possibilities of very significant lower BMD than TT ones. For example:

-If women are less than 80 Kg ,they were highly associated with lower spinal BMD (**p=0.000**). If they are 90 Kg or more, they're associated with lower BMD at the spine (**p=0.044**), hip (**p=0.002**), and femoral neck (**p=0.002**). However, CT short women (with height less than 150 cm) showed nothing , except when they weighed

90 Kg or more. In this case, a significant lower hip BMD was observed when compared to those having the TT genotype (**p=0.042**).

Chapter 4
Discussion

In recent studies, compelling data from different studies indicate that BMD is – at least in part – genetically determined. Morrison and co-workers showed that common allelic variants in the gene encoding the vitamin D receptor ***(VDR)*** can be used to predict differences in bone densities, claiming that the VDR genetic marker accounted for up to 75% of the variation in the heritable component of the peak bone mass (Morrison etal. 1992,1994). At the same time, Miyao et al demonstrated that the allelic polymorphism in the ***MTHFR*** gene was associated with reduced BMD in Japanese women (Miyao etal. 2000). Consequently, the hypothesis that polymorphisms in the VDR gene and MTHFR gene affect BMD raised great interest. Several studies have confirmed the relationship between the VDR or MTHFR genotypes and BMD – although some are contradictory – (Tokita etal.1996, Salamone etal.1996, Houston etal.1996, Chen etal.2001, Kim etal.2002, Jorgensen etal.2002, Bathum etal.2004), while other studies showed negative findings (McClure etal.1997, Eccleshall etal.1998, Graafmans etal.1997, Rauch etal.1997, Correa etal.1999,… and others).

In the present study, we analyzed the *Bsm-I* and *Fok-I* polymorphisms of the VDR gene, in addition to the *MTHFR* polymorphism (C677T), and addressed the question of whether there is association between these polymorphisms and BMD in the Palestinian population. Furthermore, whether this association is affected by

gene-gene interaction, and gene-environmental interaction (including years since menopause, height, and weight) was also analyzed.

*** Genotype Distribution:**

The observed VDR ***Bsm-I*** genotype distributions were -to some extent- similar to those previously reported by Ferrari and co-workers (Ferrari etal,1995), to those reported in the Caucasian (Rizzoli etal,2001), and Danish populations (Tofteng etal.2002). In addition,the VDR ***Fok-I*** genotype distributions observed in our study were nearly in agreement with those reported in the Taiwanese (Chen etal, 2002), and American populations (Harris etal,1997). Considering the ***MTHFR*** genotypic distribution, the majority of the population were heterozygous , some had the TT homozygous genotype, while there was a relative lack of the CC genotype .

*** Genotype Association with BMD:**

Our finding that the BB genotype of the ***Bsm-I polymorphism*** was significantly associated with lower BMD at the total hip than the bb genotype supports the finding by Langdahl and co-workers (Langdahl etal,2000). Since among spinal osteoporotic women, a significant lower BMD was observed in Bb than in bb genotypes which means that once a woman has osteoporosis, the severity of her bone loss will be great if her genotype is Bb. This also agrees with the findings by Kikuchi etal,1999.

After adjustment for age since menopause, weight, and height, we found that in all cases of BMD significant changes, the "B" allele (opposing the "b" allele) is associated with low BMD. This is consistent with many other studies including

Tokita et al.1996, Sainz et al.1997, Krall et al.1995, Vandevyver et al.1997, Viitanen et al.1996, Fleet et al.1995, Geusens et al.1997, Lazaretti-Castro et al.1997, Hauache et al.1998, Kikuchi et al.1999, Ferrari et al.1998 and Gomez et al.1999. These results – however – contrasts other findings by Salamone et al1996, Hauston et al.1996, Tamai et al.1997and Lorentzon et al.2001, where the "B" allele was the one associated with high BMD.

Concerning years after menopause, our data show that after 15 years of menopause, women having the BB genotype had significant lower hip BMD than those having the bb genotype. This is in contrast with those of Vandevyver etal, who showed that the relationship between the Bsm-I VDR gene polymorphism and BMD in the elderly, postmenopausal women, is not as strong as shown by others in young, premenopausal women (Vandevyver etal, 1997). Evidently, our observation is in good agreement with the finding of Krall and co-workers, who observed an association between the VDR genotype with baseline femoral neck BMD in late, but not in early, menopause, where adjusted femoral neck BMD in the BB group was 7% lower than bb and 8% lower than Bb among women in late menopause (10 years). Among women in early menopause (<10 years), femoral neck BMD was similar in all genotypes (Kral etal,1995).

These observations are consistent with the view that genetic factors may have a stronger influence on the achievement of *the peak bone mass* than on the rate of bone loss leading to the hypothesis that in the elderly other factors such as age, years since menopause, weight, height, Ca intake, vitamin D, and exercise are more

important in maintaining the BMD than genetic influences (Vandevyver etal,1997). Testing this hypothesis, we studied VDR genotypes in our subjects, after grouping them according to their weight and height, irrespective of their genotypes, reflecting the fact that in healthy individuals, weight accounts for most of the interindividual variability in bone mass. Very interesting , it became evident for BB, or Bb tall women ,whose height was 160 cm or more, and weigh 80 Kg or more, that their spinal BMD was very much significantly lower than bb women of the same height and weight. Conversely, nothing was observed on BB or Bb tall women weighing less than 80 Kg , nor on BB or Bb women less than 160cm height weighing 80 Kg or more. The apparent conclusion from this finding is that **once a tall woman knows that her genotype is BB or Bb, she must take care not to reach 80 Kg, since this may make her at high risk to develop osteoporosis.**

The observation that other factors can mask the influence of the VDR genotypes on BMD was also shown by Dawson-Hughes and coworkers (Dawson-Hughes etal,1995). They showed that, compared with women with the bb genotype, women with the BB genotype of the VDR gene have reduced calcium absorption efficiency on low calcium intake, consistent with a functional defect in the intestinal VDR. The impact of this heritable difference is reduced at higher calcium intakes (Vandevyver etal,1997). Furthermore, in another study done by Kiel & co-workers , it was concluded that calcium supplementation was associated with greater BMD only for those with the bb genotype. Clearly, the Bsm-I RFLP is in an intron of the VDR gene and is not known to alter the protein product ; however,

there is early evidence that the rate of VDR gene transcription or stability of the transcript may differ according to genotype. While post-transcriptional modification related to the Bsm-I RFLP is possible, additional work is needed to identify the mechanism to explain this finding. At present, one likely interpretation is that the b allele is in disequilibrium with a mutation within the VDR gene which promotes calcium absorption. Further understanding of the biologic mechanisms underlying these genetic differences will help define the role of the VDR gene in the inheritance of BMD (Kiel etal,1997).

Our data concerning the ***Fok-I RFLP*** , showed that there was no significant difference in BMD at any of the three skeletal sites related directly to any of the Fok-I genotypes. This agrees ,to some extent, with the findings done by a number of previous studies as Eccleshaa etal.1998, Correa etal.1999, Lucotte etal.1999, and Ferrari etal. 1998b, and disagrees with others like Harris etal.1997, Gross etal.1996, Aria etal.1997, Ames etal.1999, Lucotte etal.1999,and Gennari etal. 1999), who observed a trend for lower BMD in the f-allele compared to the F-allele. Even after adjustment for years after menopause, weight, and height, no associations were mentioned , except that considering the height alone (short FF women < 150 cm , had significantly lower femoral neck BMD than ff women), or the height and weight together (if FF women, whose height was less than 160 cm had a weight of 70 Kg or more, they showed a significant lower femoral neck BMD than those having the ff genotype (p=0.045, ANOVA)). This trend in BMD

loss contrasts the trend found in other previous studies, although the p-value mentioned here (0.045) isn't highly significant.

This guides us to a conclusion that the Fok-I polymorphism isn't very much significantly associated with BMD. However, cross-genotyping with other polymorphic regions (as mentioned later) may provide a further insight into the complex determination of BMD.

Clearly, the Fok-I polymorphism at the translation initiation site in exon 2 of the VDR gene results in a change in the VDR structure. Since the F- allele lacks the first ATG, the mRNA translation would probably initiate from the second ATG downstream, thus making the F- allele VDR three amino acids shorter than the f-allele VDR. Arai et aI. (1997) have reported that the shorter variant of VDR (F-VDR) exhibited greater transcriptional activity in transfected HeLa cells which may explain higher BMD in FF individuals. Conversely, Gross et al. (1998) did not find functional differences between F-VDR and f-VDR forms. Thus, the molecular mechanism of the start codon polymorphism role remains unclear, and the differential activity of "F" compared with "f "alleles remains to be proven, but the change in VDR amino acid sequence does provide a rational basis from which to consider how the VDR SCP polymorphism might affect BMD(Zajickova et al, 2002).

Our results with the ***MTHFR genotypes***, showed that the homozygous TT and CC were associated with significantly higher spinal BMD compared to CT . Only 3 women (from the total 345) had the CC genotype and are normal. However, since

the number is too small, we cannot draw any conclusion in association with the CC genotype. Therefore, the statistical analysis for the differences in BMD with the MTHFR gene in association with nongenetic interactions were tested only between the CT and TT individuals. Accordingly, different interesting associations became evident : where during early menopause (not exceeding 20 years), CT women had significant lower BMD at the spine, hip, and femoral neck than TT women. Considering weight and height, the risk of developing osteoporosis appeared to be great in tall women of 160 cm or more, weighing 70 Kg or more, and in short women weighing 90 Kg or more.

Clearly, in all MTHFR associations, the TT genotype appeared to be the one associated with higher BMD as compared to the CT genotype. This finding , however , is contradictory with the findings by Miyao et al (2000), and Abrahamsen et al (2003, 2005) who reported an association between the TT genotype and low BMD. It also contradicts the findings of Bathum et al(2004) and Villadsen et al (2004) who reported a significant association between TT genotype and increased risk of fracture. Conversely, our results agrees with those of Macdonald et al. (2004), where participants with the TT genotype (and high riboflavin intake) had higher BMD than the CC and CT groups . Furthermore, our findings agrees ,to some extent, with the study done by Jorgensen etal (2002), in which they reported a reduced risk of fractures in postmenopausal women heterozygous, or homozygous for the T677 polymorphism (although they didn't confirm an association with BMD). There is no clear direct explanation for all

these discrepancies. However, **several factors could be involved.**

First, it is important to note that the studies done by Bathum etal. and Villadsen et al. associated the TT genotype with fracture risk, independent of the BMD. Based on the biological hypothesis, one could argue that the increased risk of fracture is not exclusively mediated through BMD , especially that the effects of the TT genotype on fracture risk exceed those expected from BMD alone (Abrahamsen etal,2005) , but also reduced bone quality may play a role. The TT genotype is associated with elevated levels of circulating homocysteine (p-HCy), and homocysteine is known to interfere with the formation of cross-links in collagen, an essential process in bone formation. The increased fracture risk could therefore be explained by reduced bone strength due to abnormal collagen formation in individuals homozygous for the T- allele, alone or in combination with reduced BMD (Villadsen etal,2004). Furthermore, Jorgensen et al. proposed the hypothesis that the effect of TT genotype on fracture is mediated through factors affecting the risk of falling , independent of the BMD values.(Jorgensen etal,2002).

Second, new studies have drawn attention to a possible role of the vitamin B complex in overcoming the effects of MTHFR genotype on the skeleton. Specifically, the TT genotype may lead to diminished BMD only in the presence of low intake of one or more members of the vitamin B complex. If so, fractures due to MTHFR TT genotype could be preventable by vitamin B supplements (Abrahamsen etal,2005). Interestingly, recent dietary reports from the Aberdeen Osteoporosis Screening Study showed a significant interaction between riboflavin

and MTHFR genotype in determining femoral BMD, where participants with the TT genotype and high riboflavin intake had higher BMD than the CC and CT groups (Macdonald etal,2004).

The total Homocysteine (tHcy) of individuals homozygotic for the T-allele of the MTHFR (C677T) mutation is only about 2 to 2.5 μM higher than the normal mean fasting level of 10 μM. However, several studies have shown that this comparatively small difference in tHcy between the genotypes disappears when the intake of folate is high (Jorgensen etal,2002). If the effect on BMD of the MTHFR (C677T) genotypes found by Miayao et al. is mediated through the level of tHcy, another reason why we got these results could be because of different levels of folate intake between Japanese and Palestinian women.

Third, another possible explanation, is that many of the studied polymorphisms in candidate genes have had different allele frequencies in different populations, in addition to differences in their effect on BMD and fracture risk. This may be an explanation to our results, especially that the genotype of our studied population is 89.3% CT , with only 9.9%TT and 0.9% CC.

Finally, another possible explanation, is that it isn't the C677T polymorphism in the MTHFR gene itself that increases the risk of osteoporosis , but the variants in nearby genes on chromosome 1, which are in linkage disequilibrium with this polymorphism (Villadsen etal,2004).

Interestingly, two recent linkage studies have linked BMD to a region on chromosome 1p36, the same region where the MTHFR gene is situated (Devoto

etal.2001 , Karasik etal.2002).This furthermore points towards an association between the C677T mutation and osteoporosis, although this finding could also be due to linkage disequilibrium with other osteoporosis-related gene nearby (Bathum etal,2004).

It is also very interesting to observe the effect of gene-gene interactions between the three polymorphisms on BMD and concluding that women having the haplotypes FFBb, ffBB, BBTT, CTBb, and CTFF were associated with decreased hip BMD, while FFBb, BBTT, CTBb, and CTFF women were associated with decreased femoral neck BMD. Indeed, this is the first study -worldwide- to cross link the VDR polymorphisms with the C677T-MTHFR polymorphism. Therefore, these results will provide us with a very important tool to test individuals at an early age to identify those with very high risk of osteoporosis based on their genetic background, and help them to modify their life style to minimize the risk of acquiring the disease and decrease its severity to the minimum. However, It should be considered that the functional aspects of the polymorphisms are not clear, and because association studies cannot provide any causal evidence between genotype and phenotype, functional studies are highly needed (Tofteng etal,2002).

In conclusion, this study provides significant associations between the b-allele of the Bsm-I VDR and high BMD. It also demonstrates significant associations between the CT genotype of the MTHFR with decreased BMD compared to the TT genotype. On the contrary, it showes that the Fok-I VDR RFLP alone is a weak predictor of BMD, but gene-gene interactions, and gene environmental

interactions gives many significant association results in predicting BMD. Moreover, additional studies linking interactions with other candidate genes ,and other environmental factors might bring further insights into the complex pathophysiology of a polygenic disease such as osteoporosis.

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