Chapter I

Introduction

1.1 Calcium Sensing Receptor Gene

The calcium-sensing receptor (CaSR) is a class C G-protein coupled receptor Figure (1.1), plays a key role in regulating extracellular fluid calcium levels by its actions on the parathyroid gland and kidney cell (Brown *et al.* 1993; Thompson *et al*. 2005). Activation of the CaSR by increased extracellular ionized calcium inhibits parathyroid hormone (PTH) secretion and promotes urinary calcium excretion (Brown 2002).

The CaSR (based on structural homology of seven transmembrane domains, with related sequences and functions) that includes the glutamate receptors, the gamma-amino butyric acid receptors and the recently described taste receptors and is expressed primarily in the parathyroid cells and cells lining the kidney tubule (Nelson *et al*. 2001; Staszewski *et al*. 2002). It plays a key role in calcium-regulated signaling and is essential in maintaining mineral ion homeostasis (Hendy *et al*. 2000). CaSR is expressed in the developing tooth, and might provide a mechanism for the rapid response to alterations in extracellular calcium concentrations during tooth formation (Mathias *et al*. 2001).

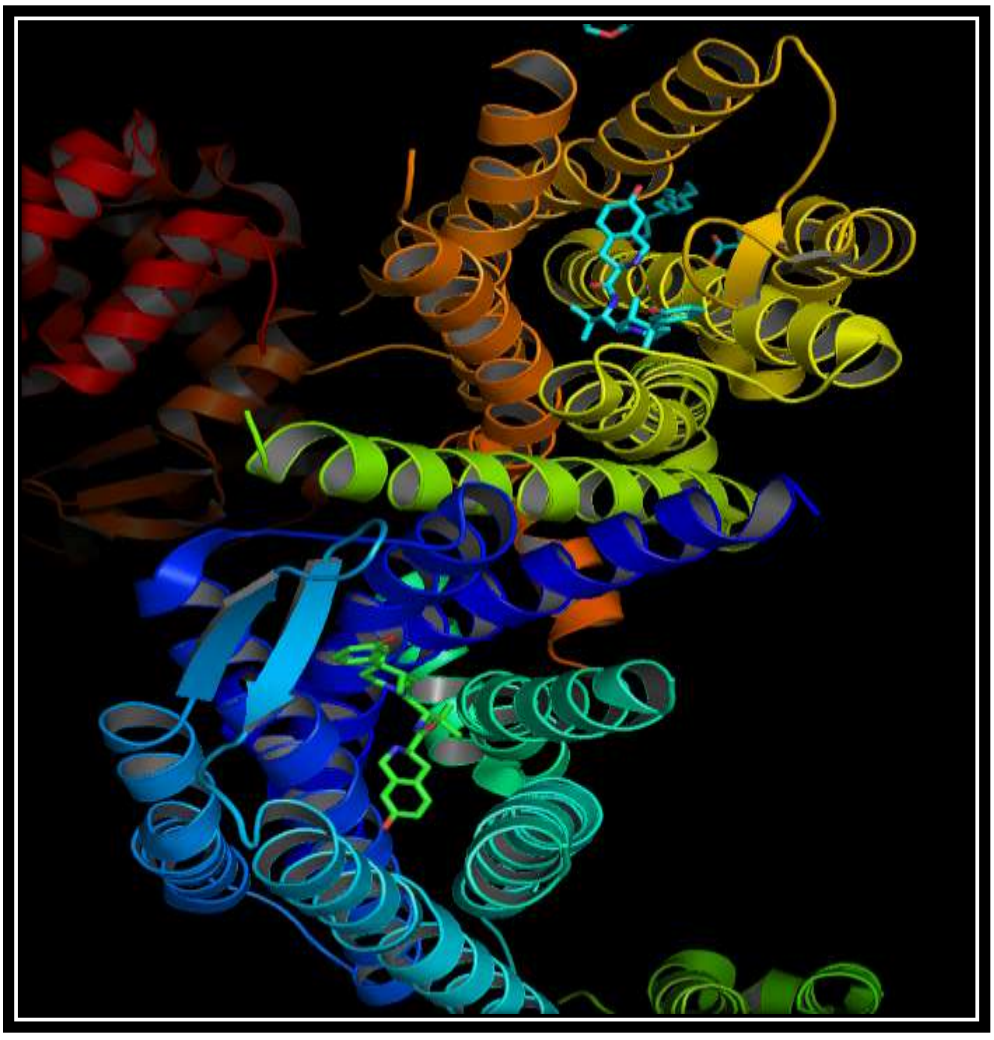

Figure 1.1: Structure of CaSR Protein (Brown *et al.* 1993; Thompson *et al*. 2005).

The function of the CaSR protein is to maintain the serum calcium concentration within a narrow physiological range. It is expressed mainly in the principal parathyroid cells and renal tubular cells, although it has also been described in many other tissues and cells (lung, terminal ileum, colon, adrenal gland, thyroid, osteoclasts). Calcium regulation takes place through two mechanisms: increased PTH secretion by the parathyroid glands and calcium reabsorption in the renal tubules (Hendy *et al*. 2000).

The CASR gene codes a polypeptide of 1078 amino acid (Brown *et al.* 1993) and the CASR gene that maps to 3q13.3-21 (Janicic *et al*. 1995).

Mutations in the human CASR gene have been reported, lead to a range of diseases associated with failure of calcium regulation. Mutations can be the cause of the monogenic diseases familial hypocalciuric hypercalcemia and neonatal severe hyperparathyroidism (Pollack *et al*. 1993). Other inactivating mutations have been found in parathyroid lip hyperplasia (Fukumoto *et al*. 2001) but no evidence for CASR point mutations was found in familial idiopathic hypercalciuria. Furthermore, evidence of a role for the CASR in more complex multigenic diseases. Recently, several single nucleotide polymorphisms in exon 7 in the CASR gene were evaluated for their role in calcium stone formation (Lerolle *et al*. 2001).

1.1.1 Calcium Sensing Receptor Gene Polymorphisms

There are three polymorphisms in the intracellular tail encoded by exon 7: 986Ala/Ser (A986S), 990Arg/Gly (R990G) and 1011Gln/Glu (Q1011E), Figure (1.2) (Table 1.1), and they encode non conservative exchanges of single amino acids. Among these polymorphisms, the A986S is the most common and widely studied variant in

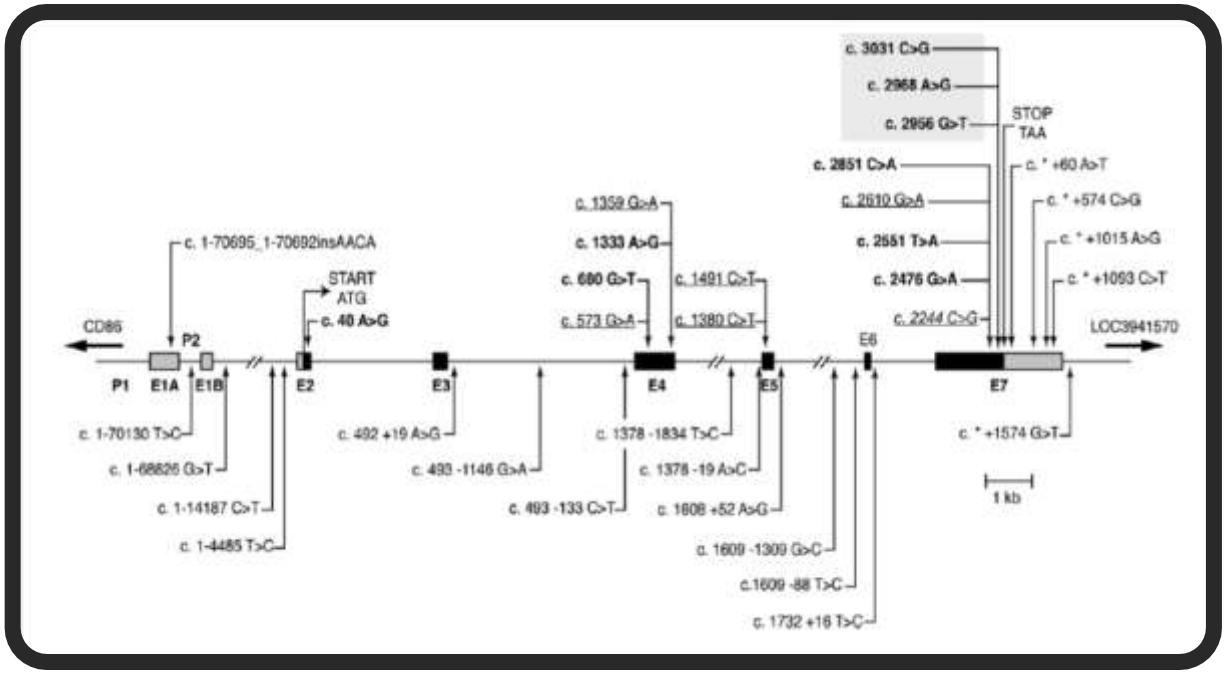

Figure 1.2: Location of the CASR single nucleotide polymorphisms (Janicic *et al*. 1995).

Table 1.1 Polymorphisms of CASR gene (Heath *et al*. 1996)

SNP #	Trivial name	Nucleotide number	Domain	Polymorphism	dSNP accession number
1	(AACA) Ins/Del	c.1-70695_ 1-70692	Exon 1A	AACA Ins/Del[2]	rs10576069
2	IVS1A-214[1]	c.1-70130	Intron 1A	T/C	rs1048213
3	IVS1B+835	c.1-68826	Intron 1B	G/T	rs7628990
4	IVS1B-13945	c.1-14187	Intron 1B	C/T	rs7629281
5	IVS1B-4243	c.1-4485	Intron 1B	T/C	rs1965357
6	IVS3+19	c.492+19	Intron 3	A/G	rs9869985
7	IVS3-1146	c.493-1146	Intron 3	G/A	rs3804592
8	IVS3-133	c.493-133	Intron 3	C/T	rs3749207
9	E191E[3]	c.573	Exon 4	G/A	–
10	K453K[3]	c.1359	Exon 4	G/A	–
11	IVS4-1834	c.1378-1834	Intron 4	T/C	rs3804594
12	V460V[3]	c.1380	Exon 5	C/T	–
13	S497S[3]	c.1491	Exon 5	C/T	–
14	IVS5+52	c.1608+52	Intron 5	A/G	rs2279802
15	IVS5-1309	c.1609-1309	Intron 5	G/C	rs3804595
16	IVS5-88	c.1609-88	Intron 5	T/C	rs4678174
17	IVS6+16	c.1732+16	Intron 6	T/C	rs2270916
18	A826T[4]	c.2476	Exon 7	G/A	–
19	C851S[4]	c.2551	Exon 7	T/A	–
20	E870E[3]	c.2610	Exon 7	G/A	–
21	A986S	c.2956	Exon 7	G/T	rs1801725
22	R990G	c.2968	Exon 7	A/G	rs1042636
23	Q1011E	c.3031	Exon 7	C/G	rs1801726
24	–[5]	c.*+60	Exon 7	A/T	rs4677948
25	–	c.*+574	Exon 7	C/G	rs6438718
26	–	c.*+1015	Exon 7	A/G	rs9740
27	–	c.*+1093	Exon 7	C/T	rs1802757

Caucasian subjects. Most studies on the A986S polymorphism focused on its effects on various bone and mineral disorders in which serum calcium and PTH concentrations had a prominent part. Since the A986S polymorphism was the most commonly observed, it may thus be a genetic determinant of serum calcium concentrations and an interesting candidate for genetic predisposition to diseases of bone and mineral metabolism. In some way, this candidate locus is likely to be a useful therapeutic and diagnostic target with clinical significance (Heath *et al*. 1996).

Most research has focused on A986S polymorphism, which results from an amino acid shift in codon A986 (alanine to serine). The polymorphism has a significant effect on extracellular calcium, and the presence of allele S is associated with increased serum calcium (Lorentzon *et al*. 2001) related to a decrease of urinary calcium excretion (Brown and Hebert 1997).

1.2 Vitamin D and Parathyroid Hormone

Vitamin D and PTH are in general recognized for their important roles in maintaining extracellular calcium and phosphorous homeostasis and in regulating bone formation and bone resorption. Vitamin D is largely obtained from the exposure to sunlight as well as from dietary and supplemental sources (Forman *et al*. 2007).

Vitamin D deficiency may increase the bone turnover and lower the bone mineral density (BMD), thus increase the fracture incidence. Vitamin D supplementation has confirmed an increase of BMD, a decrease of bone turnover and a decrease of fracture (Lips *et al*. 2011).

The active vitamin D metabolite 1-25- dihydroxy- vitamin D [1, $25(OH)_2$ D] opens up calcium channels in the gut, stimulates the formation of calcium binding

protein in the intestinal cell, and thereby stimulates the absorption of calcium and phosphate from the gut. In this way, optimal circumstances for bone mineralization are created. Mineralization in itself is a passive process, once sufficient calcium and vitamin D are available. In case if vitamin D deficiency the 1, $25(OH)_2$ D concentration may drop and less calcium will be available for bone mineralization. The PTH level will increase, stimulating the hydroxylation of $25(OH)_2$ D in the kidney to 1, $25(OH)_2$ D. The increased serum PTH stimulates bone turnover, leading to bone loss, Figure (1.3) (Lips 2001).

PTH is the regulating factor in the calcium and phosphate metabolism. PTH effects calcium and phosphate metabolism in bone and kidneys, Figure (1.4). If the calcium level in the blood is decreased then the PTH stimulates multiple pathways to raise the calcium level:

1- Stimulating bone resorption (osteocalsis) which releases extra calcium from the bone.
2- Reducing renal calcium excretion, increasing renal phosphate excretion.
3- Promoting bioactive vitamin D production by the kidney which enhances calcium and phosphate absorption by the small intestines (Potts 2005).

If the body continues to produce excess PTH, such as in patients with primary hyperparathyroidism this will effects faster bone resorption than bone formation, this will eventually lead to osteoporosis (Roschger 2007).

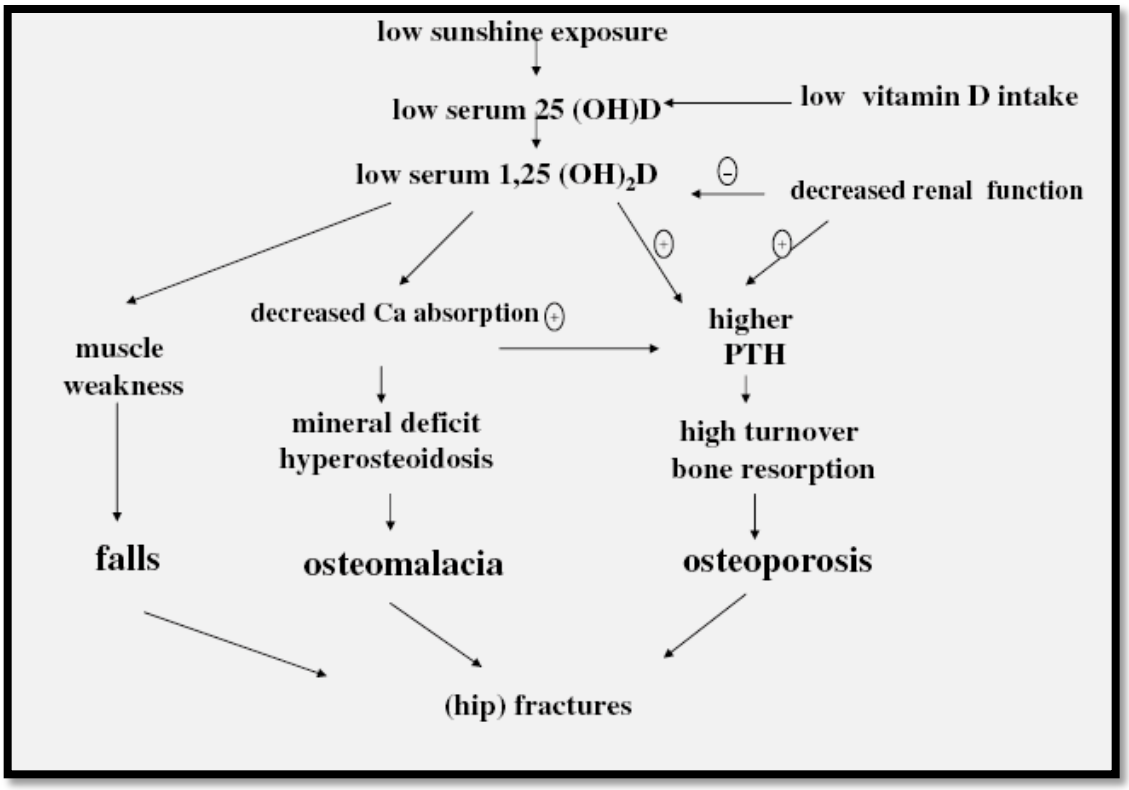

Figure 1.3 Schematic presentation of pathways from vitamin D deficiency and secondary hyperparathyroidism to osteoporotic fracture (Lips 2001).

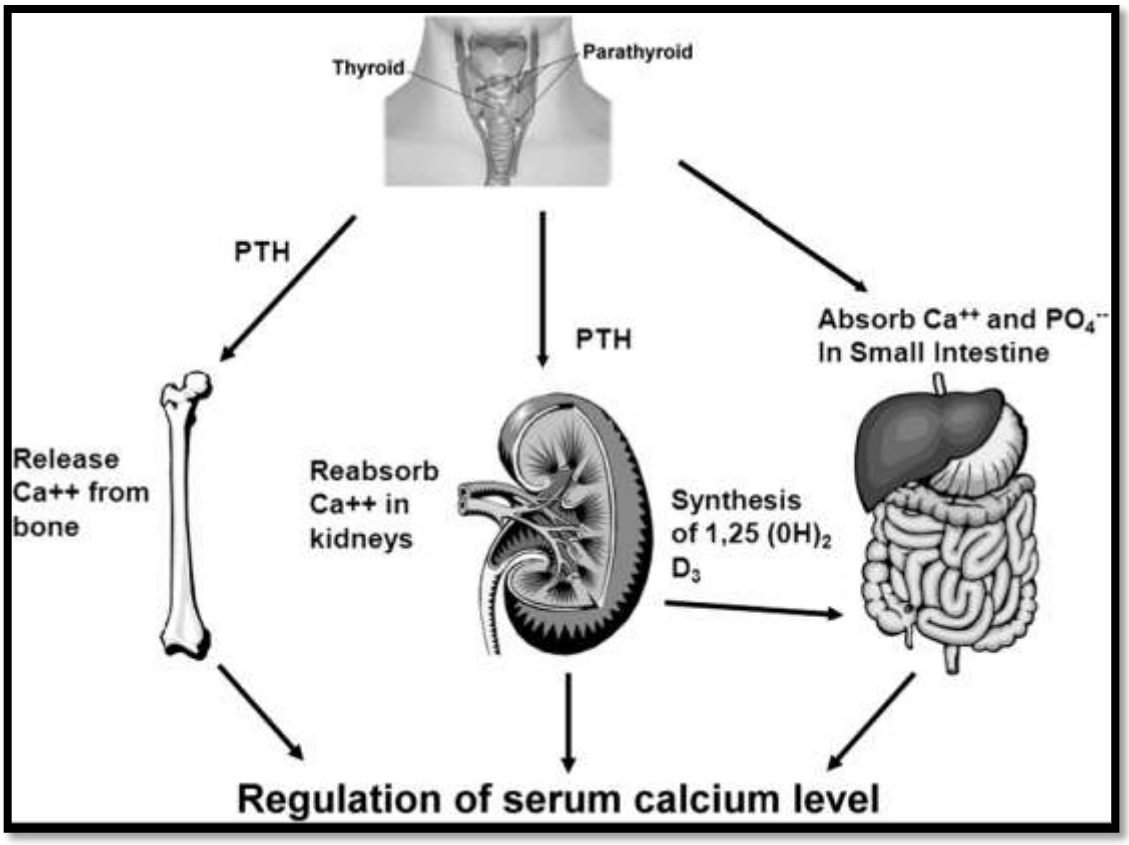

Figure 1.4 Role of PTH in regulating the balance in calcium and phosphate metabolism

(Potts 2005).

1.3 Bone

1.3.1 Bone Strength and Remodelling

Bone strength depends on the vertebral bone density and the trabecular bone structure. It is therefore of interest to investigate the relationship between the static histomorphometric measures and the vertebral bone strength (Kleerekoper *et al*. 1985).

Bone turnover markers (BTMs) reflect whole body rates of bone resorption and bone formation. Specific assays for bone turnover have been developed that have aided in the diagnosis and management of metabolic bone diseases, including osteoporosis. BTMs are now commonly used in clinical trials as measures of antiresorptive and anabolic effects. Markers of bone turnover, particularly markers of bone resorption, have been shown in some, but not all, prospective epidemiologic studies to be associated with fracture risk (Bone 2004) and this association appears to be independent of BMD (Delmas 2000).

Bone remodelling is the continuous process of bone resorption and bone formation occurring in the skeleton of vertebrates throughout their lifetime (Mundy 2000). Remodelling occurs asynchronously at multiple spatially and temporally isolated sites of the skeleton in order to remove portions of damaged or older bone, replacing this with newly formed bone (Parfitt 2002). The purpose of bone remodelling is to:

First: Repair of micro-factures that may lead to macro scale weakness fractures under repeated cyclic loading, sometimes referred to as targeted remodeling (Martin 2002).

Second: Mineral homeostasis, by providing access to the stored of calcium and phosphate, sometimes referred to as random remodelling is accomplished by groups of

bone forming cells (osteoblasts) and bone desorbing cells (osteoclasts), which work together in so-called basic multicellular units' (BMUs) (Burr 2002).

A BMU consists of cells of the osteoblastic and osteoclastic lineage at different stages of maturation. The osteoblasts are derived from mesenchymal stem cells located in the bone marrow, spleen or liver and the osteoclasts are derived from hematopoietic stem cells. Depending on the gene expression profiles and cell markers, various stages of maturation of these cell lines can be distinguished (Aubin 1998).

BTMs are dynamic variables that are affected by several environmental (e.g. climate, country), epidemiological (e.g. age, gender), and lifestyle factors (e.g. smoking status, BMI, alcohol consumption) (Glover 2009). It is important to understand the influence of these lifestyle factors on BTM levels. Furthermore, BTMs are also correlated with $25(OH)_2$ D levels, as lower $25(OH)_2$ D levels result in an increase in PTH levels, which in turn stimulates bone resorption and turnover (Glover 2008).

1.4 Calcium Sensing Receptor and Osteoporosis

The bone remodelling process is characterized by a sequence of actions starting by resorption of the mineralized matrix by osteoclasts and deposition of a new bone matrix by osteoblasts, resulting in replacement of the resorbed bone. The maintenance of bone mass is dependent on the balance between bone resorption and formation during bone remodelling. The age-related difference between the increased bone resorption and decreased formation results in bone loss and osteoporosis (Raisz 2005). Bone remodelling is physiologically finely regulated by a variety of hormones,

cytokines and growth factors that control osteoclast and osteoblast number and function (Martin and Seeman 2008).

Extracellular calcium is important to control the bone remodelling by directly regulating the parathyroid cells through the activation of the seven-transmembrane-spanning extracellular CaSR, CaSR is a member of the G-protein coupled receptor (GPCR) family (Garrett *et al*. 1995). The receptor plays a key role in maintaining extracellular calcium ion concentration through its actions on PTH secretion and renal calcium reabsorption (Brown *et al*. 1998). Activation of CaSR by extracellular calcium concentration in the parathyroid gland down regulates PTH secretion. The CASR mutations result in disorders of calcium homeostasis. Mutations of the CASR gene can be either inactivating or activating mutation. The loss-of-function mutations can cause a disease such as autosomal dominant familial hypocalciuric hypercalcemia (FHH). The Gain-of-function CASR mutations are related with autosomal dominant hypocalcemia (ADH) and normal PTH levels (Raue *et al*. 2006). Clinical observations provided support for the central role of the CaSR in calcium homeostasis through regulation of PTH secretion and renal calcium treatment. Recent genetic and biochemical data specify that the CaSR plays an important role in the control of bone cells.

Osteoporosis is a multi-factorial disorder characterized by low BMD and micro architectural deterioration of bone, resulting in loss of bone strength and thus increased fracture risk. Feebleness fractures are the most important and disabling consequences of osteoporosis and result in a loss of functional ability, serious morbidity, elevated mortality, and high socio-economic burden (Johnell and Kanis. 2006). Due to increased life expectancy, the incidence of fractures is increasing over time, thereby

increasing the population burden of fractures (Holt *et al.* 2009). Several studies of different populations have established the importance of clinical risk factors as determinants for low bone mass, and fragility fractures no one risk factor alone is able to predict bone mass or risk of fractures on its own individual merits.

1.5 Aims of the Study

1- To determine the genotype frequency distribution of calcium-sensing receptor gene rs1801725 (A986S) among adult Saudi males and females.

2- To study the association of calcium-sensing receptor gene rs1801725 (A986S) polymorphism with BMD in healthy Saudi males and females.

3- To investigate of the potential association of calcium sensing receptor gene rs1801725 (A986S) polymorphism with vitamin D deficiency and secondary hyperparathyroidism.

CHAPTER TWO

MATERIALS AND METHODS

Chapter II

Materials and Methods

2.1 Materials

2.1.1 Subjects

The study was carried out by recruiting 50 Saudi males and 63 Saudi females. They were convenience samples, quantitative cross sectional exploratory study, selected from subjects attending the center of excellence for osteoporosis research (CEOR), with age range between 20 and 60. Each subject was medically examined and interviewed using a standardized questionnaire. It contain questions on socioeconomic status, lifestyle, smoking habits, level of physical activity in leisure time, sun exposure, and the use of vitamins and medications. Dietary intakes of calcium and vitamin D supplementation were also recorded. Exclusion criteria were: known renal disease, thyroid disorders, diabetes mellitus, pregnancy, patients on medications that affects bone metabolism such as steroids and antiepileptic medications.

The practical works of this research were conducted in the center of excellence for osteoporosis research (CEOR), King AbdulAziz University, Jeddah, Saudi Arabia.

2.1.2 Kits

2.1.2.1 Genomic DNA Extraction Kit

The DNA extraction kit Gentra Puregene Blood Kit was purchased from QIAGEN Inc, USA. The kit contains RBC lysis solution, cell lysis solution, protein precipitation solution and DNA hydration solution; all reagents were stored at room temperature (15–25°C).

2.1.2.2 Polymerase Chain Reaction (PCR) Kit

Amplification of genomic DNA for subjects were performed by using Promega Go Taq® Hot Star Green Master Mix Kit (100 units), USA. The kit contains Go Taq® Hot Star Green Master Mix, 2X and nuclease free water.

2.1.2.3 Restriction Enzymes

The restriction enzymes are *SacI* restriction enzyme and *HinP1I (Hin6I)* restriction enzymes were carried out by using Thermo Scientific FastDigest restriction enzyme, Fermentas, U.S.A.

2.1.2.4 Primers

The primers for SNP A986S (rs1801725) were ordered from Metaboin international AG, Germany. The concentration was 100 pmol/ µl.

1. Forward primer 5´- CTT TGA TGA GCC TCA GAA GAG C -3´, the oligo number: 10706B3- 9642C01.

2. Reverse primer 5´- ACA ACT CTT CAG GGT CCT CC -3´, the oligo number: 10706B3-9642D01.

2.1.3 Reagents

Agarose, ethanol, SYBR green, O'RangeRuler 50 bp DNA Ladder and TBE buffer (10 X) were all purchased from Fermentas, U.S.A.

2.2 Methods

2.2.1 Anthropometric Measurements

Anthropometric measurements were performed in the morning, before breakfast, with the subjects wearing light clothes and bare footed. Weight and height measurement were performed using a digital scale. The body mass index (BMI) was calculated by dividing the body weight (in kilograms) by the square of the height (in meters).

2.2.2 Sample Collection

Venous blood samples were obtained from the antecubital vein after 9 hours overnight fasting; 7 ml of blood was withdrawn into a plain vacutainer tube for serum preparation. Then 3 ml of blood was withdrawn into EDTA vacutainer tube to be used for DNA extraction.

2.2.3 Bone Mineral Densitometry Measurements

BMD (g/cm^2) was determined for the anteroposterior lumbar spine (L1-L4) and the neck femurs by dual-energy X-ray absorptiometry (DXA) (LUNAR Prodigy Model, Lunar Corp., Madison, WI).

BMD values were classified according to WHO criteria: a T-score between -1 and -2.5 is indicative of osteopenia, while a T-score of -2.5 and below reflects osteoporosis: a T-score of -1 and above is considered normal.

2.2.4 Genomic DNA Extraction from Whole Blood

Three hundred microliter of the whole blood was added to 1.5 ml micro centrifuge tube containing 900 µl red blood cell lyses solution. The tube was then mixed by inverting it 10 times. The mixture was incubated at room temperature for 1 min, then spin briefly for 20 s at 13000-16000 ×g. The supernatant were then carefully discarded by pipetting. After that cell lyses solution (300 µl) was added to the sample and mixed by vortex for 10 s. Then protein precipitation solution (100 µl) was added and mixed by vortex for 20 s. The mixture centrifuged for 1 min at 13000–16000×g. Isopropanol (300 µl) was added into a 1.5 ml micro centrifuge. Then the supernatant from the previous step was added and mix by inverting 50 times until the DNA was visible as threads. This is followed by spinning the mixture for 1 min. Then the supernatant were carefully discarded, and the tube was drained by inverting it on a clean piece of absorbent paper. Next 70% ethanol (300 µl) was added (to wash the DNA pellet) and the mixture was inverted several times. Then centrifuged for 1 min at 13000-16000 ×g. Subsequently the supernatant were carefully discarded, and the tube was drained by inverting it on a clean piece of absorbent paper. DNA hydration solution (100 µl) was added and vortex for 5s

at medium speeds and then incubated at 65ºC for 5 min to dissolve the DNA. After that the DNA was incubated at room temperature (15-25ºC) overnight.

2.2.5 Determination of DNA Concentration

A UV spectrophotometer (Gene Quant 1300, Model No. 80-2120-02, England) was used to determine DNA concentration by the quantitative method that was based on the measurement of the optical density. The DNA concentration was determined by reading the absorbance at wavelength of 260nm.

2.2.6 Primers Dilution

The primers have been centrifuged for 30 second at 10,000 xg. Then RNAse free water (327 µl) was added for the forward primer and mixed well to give a final concentration of 100µM, after that the primers were left to stand for 15 minutes. Finally, an aliquot were made in eppendorf tubes to avoid contamination. The primers were then stored at - 20ºC.

2.2.7 Polymerase Chain Reaction (PCR)

The fragments that contains A986S (rs1801725) SNP was amplified by using the Forward primer 5´- CTT TGA GCC TCA GAA GAG C -3´, and Reverse primer 5´- ACA ACT CTT CAG GGT CCT CC -3´. PCR amplification products were carried out in a final volume of 50 µl [25 µl HotStar Taq Master Mix, 6 µl (2µg/µl) genomic DNA, 15 µl RNase free water, and 2 µl of each primer] in athermal cycler (BIO-RAD, DNA Engine Tetrad2 Peltier Thermal Cycler, serial number: AL116086, U.S.A.). The

amplification conditions were as follows: 94ºC for 5 min, followed by 30 cycle of 30s each at 94ºC, 58ºC, and 72ºC, and ending with a single 7 min extension step at 72ºC.

After the thermal cycle have been terminated, 5 µl of each PCR products with 2 µl loading dye was loaded into the wells of 2 % agarose gel to ensure that the amplification had occurred. The remaining PCR product was stored at - 20ºC.

2.2.8 Electrophoresis

2.2.8.1 Preparation of 2% Agarose Gel

In order to prepare 2% agarose gel. In a conical flask, 2 g of agarose was diluted into 100 ml of 1X TBE buffer than placed in a microwave until the agarose completely dissolved. Then the agarose left to cool on the bench for 5 minutes down to about 60ºC, while the agarose cooling, the gel casting tray was prepared clean and ready on a level surface and two spacers were inserted firmly between the edges of the casting tray, sample comp then was insert in its correct position. In order to visualize the band clearly 10 µl of SYBR green was added carefully, and then swirled to mix. Then the gel was poured slowly into the casting tray. After that, any bubbles were pushed away to the far side of the try by using a disposable tip. Finally the gel left the polymerized for at least 30 minutes.

2.2.8.2 Running Gel

After the gel was allowed to set for half an hour then the combs and the two spacer were removed gently. The gel casting trays of the electrophoresis were filled with 1X TBE buffer. After that, (8µl DNA ladder of the 50 bp + 8µl RNase free water) were

loaded into the well, then the samples were loaded in the rest of the wells. The samples were loaded slowly and carefully.

Electrophoresis lead was attached firmly and was connected to the power supply generator (ensuring that the DNA will migrate from the negative to the positive terminal). The electrophoresis was started at a voltage of 120 volts for 45 minutes using power supply (BIO-RAD, Model No. Power Pac 300, USA). The distance DNA migrated in the gel was judged by visually monitoring migration of the tracking dyes.

2.2.8.3 Visualization of the DNA bands

DNA band were visualized by previous staining of the gel with SYBR green (10 µl) from 10X stock solution. This fluorescent dye intercalates between bases of DNA and it was incorporated into the gel so that staining occurs during electrophoresis. The gel then was placed on a gel documentation system (BIO-RAD, Model No. Universal Hood Π, USA) to examined, visualized and then photographed.

2.2.9 Genotyping

2.2.9.1 Genotyping of A986S SNP in CASR gene by *Hin6I*

The genotypes for this SNP were determined by restriction fragment length polymorphism (RFLP) procedure. The PCR products were digested by combining the following components in a 200 µl microfuge tube to a total volume of 30 µl:

PCR product	5 µl (0.2 µg/µl)
RNase free water	22 µl
10X NE Buffer	2 µl
Restriction enzyme (*hin6I*)	1 µl

Samples were incubated at 37ºC for 5 min then inactivated the enzyme by heating at 80ºC for 10 min and after that; the genotypes were resolved after running it on 2% agarose gels electrophoresis.

2.2.9.2 Genotyping of A986S SNP in CASR gene by *SacI*

The genotypes for this SNP determined by restriction fragment length polymorphism (RFLP) procedure. They prepared in a total volume of 30 µl as follow:

PCR product	5 µl (0.2 µg/µl)
RNase free water	22 µl
10X NE Buffer	2 µl
Restriction enzyme (*SacI*)	1 µl

Then the tube incubated at 37ºC for 30 min then inactivated the enzyme by heating at 65ºC for 5 min and after that, the genotypes were resolved after running it on 2% agarose gels electrophoresis.

2.3 Biochemical analysis

Calcium was measured using Filter photometric (VTROS 250, USA). Bone marker serum osteocalcin (s-OC) was measured using ECLIA Elecsys autoanalyzer [Roche Diagnostics GmbH, D-68298 Mannheim, Germany]. Serum bone-specific alkaline

phosphatase (s-bone ALP) was measured with a Metra Biosystem immunoassay kit in a microtiter strip (Alkphase-B, Metra Biosystems, Inc., Mountain View, CA, USA). Serum procollagen type 1 N-terminal propeptide (s-PINP) was measured using ECLIA Elecsys autoanalyzer [Roche DiagnosticsnGmbH, D-68298 Mannheim, Germany]. Serum crosslinked C-terminal telopeptide of type 1 collagen (s-CTX) was measured by Elecsys β-CrossLaps assay using ECLIA Elecsys autoanalyzer. All analysis was conducted at the CEOR, King AbdulAziz University, Jeddah, Saudi Arabia.

2.4 Hormones analysis

Estradiol, free hormone (LH), parathyroid hormone (PTH), thyroid stimulating hormone (TSH), thyroid test (T3 and T4) where analyzed at CEOR, King AbdulAziz University, Jeddah, Saudi Arabia.

2.5 Statistical Analysis

The clinical and laboratory data recorded on an investigative report form. Statistical analysis of the data performed using statistical package for social sciences (SPSS for windows, version 18) (SPSS Inc., Chicago, IL, USA). Descriptive data were given as mean ± standard deviation (SD). Correlation between different variables was assessed using Pearson's or Spearman's correlation as appropriate. P values < 0.05 were considered significant. Differences in biochemical values relative to genotype were tested by one-way ANOVA and Tukey post-hoc comparisons. Hardy-Weinberg Equilibrium was used to determine allele frequency and genotype frequency in a population.

CHAPTER THREE

RESULTS

Chapter III

Results

3.1 Subjects

In this study, 113 Saudi volunteers (50 males and 63 females) were involved. All study subjects were of Saudi origin without any known ancestors of other ethnic origins. All the participants underwent complete physical examination and routine biochemical analysis of blood. Allele and genotype frequencies for the CASR gene A986S polymorphism were determined, the association of the CASR gene A986S polymorphism with BMD, vitamin D deficiency and secondary hyperparathyroidism were evaluated.

3.2 Physical and biochemical characteristics

The clinical characteristics including the serum concentration of calcium and BMD and PTH and 25(OH) D and other Physical and biochemical characteristics in Table 3.1. The subjects were divided into two groups according to gender. The number of males was 50 and females were 63 subjects. The BMD at lumbar spine, 1.09±0.15 g/cm^2 in males and 1.04±0.18 g/cm^2 in females.

Table 3.1: Physical and biochemical characteristics for the male and female subjects

Groups		Males (n=50)		Females (n=63)	
		Mean	SD	Mean	SD
	Age (years)	43.32	13.87	44.40	13.22
	Weight (kg)	83.15	18.59	73.28	15.36
	Height (cm)	167.35	7.24	154.58	6.91
	BMI (kg/m^2)	29.67	6.24	30.89	7.12
	BMD lumbar spine (g/cm^2)	1.09	0.15	1.04	0.18
	BMD neck femur (g/cm^2)	0.97	0.13	0.94	0.14
	s- Calcium (mmol/L)	2.34	0.11	2.32	0.12
	ALP (U/L)	93.76	30.74	88.57	28.92
	s-OC (ng/ml)	26.24	11.12	51.50	212.46
	p-CTX (ng/ml)	335.08	169.36	285.40	149.20
	s-P1NP (ng/ml)	44.31	17.65	45.50	18.96
	s-BALP (ng/ml)	20.16	9.67	19.75	8.24
	s-PTH (pmol/L)	5.12	2.42	6.08	3.58
	s-25-(OH)D (nmol/L)	31.93	14.04	35.21	30.77

SD: Std. Deviation, ALP: alkaline phosphatase,

s-OC: marker serum osteocalcin, s-CTX :crosslinked C-terminal telopeptide of type1 , s-PINP: collagen serum procollagen type 1 N-terminal propeptide , PTH: Parathyroid hormone.

Mean PTH level in males is 5.12±2.42 pmol/L and 6.08±3.58 pmol/L in females. Mean vitamin D Level in males is 31.93±14.04 nmol/L and 35.21±30.77 nmol/L in females.

3.3 Correlation between calcium level and other variables

The subjects were divided into two groups according to gender. The calcium correlation with physical and biochemical parameter were done by using Pearson correlation. The overall results showed in male group that there is a significant negative correlation between calcium and weight (*P*=0.024) (Table 3.2). There is a significant negative correlation between calcium and BMI (*P*=0.014).

The results showed in female group that there is a significant correlation between calcium and 25-(OH) D (*P*=0.029) (Table 3.3).

3.4 Correlation between PTH and other variables

The subjects were divided into two groups according to gender. The PTH correlation with physical and biochemical parameter were done by using Pearson correlation. The PTH and age, PTH and BMI is highly significant correlation (*P*=0.001) and (*P*=0.008) respectively in males group (Table 3.4). The correlation between PTH and weight is significant (*P*=0.014).

In female group the PTH correlation with BMI is significant (*P*=0.015) (Table 3.5). There is no correlation between PTH and other physical and biochemical parameter.

Table 3.2: Correlation between calcium and other variables in male group

Variables	Calcium	
	Pearson correlation	***P* value**
Age (years)	-0.272	0.056
Weight (kg)	-0.319	0.024*
Height (cm)	0.018	0.900
BMI (kg/m^2)	-0.346	0.014*
BMD lumbar spine (g/cm^2)	-0.134	0.353
BMD neck femur (g/cm^2)	0.173	0.244
ALP (U/L)	0.123	0.396
PTH (pmol/L)	-0.269	0.059
25-(OH)D (nmol/L)	-0.094	0.515

Asterisk indicates statistical significance at:* $P < 0.05$.

ALP: alkaline phosphatase, PTH: Parathyroid hormone.

Table 3.3: Correlation between calcium and other variables in female group

Variables	Calcium	
	Pearson correlation	***P* value**
Age (years)	0.178	0.163
Weight (kg)	-0.126	0.327
Height (cm)	-0.145	0.255
BMI (kg/m^2)	-0.060	0.638
BMD lumbar spine (g/cm^2)	0.244	0.054
BMD neck femur (g/cm^2)	0.234	0.086
ALP (U/L)	0.065	0.614
PTH (pmol/L)	-0.153	0.232
25-(OH) D (nmol/L)	0.276	0.029*

Asterisk indicates statistical significance at:* $P < 0.05$.

ALP: alkaline phosphatase, PTH: Parathyroid hormone.

Table 3.4: Correlation between PTH and other variables in male group

Variables	PTH	
	Pearson correlation	***P* value**
Age (years)	0.474	0.001**
Weight (kg)	0.344	0.014*
Height (cm)	-0.034	0.813
BMI (kg/m^2)	0.369	0.008**
BMD lumbar spine (g/cm^2)	-0.147	0.307
BMD neck femur (g/cm^2)	-0.273	0.063
ALP (U/L)	0.220	0.124
s- Calcium (mmol/L)	-0.269	0.059
25-(OH)D (nmol/L)	-0.169	0.241

Asterisk indicates statistical significance at:* $P < 0.05$, ** $P < 0.01$

ALP: alkaline phosphatase, PTH: Parathyroid hormone.

Table 3.5: Correlation between PTH and other variables in female group

Variables	PTH	
	Pearson correlation	***P* value**
Age (years)	0.140	0.273
Weight (kg)	0.246	0.052
Height (cm)	-0.207	0.104
BMI (kg/m^2)	0.305	0.015*
BMD lumbar spine (g/cm^2)	0.064	0.619
BMD neck femur (g/cm^2)	-0.048	0.727
ALP (U/L)	0.216	0.089
s- Calcium (mmol/L)	-0.153	0.232
25-(OH)D (nmol/L)	-0.240	0.058

Asterisk indicates statistical significance at:* $P < 0.05$.

ALP: serum bone-specific alkaline phosphatase, PTH: Parathyroid hormone.

3.5 Correlation between 25-(OH) D and other variables

The 25-(OH) D correlation with physical and biochemical parameter were done by using Spearman correlation and the subjects were divided into two groups according to gender. There is negative significant correlation between 25-(OH) D and alkaline phospatase (P=0.017) in male group (Table 3.6) and no correlation between 25-(OH) D and other parameter.

In female group there is significant correlation between 25-(OH) D and age, 25-(OH) D and alkaline phosphatase, 25-(OH) D and calcium (*P*=0.022), (*P*=0.013) and (*P*=0.029) respectively (Table 3.7).

3.6 Correlation between BMD and other variables

The correlation between BMD at lumbar spine, dual femur and other physical and biochemical was done by Pearson correlation and divided to two groups according to gender. There is significant correlation between BMD lumbar spine and height (*P*=0.017) and highly significant between BMD lumbar spine and weight (*P*=0.008) in males group (Table 3.8).

In females groups there is a significant correlation between BMD lumbar spine and calcium (*P*=0.024) and no significant differences between other variables. There is a highly significant between BMD neck femur and weight (0.007) (Table 3.9).

Table3.6: Correlation between 25-(OH) D and other variables in male group

Variables	25-(OH)D	
	Spearman correlation	***P* value**
Age (years)	0.140	0.334
Weight (kg)	-0.086	0.553
Height (cm)	0.065	0.654
BMI (kg/m^2)	-0.123	0.397
BMD lumbar spine (g/cm^2)	0.074	0. 610
BMD neck femur (g/cm^2)	-0.028	0.852
ALP (U/L)	-0.336	0.017*
s- Calcium (mmol/L)	-0.094	0.515
PTH (pmol/L)	-0.169	0.241

Asterisk indicates statistical significance at:* $P < 0.05$.

ALP: alkaline phosphatase, PTH: Parathyroid hormone.

Table 3.7: Correlation between 25-(OH) D and other variables in female group

Variables	25-(OH) D	
	spearman correlation	***P* value**
Age (years)	0.288	0.022*
Weight (kg)	0.005	0.968
Height (cm)	-0.209	0.101
BMI (kg/m^2)	0.090	0.482
BMD lumbar spine (g/cm^2)	-0.039	0.763
BMD neck femur (g/cm^2)	-0.151	0.270
ALP (U/L)	0.310	0.013*
s- Calcium (mmol/L)	0.276	0.029*
PTH (pmol/L)	-0.240	0.058

Asterisk indicates statistical significance at:* $P < 0.05$.

ALP: alkaline phosphatase, PTH: Parathyroid hormone.

Table 3.8: Correlation between BMD and other variables in male group

Variables	BMD lumbar spine (L1-L4)		BMD neck femur	
	Pearson correlation	***P* value**	**Pearson correlation**	***P* value**
Age (years)	-0.019	0.861	-0.214	0.148
Weight (kg)	0.345	0.008**	0.166	0.265
Height (cm)	0.290	0.017*	-0.035	0.816
BMI (kg/m^2)	0.254	0.064	0.206	0.164
ALP (U/L)	-0. 211	0.098	-0.219	0.139
s-Calcium (mmol/L)	-0. 134	0.525	0.173	0.244
PTH (pmol/L)	-0.147	0.373	-0.273	0.063
25-(OH)D (nmol/L)	0.162	0.385	0.054	0.719

Asterisk indicates statistical significance at:* P < 0.05, ** P <0.01

ALP: alkaline phosphatase, PTH: Parathyroid hormone.

Table 3.9: Correlation between BMD and other variables in female group

Variables	BMD lumbar spine (L1-L4)		BMD neck femur	
	Pearson correlation	***P* value**	**Pearson correlation**	***P* value**
Age (years)	-0.157	0.216	-0.124	0.369
Weight (kg)	0.053	0.215	0.360	0.007**
Height (cm)	0.143	0.125	0.204	0.136
BMI (kg/m^2)	-0.027	0.695	0.237	0.082
ALP (U/L)	-0.186	0.163	-0.048	0.729
s-Calcium (mmol/L)	-0.244	0.024*	-0.234	0.086
PTH (pmol/L)	0.064	0.770	-0.048	0.727
25-(OH)D (nmol/L)	-0.029	0.746	-0.085	0.536

Asterisk indicates statistical significance at:* $P < 0.05$, ** $P < 0.01$

ALP: alkaline phosphatase, PTH: Parathyroid hormone.

3.7 Analysis of the rs1801725 SNP in the CASR gene

PCR technique was used to amplify the region that contains rs1801725 SNP for all the collected samples (n=113). The amplified fragment showed a size of 218bp.

In restriction enzyme *SacI* the normal (AA) genotype produced one band of size 218bp. The heterozygous (AS) genotype produced three fragments of size 218, 198 and 20bp. The homozygous (SS) genotype produced two band of size 198bp and 20bp (Figure 3.1).

In restriction enzyme *Hin6I* the normal (AA) genotype produced two band of size 195bp and 23bp. The heterozygous (AS) genotype produced three fragments of size 218, 195 and 23bp. The homozygous (SS) genotype produced one band of size 218bp (Figure 3.1).

3.8 Genotypes and allele frequencies of rs1801725 in males and females

Genotype frequencies of calcium sensing receptor gene rs1801725 in males showed 26% (n=13) normal (AA), and 56% (n=28) heterozygous (AS) and 18% (n=9) homozygous (SS). Allele frequencies for A and S were 54% and 46% respectively. The genotype distribution is within Hardy-Weinberg equilibrium (Goodness of fit X^2= 0.80, df =2, *P* =0.00001).

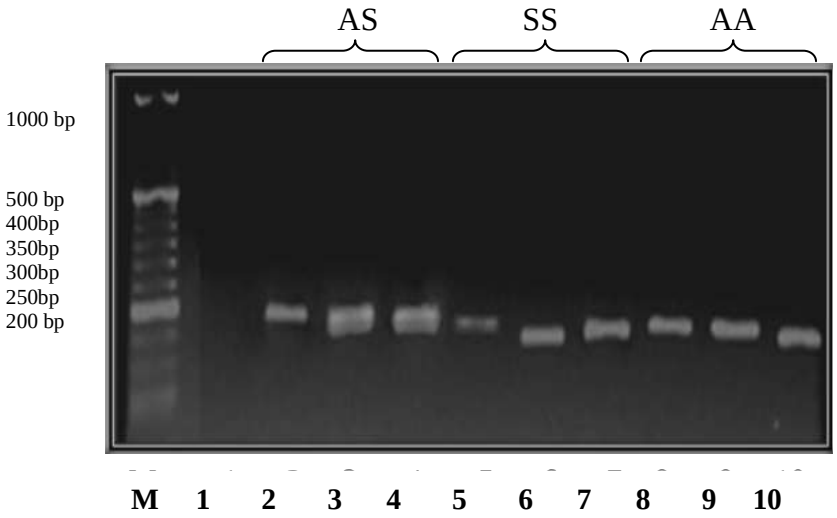

Figure 3.1 Photograph of a 2% agarose gel showing the result of *SacI* and *Hin6I* digestion. Lane M: DNA marker. Lane 1: control. Lane 2: uncut PCR product of size 218bp. Lane 3: heterozygous (AS) genotype that shows two band 218 and 198bp in *SacI*. Lane 4: heterozygous (AS) genotype that shows two band 218 and 195bp in *Hin6I*. Lane 5: uncut PCR product of size 218bp. Lane 6: homozygous (SS) genotype that shows one band 198 in *SacI*. Lane 7: homozygous (SS) genotype that shows one band 218 in *Hin6I*. Lane 8: uncut PCR product of size 218bp. Lane 9: normal (AA) genotype that shows one band 218bp in *SacI*. Lane 10: normal (AA) genotype that shows one band 195bp in *Hin6I*.

In females, the result showed 49.21% (n= 31) normal (AA), and 36.51% (n=23) heterozygous (AS), and 14.29% (n=9) homozygous (SS). Allele frequencies for A and S were 67.46% and 32.54% respectively. The genotype distribution is within Hardy-Weinberg equilibrium (Goodness of fit X^2= 1.78, df= 2, *P*= 0.07).

Genotype and allele frequencies of the males and females are presented in (Table 3.10).

3.9 Association of CASR rs1801725 polymorphism with BMD

The result of association between rs1801725 SNP and BMD is summarized in (Table3.11). There were no significant differences in BMD observed between the AS, SS genotype and the AA genotype carrier.

3.10 Association of CASR rs1801725 polymorphism with 25-(OH) D

The result of association between rs1801725 SNP and 25-(OH) D is summarized in (Table3.12). There were no significant differences in 25-(OH) D observed between the AS, SS genotype and the AA genotype carrier. The AS carriers showed higher levels of 25-(OH) D (31.96±23.65 nmol/L) compared to the SS genotype carriers (25.20±12.43 nmol/L).

Table 3.10 Genotypes and allele frequencies of rs1801725 in males and females

Calcium polymorphism	Frequencies (%)	
	Male (n=50)	Female (n=63)
AA	26.00 (n=13)	49.21 (n=31)
AS	56.00 (n=28)	36.51 (n=23)
SS	18.00 (n=9)	14.29 (n=9)
AS+SS	74.00 (n=37)	50.79 (n=32)
Alleles		
A	54.00	67.46
S	46.00	32.54

Table 3.11 Association of CASR rs1801725 polymorphism with BMD

BMD	A986S genotype			*P*-value
	AA	AS	SS	
BMD lumbar spine (g/cm^2)	1.071 (0.18)	1.076 (0.16)	1.03 (0.15)	0.98^{AS} 0.78^{SS}
BMD neck femur (g/cm^2)	0.93 (0.16)	0.96 (0.12)	0.98 (0.13)	0.72^{AS} 0.50^{SS}

Data are mean (SD), *probability that AS and SS group mean differs from AA group, by ANOVA and Tukey multiple comparison test.

Table 3.12 Association of CASR rs1801725 polymorphism with 25-(OH) D

Genotypes	patients		*P*-value
	Mean	SD	
AA	39.34	28.61	ref
AS	31.96	23.65	0.312
SS	25.20	12.43	0.102

SD: std.deviation, *probability that AS and SS group mean differs from AA group, by ANOVA and Tukey multiple comparison test.

3.11 Association of CASR rs1801725 polymorphism with PTH

The result of association between rs1801725 SNP and PTH in secondary hyperparathyroidism patients is summarized in (Table3.13). There were no significant differences in PTH observed between the AS, SS genotype and the AA genotype carrier.

Table 3.13 Association of CASR rs1801725 polymorphism with PTH

Genotypes	patients		*P*-value
	Mean	SD	
AA	7.56	2.38	ref
AS	7.78	3.90	0.939
SS	7.50	2.47	0.997

SD: std.deviation, *probability that AS and SS group mean differs from AA group, by ANOVA and Tukey multiple comparison test.

CHAPTER FOUR

DISCUSSION

Chapter IV

Discussion

This study is the first to examine the effect of the CASR A986S polymorphism in men worldwide.

Vitamin D deficiency in Saudi males and females is ~85% and it is increasing due to life style along with other factors including genetics. Studies conducted by Sadat *et al.* showed that the concentration of vitamin D was ≤50 nmol/l among 28-37% of 200 randomly selected healthy men living in Alkhobar (Sadat *et al*. 2009). Furthermore, another low concentration of vitamin D was detected in both males and females, even though they are exposed to sunlight and having adequate dairy products (Elsammak *et al.* 2011). Vitamins D deficiency has been studied in Middle East and turkey. Table4.1 demonstrates some of these studies in some countries. We thought it would be interesting to look at the matter from the genotype point of view, so we determined the frequency distribution of CASR rs1801725 A986S among adult Saudi males and females and studied the association of the gene polymorphisms

Table 4.1: Vitamin D deficiency in some countries

Countries	Title of Study	25-$(OH)_2$D level	References
Saudi Arabia	• High prevalence of vitamin D deficiency in the sunny eastern region of Saudi Arabia	25-$(OH)_2$ D level were [25.25 nmol/L in male and 24.75 nmol/L in female].	(Elsammak *et al*. 2011).
Oman	• Vitamin D status in healthy Omani women of childbearing age • Vitamin D Status in Pregnant Omanis	$25(OH)_2$ D level <50 nmol/L Vitamin D deficiency was present in 34 (33%) of patients ($25(OH)_2$ D <25 nmol/L), 'at risk' levels were found in 67 (65%) patients ($25(OH)_2$ D 25–50 nmol/L); two patients (1.9%) had values between 50 and 75 nmol/L.	(Al-Kindi, 2011). (Al Kalbani *et al*. 2011)
United Arab Emirates	• Efficacy of daily and monthly high-dose calciferol in vitamin D-deficient nulliparous and lactating women	$25(OH)_2$ D < 50 nmol/L	(Saadi *et al*. 2007)
Qatar	• Vitamin D status in health care professionals in Qatar	Vitamin D concentration was lower in females (25.8 nmol/L) than in males (34.3 nmol/L).	(Mahdy *et al.* 2010)
Turkey	• Sunlight exposure and vitamin D deficiency in Turkish women	$25(OH)_2$ D were 15.2± 12.1 nmol/L	(Alagöl *et al.* 2000)

with vitamin D deficiency and secondary hyperparathyroidism.

Based on these data the frequency distribution of calcium sensing receptor gene in males showed 26% normal (AA), 56% heterozygous (AS) and 18% homozygous (SS). Allele frequencies for A and S were 54% and 46% respectively. In females, 49.2% normal (AA), 36.5% heterozygous (AS) and 14.29% 14.3% homozygous (SS). Allele frequencies for A and S were 67.5% and 32.5% respectively. In comparing it to previous works José *et al*. showed that the frequency distribution of calcium sensing receptor gene was 69% for AA, 27% for AS and 4% for SS, with a prevalence of 82% for allele A and 18% for allele S (José *et al*. 2006). In Hungarian postmenopausal women was 25.2% for presence S allele and 74.8% for absence of S allele (Takacs *et al.* 2002). We noticed that the frequency of distribution of the normal allele is higher in both findings than our finding which allele frequencies for A and S were 67.5% and 32.5% respectively. Consequently, the percentage of polymorphism in Saudi population is higher. If it's not due to genetic effect, it could be either due to life style or age differences since Takacs *et al.* and José *et al*. conducted their studies on postmenopausal women. On the other hand our average age for male and female was 40 years.

We found no association of CASR rs1801725 polymorphism with BMD. There are several studies that relate A986S polymorphism to BMD and are consistent with our data. Takacs *et al*. was did not find an association between CASR A986S polymorphism and BMD in Hungarian postmenopausal women (Takacs *et al.* 2002). Young *et al.* assessed bone mass at baseline and after 2 years of calcium therapy in a group of 135 postmenopausal women, observing no relationship in either case. Furthermore, in Italian women no differences were observed (Cetani *et al*. 2003). In a

study of 1252 postmenopausal Australian women, they found no relation of the polymorphism with bone mass and the presence of fractures due to fragility. In healthy Chinese premenopausal women (Bollerslev *et al*. 2004) there were no significant differences in the BMD or bone size of either the spine or hip between CASR polymorphisms (Mo *et al*. 2004).

Same SNP of CASR was studied by Lorentzon *et al.* in young healthy girls however showed contradictory result. The study was performed on subjects with S allele who had lower BMD at the lumbar spine and total body (Lorentzon *et al.* 2001). They found a correlation between CASR allele and BMD. Nevertheless Lorentzon *et al*. wasn't confident about the fact that CASR polymorphism had a direct effect on BMD. The effect could be due to the amount of physical activity because the association between CASR genotype and BMD disappeared after modifying the physical activity. In this study, there were no differences between the physical activities between the groups on the other hand, the average age was different. Up to 60-80% of age-specific variation in BMD is due to genetic factor (Kelley *et al.* 1995). Therefore, the contradictory result could be either due to life style in Saudi Arabia, smoking habits etc.

There were no significant association observed between vitamin D levels and CASR A986S polymorphism. The AS carriers showed higher levels of vitamin D (31.96±23.65 nmol/L) compared to the SS genotype carriers (25.20±12.43 nmol/L).

In this study there were no significant association observed between CASR A986S polymorphism and PTH. Which was consistent with the finding of Miedlich *et al.* and He *et al.* for whom have reported no correlation of PTH with CaSR A986S variant (Miedlich *et al.* 2001 and He *et al.* 2012).

Differences in the genetic background of mixed populations may alter the outcome of association studies. However, our study population was homogeneously of Saudi origin. Not only the multifactorial genetics but also environmental factors have an influence on BMD. If the genetic effect was weak, the environmental factors may have masked the actual genetic influence of the CASR gene in our association study.

In summary, we found no evidence to support a relationship between CASR A986S polymorphism with BMD, vitamin D and PTH in adult Saudi males and females. There are a number of limitation associated with this study one of them is a small size of the population studied that would have influence on the statistical power of the analysis.

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