Studies on the interaction between the estrogen and vitamin D endocrine system

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Studies on the interaction between the estrogen and vitamin D endocrine system

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List of abbreviations

$1,25\text{-}(OH)_2D_3$	1α,25-Dihydroxyvitamin D_3
$25\text{-}(OH)D_3$	25-Hydroxyvitamin D_3
Alp	Alkaline phosphatase
BMD	Bone mineral density
Ca	Calcium
CaBP	Calcium binding protein
cAMP	cyclic-3',5' Adenosine monophosphate
DBP	Vitamin D binding protein
DEXA	Dual energy X-ray absorptiometry
DNA	Deoxyribonucleic acid
E_2	17β-Estradiol
ECaC1/2	Epithelial calcium channel 1/2
ED_{50}	Median effective dose
ERα/β	Estrogen receptor alpha/beta
IGF	Insulin-like growth factor
IGFBP	Insulin-like growth factor binding protein
kDa	kilo Dalton
α MEM	alpha Minimal essential medium
OVX	Ovariectomy
PBMC	Peripheral blood mononuclear cells
PCR	Polymerase chain reaction
PMCA	Plasma membrane calcium ATPase
PTH	Parathyroid hormone
RFLP	Restriction fragment length polymorphism
RIA	Radioimmunoassay
(m)RNA	(messenger) Ribonucleic acid
Sham	Sham-operated
SNP	Single nucleotide polymorphism
TGF α/β	Transforming growth factor alpha/beta
VDR	Vitamin D receptor

Chapter 1

INTRODUCTION

1.1 Calcium: Physiological functions and homeostasis

Calcium plays an essential role in intracellular and extracellular processes. It is well known that calcium functions as an intracellular second messenger. Extracellular calcium is important for the maintenance of the normal mineralization of bone, the stability of plasma membranes and the regulation of the permeability of plasma membranes to sodium ions. Calcium is also an essential component in the coagulation cascade. Because of the involvement in these critical processes, it is essential that the calcium concentration in the extracellular fluids and in the cytosol be maintained with great constancy. The extracellular ionized calcium concentration is maintained at $\sim 10^{-3}$ M, which is about 10.000-fold higher than the concentration of calcium in the cells. Two mechanisms are thought to maintain the low intracellular calcium concentration. Cytosolic calcium is to some extent buffered by binding to other cytoplasmic constituents, including the mitochondria and microsomes, and cells contain specific calcium binding proteins (CaBP), which may serve as a buffer and/or a calcium transport protein in the cytosol. The extracellular pool of calcium, which is about 1 gram, is in dynamic equilibrium with calcium that enters and exits the organism via the intestines, bones and renal tubules. In this respect, the skeleton is the most important storehouse of calcium as it contains about 99% of total body calcium in the form of hydroxyapatite. About 1% of the skeletal content is freely exchangeable with the extracellular fluids. In order to control the important calcium homeostasis, the fluxes between the three body compartments are strictly regulated via the classic calciotropic hormones, 1α,25-dihydroxyvitamin D_3 (1,25-$(OH)_2D_3$), parathyroid hormone (PTH) and calcitonin. However, over the last years several studies have indicated that 17β-estradiol (E_2) may also be involved in the control of the calcium fluxes between the body compartments.

1.2 Bone metabolism

Broadly, there are two types of bone: cortical bone and trabecular bone. There are structural and functional differences: Cortical bone is for 80%-90% calcified and fulfills mainly a mechanical and protective function, while trabecular bone is for 15-25% calcified (the remainder being occupied by bone marrow, blood vessels, and connective tissue) and has a metabolic function. The mineralized matrix is composed of hydroxyapatite crystals fixed to collagen fibers (predominantly collagen type I, 90% of the total protein), and noncollagenous proteins. The collagen fibers are usually oriented in a preferential direction, giving bone a lamellar structure.

Three major cell types are found in bone, namely osteoclasts, osteoblasts and osteocytes[1-4]. Osteoclasts are large, multinucleated bone–resorbing cells derived from hematopoietic precursors of the monocyte/macrophage lineage. They are formed by the fusion of mononuclear cells. At the conclusion of the resorbing phase of bone, osteoclasts lose their ruffled border, detach from the surfaces of mineralized bone matrix and move away and/or undergo apoptosis.

Osteoblasts originate from pluripotent mesenchymal stem cells, which can also differentiate into chondrocytes, adipocytes, myoblasts, and fibroblasts. Active osteoblasts are found within a matrix which they synthesize, and which subsequently mineralizes. The area between the osteoblast and the mineralization front is the so-called osteoid tissue. Toward the end of the secreting period, the osteoblasts become either a flat lining cell or an osteocyte.

Osteocytes are encased in the calcified matrix in osteocyte lacunae. The cells have multiple long processes, which are in contact with processes of other osteocytes or bone lining cells. These processes form a network of thin canaliculi. The osteocyte probably plays a role as mechanosensor in bone.

Bone is continuously remodeled throughout life. In the normal adult skeleton, bone formation occurs only where bone resorption has previously occurred. Under physiological conditions there is a balance between bone resorption and bone formation at discrete sites, termed bone-remodeling units (BRU)[5]. The regulation of bone turnover involves a complex interplay between mechanical stimuli, systemic hormones and locally produced cytokines and growth factors. The most important factors involved in regulation of bone remodeling are summarized in Table 1.1, and will, except for 1,25-$(OH)_2D_3$ and E_2, not be discussed in further detail.

Table 1.1 *Hormones and skeletal growth factors that regulate bone remodeling*

Systemic hormones	*Bone derived factors*
Parathyroid hormone	Insulin-like growth factors
Calcitonin	Transforming growth factor β family of peptides, including bone morphogenetic proteins
Insulin	Fibroblast growth factors
Growth hormone	Platelet-derived growth factors
1,25-Dihydroxyvitamin D_3	Cytokines of the interleukin, tumor necrosis factor and colony-stimulating factor families
Sex steroids	Receptor activator of NK-κB ligand (RANKL)
Glucocorticoids	Osteoprotegerin
Thyroid hormone	

In osteoporosis, defined as a systemic skeletal disease, characterized by low bone mass and microarchitectural detoriation of bone tissue with a consequent increase in bone fragility and susceptibility to fracture[6], there is an increased bone turnover and an imbalance between bone formation and bone resorption, favoring bone resorption. The risk for osteoporotic fractures is determined by both quantity and quality of bone[7].

Bone mass is best reflected by bone mineral density (BMD)[8]. This is currently the sole factor that can be accurately measured by several non-invasive techniques, such as dual energy X-ray absorptiometry (DEXA), single photon absorptiometry and ultrasound. BMD is at this moment the best available indicator of fracture risk.

Table 1.1 *Diagnostic criteria for osteoporosis*

Diagnosis	Criteria
Normal	A value for BMD not more than 1 SD below the average value of young adults (T-score > -1)
Osteopenia	A value for BMD more than 1 SD below the young adult average, but not more than 2.5 SD below (T-score > -2.5 and < -1)
Osteoporosis	A value for BMD more than 2.5 SD below the young adult average value (T-score < -2.5)
Established Osteoporosis	A value for BMD more than 2.5 SD below the young adult average value and the presence of one or more fragility fractures

T-score: number of standard deviation (SD) difference in bone mineral density (BMD) from the young adult average (premenopausal mean)

Bone quality is determined by structural and material properties (e.g. degree of mineralization, collagen cross linking, and microdamage) and the rate of bone turnover[9]. Bone turnover can be estimated by measuring bone matrix components released into the circulation. Biochemical markers of bone turnover represent either the enzymes involved in bone formation and bone resorption or the formation and degradation products of bone matrix metabolism (Table 1.3). This introduction will further point out the mechanism of action and function of the sex hormone E_2 and 1,25-$(OH)_2D_3$ with respect to calcium and bone metabolism.

Table 1.3 Most frequently used markers of bone turnover

Bone formation	**Bone resorption**
Alkaline phosphatase	Hydroxyproline
Osteocalcin	Deoxypyridinoline and pyridinoline
Procollagen peptides	Tartrate-resistant acid phosphatase
	Galactosyl-hydroxylysine

1.3 Introduction on estrogen metabolism, mechanism of action and function

1.3.1 Synthesis, transport and metabolism of 17β-estradiol

E_2 is synthesized from cholesterol (Figure 1.1). The last step in the synthesis of E_2 is the aromatization of testosterone. The aromatase enzyme converts testosterone to E_2 and androstenedione to estrone. In women with intact ovarian function E_2 is mainly produced by granulosa cells in the follicles. In the absence of normal ovarian function, such as in postmenopausal women, estrogens are mainly synthesized from androgens produced by the adrenal gland. Aromatase activity is also found in peripheral tissues such as fat tissue, placenta, brain tissue and fibroblasts, and in osteoblast and breast tumor cell lines[10-14]. Aromatase activity in ovaries is under influence of luteinizing and follicle stimulating hormone. Recently, Kinuta et al. have suggested that also 1,25-$(OH)_2D_3$ plays an important role in estrogen biosynthesis in both female and male gonads[15]. Up to 70% of the total circulating E_2 is bound weakly to sex hormone-binding globulin or albumin[16]. Estrogens are primarily metabolized in the liver, secreted in the bile, reabsorbed into the blood and excreted by the kidney.

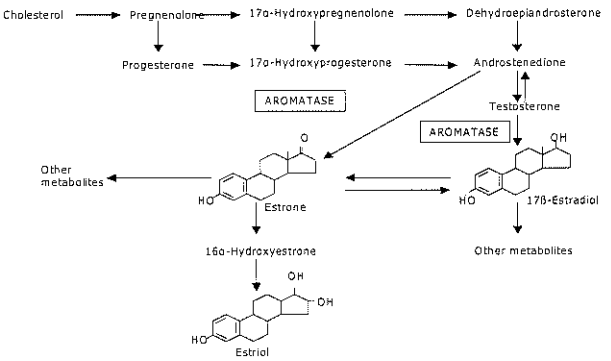

Figure 1.1 Both testosterone and estrone are substrates for 17β-estradiol synthesis. Testosterone is converted by aromatase, whereas estrone is metabolized by a 17β-hydroxysteroid dehydrogenase.

1.3.2 Biological actions of 17β-estradiol

E_2 not only acts on its classic target organs, vagina, uterus and mammae, but also on the cardiovascular system and central nervous system. Furthermore estrogens influence the synthesis of growth factors and their receptors (e.g. insulin-like growth factor (IGF)-I and II, transforming growth factor (TGF) α and β, IGF-I receptor and epidermal growth factor receptor), steroid hormone receptors (e.g. ERα and ERβ, vitamin D receptors (VDRs),

glucocorticoid receptors), peptide hormones (prolactin, luteinizing hormone, follicle stimulating hormone) and protooncogenes (c-fos, c-jun, c-myc, ras)[17]. Besides 1,25-$(OH)_2D_3$ and PTH, E_2 is also recognized as an important regulator of calcium homeostasis, acting on bone, intestine and kidney[18]. The effects of E_2 are primarily mediated by the estrogen receptor (ER), a member of the nuclear receptor superfamily[17]. The ER exists as two subtypes, ERα and ERβ. The presence of ERα and ERβ has been described in numerous tissues, like bone, kidney and intestines[17]. In bone, ERs are expressed in osteoblasts, osteocytes[18-21] and osteoclast precursors[22].

1.3.2.a Estrogen effects on bone

E_2 maintains the balance between bone formation and bone resorption[23,24]. A major action of E_2 on bone is to inhibit bone resorption, mostly by indirect actions of E_2. The antiresorptive effect of E_2 might be mediated by induction of osteoclast apoptosis[25], by down-regulation of bone resorption-inducing cytokines, like interleukin 1, interleukin 6, tumor necrosis α (TNFα), macrophage colony stimulating factor, granulocyte macrophage colony-stimulating factor and prostaglandin-E_2[26-30] and receptor activator of NF-κB (RANK)[31,32], or by up-regulation of TGFβ, which is generally considered to suppress osteoclast activity and to increase osteoclast apoptosis[33-35], although recent studies suggested that TGF-β might also play a central role as co-stimulator for osteoclast formation[2].

In addition to inhibition of bone resorption, a direct effect of E_2 on osteoblast proliferation and/or differentiation may play a role in the beneficial effect of E_2 on bone mass. The effects of E_2 on osteoblasts may be dependent on the stage of osteoblast differentiation. Di Gregorio et al. demonstrated that E_2 attenuates the self-renewal of early osteoblast progenitors in murine bone marrow by approximately 50%[36] *in vitro* and *in vivo*. E_2 decreases cell proliferation and differentially regulates extracellular matrix expression in human fetal osteoblast cells[37]. Thus, E_2 may control rate of bone turnover by suppression of osteoblastogenesis and thereby suppression of osteoclast activation.

Other studies demonstrated that E_2 might have a direct anabolic effect on osteoblasts. A stimulatory effect of E_2 on proliferation and differentiation of osteoblast-like cells from male and female trabecular bone has been reported[38]. Also direct stimulating effects on *in vitro* alkaline phosphatase activity by osteoprogenitor cells have been shown[39,40]. E_2 *in vitro* directly augments the proliferation and differentiation, ERα expression, and mRNA expression of genes for alkaline phosphatase, collagen type I, TGFβ, BMP-2, and cbfa1 in mesenchymal stem cells isolated from mouse bone marrow[41]. These direct anabolic effects of E_2 are

thought to be predominantly directed via ERα[41,42]. The effects of E_2 on osteoblasts may be mediated by nitric oxide[43-45], synthesis of which is stimulated by E_2[46,47].

Also modulating effects of E_2 on effects of PTH and 1,25-$(OH)_2D_3$ on bone have been suggested[48]. E_2 may reduce the end organ responsiveness to PTH[49-52] and enhance the effect of 1,25-$(OH)_2D_3$[53] on alkaline phosphatase activity[54], possibly by up-regulation of the VDR in osteoblastic cells[55].

Finally, E_2 may play an important role in bone metabolism and modulation of the sensivity of bone cells to mechanical stress[56] by controlling osteocyte apoptosis and the maintenance of osteocyte viability[57].

Several *in vivo* studies have shown bone preservative effects of E_2. In rats, E_2 has been shown to inhibit the decrease in trabecular number, to increase the trabecular thickness, to reduce osteoclast number and resorption activity, and to reduce mineral apposition and bone formation rates[58].

After menopause, when serum E_2 falls, bone resorption outpaces bone formation. This may ultimately result in osteoporosis. In postmenopausal women hormone replacement therapy reduces the resorptive activity at the BRU level[59]. This may result in increased BMD at the femoral neck and lumbar spine[60-63], and may reduce the risk of osteoporotic fractures[64].

Also in men estrogens play an important role in bone metabolism[65]. Men lacking the enzyme aromatase due to a gene mutation have low bone density and delayed bone maturation[66]. Treatment of these patients with E_2 improves bone mineral density (BMD) and closure of the epiphyses[67,68].

1.3.2.b Estrogen effects on intestines

Arjmandi et al. showed that E_2 *in vitro* enhances calcium uptake in isolated intestinal epithelial cells[69]. Several studies have focused on the *in vivo* effect of E_2 on intestinal calcium absorption. The only longitudinal study of the effect of natural menopause on intestinal calcium absorption published, shows that menopause is associated with a small fall in intestinal calcium absorption[70]. There was also an effect of age independent of menopause. After ovariectomy, intestinal calcium absorption was also found to fall[71], but in two cross-sectional studies no effect of menopause was found[72,73]. The effects of estrogen deficiency and replacement could be dependent on calcium intake[74], which may explain the different outcome of the studies. Both direct[75,76] and indirect effects[71,77-81] on intestinal calcium absorption have been suggested.

1.3.2.c Estrogen effects on kidneys

E_2 deficiency is associated with a rise in renal calcium excretion, which is reversed by E_2 replacement[82,83]. It is suggested that this rise is partly due to increased filtered load of calcium but also to reduced tubular reabsorption of calcium[84]. The increased renal calcium conservation after estrogen replacement therapy may be indirect via an increase in serum PTH, increased responsiveness to PTH[80] or a direct effect of E_2[85-89] independent of PTH action. Recently, van Abel et al. demonstrated in 1α-hydroxylase knock-out mice that E_2 increases calcium channel (ECaC1) mRNA expression in the kidney[89].

1.4 Introduction on vitamin D metabolism, mechanism of action and function

1.4.1 Synthesis, transport and metabolism of vitamin D

The seco-steroid vitamin D_3 is either synthesized from 7-dehydrocholesterol in skin exposed to ultraviolet irradiation[90] or is obtained from diet (fatty fish, fish oil, vitamin D_3 fortified food). In the liver the inactive precursor vitamin D_3 is converted to the hormonal precursor 25-hydroxyvitamin D_3 (25-(OH)D_3) by the cytochrome P_{450}-enzyme 25-hydroxylase[91-92]. 25-(OH)D_3 enters the blood stream and is the major circulating form of vitamin D. Because the synthesis of 25-(OH)D_3 is not tightly regulated, an increase in the subcutaneous production of vitamin D_3 or ingestion of vitamin D will result in an increase in circulating 25-(OH)D_3.
25-(OH)D_3 is predominantly converted into the most active metabolite 1,25-$(OH)_2D_3$ by the cytochrome P_{450}-mono-oxygenase 25-(OH)D-1α-hydroxylase in the distal convoluted tubule and to a lesser extent in the proximal convoluted and straight tubules of the kidney (Figures 1.2 and 1.3)[93]. This enzyme is under stringent control. PTH, but also other factors (low serum calcium, E_2, IGF-I, calcitonin) have a stimulatory effect on 1,25-$(OH)_2D_3$ synthesis in the kidney[94-100]. 1,25-$(OH)_2D_3$ stimulates the conversion of 25-(OH)D_3 into 24,25-$(OH)_2D_3$ by the renal enzyme 24-hydroxylase[99] and inhibits the 25-(OH)D_3-1α-hydroxylase enzyme and thereby limits its own synthesis[92]. Furthermore 1,25-$(OH)_2D_3$ has a direct inhibitory effect on the production and secretion of PTH by the parathyroid glands[93,101,103]. Catabolism of 1,25-$(OH)_2D_3$ occurs through sequential oxidations at the C-24 or C-26 position of the molecule in essentially all its target tissues[103,104]. A third metabolic pathway that metabolizes 1,25-$(OH)_2D_3$ into 1,25-$(OH)_2$-3-epi-D_3 has recently been demonstrated in human keratinocytes and colon carcinoma cells, rat osteoblastic cells and bovine parathyroid cells[105-107]. Over the years data have accumulated that metabolites formed in the C-24 hydroxylation

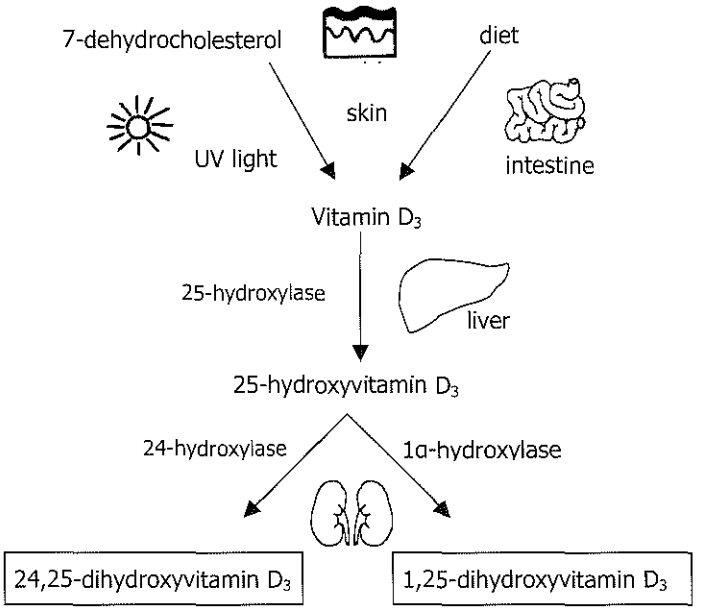

Figure 1.2 Metabolic activation of vitamin D.

cascade[108-110] and 3-epi metabolites[111] have biological activity. All metabolites are ultimately eliminated by biliary excretion.

Vitamin D_3 and its metabolites circulate in the blood primarily after binding to the vitamin D binding protein (DBP). In plasma, only 0.4% of $1,25\text{-}(OH)_2D_3$ and 0.04% of $25\text{-}(OH)D_3$ is present in an unbound form. More than 85% is bound to DBP. The rest is bound to albumin (about 12-15%) and low density lipoproteins[112].

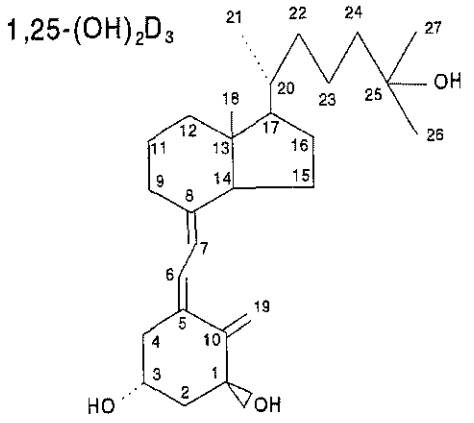

Figure 1.3 Chemical structure of the seco-steroïd 1,25-dihydroxyvitamin D_3 (1,25-$(OH)_2D_3$)

1.4.2 Biological actions of vitamin D

Like E_2, 1,25-$(OH)_2D_3$ exerts its action via a nuclear receptor, the vitamin D receptor (VDR)[113]. The VDR was originally found in the classic vitamin D target organs involved in mineral homeostasis: the intestine, bone, kidney, and the parathyroid glands. Via VDRs in non-classic vitamin D target tissues, 1,25-$(OH)_2D_3$ is also involved in immunomodulation, control of other hormonal systems (growth hormone, prolactin), inhibition of cell growth and induction of cell differentiation[114-117].

1.4.2a Vitamin D and intestinal calcium transport

The major biological function of 1,25-$(OH)_2D_3$ is to maintain calcium homeostasis by increasing the efficacy of the intestinal cells to absorb dietary calcium. Intestinal calcium absorption is related to serum 1,25-$(OH)_2D_3$ concentrations[118]. Possible targets involved in vitamin D-dependent active duodenal calcium absorption are the entry of calcium through the plasma membrane into the enterocyte, the intracellular transfer by calbindin-D_{9K}[119-121], and the calcium extrusion by the plasma membrane calcium ATPase (PMCA)[122-126]. Recently, apical membrane calcium influx channels have been described in rabbit[127], rat[128] and human[129] intestinal epithelial cells. These epithelial calcium channels are vitamin D

dependent[130,131] and have been suggested to be important in regulating intestinal calcium absorption[127,129].

Calbindins (CaBP) occur in high concentration in several calcium-transporting tissues, like intestine, kidney and placenta, in which the expression is vitamin D dependent[132,133]. They are also found in lower concentrations in other tissues, such as the brain, pancreas and parathyroid gland, in which the expression is vitamin D independent[134-136]. They have been shown to bind calcium with high affinity. Serum 1,25-$(OH)_2D_3$ levels have been shown to correlate with intestinal CaBP-D_{9K} levels in rats[119] and humans[137], renal CaBP-D_{28K} levels in rats[119] and with the rate and time course of calcium transport from the microvillar region to the basolateral membrane[116,134,138,139]. In this respect, CaBP is thought to serve as both a calcium translocater and a cytosolic calcium buffer[140,141].

The extrusion of Ca^{2+} from the enterocyte cytosol into the lamina propria is mediated by the plasma membrane calcium pump (PMCA) and the sodium/calcium exchanger. PMCA (MW 135 kDa) transports one Ca^{2+} per ATP[137]. The activity is for about 50% dependent on calmodulin, which increases both affinity of the pump for calcium and the maximum rate of Ca^{2+} transport[138,143,144]. PMCA has an approximately 2.5 fold greater affinity for Ca^{2+} than does CaBP-D_{9K}[145], which has about a fourfold greater affinity for Ca^{2+} than do components of the brush-border[146,147]. Like the calbindins, expression of PMCA in rats is vitamin D dependent[123,124,148,149]. It is currently unknown whether expression of PMCA is also vitamin D dependent in humans. So far, in one study serum 1,25-$(OH)_2D_3$ levels were not correlated with PMCA expression[137].

The sodium/calcium exchanger has been estimated to account for 20% of calcium extrusion in rat duodenum. It is dependent on the Na^+ gradient and is responsive to a negative intracellular potential[150], while it is not vitamin D dependent[143].

Several studies have suggested that the effect of 1,25-$(OH)_2D_3$ on intestinal calcium transport is modulated by estrogen status[72,79-81] and by the enterocyte VDR level[151]. However, there is increasing *in vitro* evidence that 1,25-$(OH)_2D_3$ also stimulates rapid intestinal calcium transport (transcaltachia) via nongenomic pathways, i.e. paracellular passive transfer[152] and transcellular transfer via endocytotic-exocytotic vesicular transport[153-155]. The actual contribution of these nongenomic paracellular and vesicular pathways to 1,25-$(OH)_2D_3$ mediated intestinal calcium absorption remains to be elucidated.

1.4.2b Vitamin D and bone

Vitamin D is essential for normal growth and bone mineralization[156]. Vitamin D deficiency results in rickets during childhood and osteomalacia in adults. 1,25-$(OH)_2D_3$ exerts its action on osteoblasts via the VDR and regulates their growth and differentiation[157]. The synthesis of bone matrix proteins (e.g. osteocalcin, alkaline phosphatase, type I procollagen) and the synthesis of certain cytokines and growth factors (e.g. TGFβ, IGF-I, IGFBPs, RANKL) by osteoblasts are regulated by 1,25-$(OH)_2D_3$[158-168]. Recently, 1,25-$(OH)_2D_3$ has been shown to increase the plasma membrane calcium pump protein and function located on the apical plasma membrane of osteoblast-like cells[169]. In this way, 1,25-$(OH)_2D_3$ might induce calcium uptake by osteoblasts, which is necessary for bone mineralization.

1,25-$(OH)_2D_3$ also maintains normal serum calcium and phosphate by inducing bone resorption through enhancement of osteoclastogenesis[170] through stimulation of the differentiation and fusion of osteoclast progenitors into osteoclasts. Furthermore 1,25-$(OH)_2D_3$ stimulates osteoclastic activity. 1,25-$(OH)_2D_3$ is thought to mediate its resorptive action via stimulated secretion of specific factors by the osteoblasts (see Chapter 1.2)[171,172]. In view of the reported VDR mRNA transcripts in osteoclasts, 1,25-$(OH)_2D_3$ might also exert direct effects on active bone resorbing cells[173].

Finally, 1,25-$(OH)_2D_3$ may regulate serum calcium concentration by downregulation of calcitonin receptor expression in newly formed osteoclasts[174]. Calcitonin is secreted by C-cells in the thyroid gland and reduces the number and activity of osteoclasts[175].

Several groups have investigated the effect of vitamin D_3 or 1,25-$(OH)_2D_3$ suppletion on osteoporotic fracture risk. Ooms et al. showed that vitamin D_3 suppletion in elderly women increased femoral neck BMD[176]. However, no beneficial effect of vitamin D_3 suppletion was measured on hip fracture risk[177-179] and vertebral fracture risk[177]. Treatment with 1,25-$(OH)_2D_3$ in postmenopausal osteoporotics with vertebral fractures increases spine density[180-181], and reduces the incidence of vertebral fractures in some studies[182], but not others[183].

Two clinical trials have been directed to the effect of combined vitamin D and calcium suppletion in elderly women on osteoporotic fracture risk. In both studies a reduction of non-vertebral fractures was found[184,185]. Chapuy et al. also demonstrated a beneficial effect on hip fracture risk[184].

The differences in effects of vitamin D and/or calcium suppletion in these trials may be related to differences in preexistent calcium and vitamin D intake in the study populations. For example, in the French study population[184] dietary calcium intake was on average lower than in the Dutch study population[176,179]. In view of these differences in calcium and vitamin

D intake, vitamin D and calcium suppletion are given in both placebo and treatment groups in clinical trials with agents directed to prevention of osteoporotic fractures.

1.4.2c Vitamin D and parathyroid glands

PTH and 1,25-$(OH)_2D_3$ mutually regulate their synthesis via a negative feedback loop. PTH stimulates 1,25-$(OH)_2D_3$ production and calcium reabsorption in the kidney. 1,25-$(OH)_2D_3$ in turn decreases the synthesis and secretion of PTH directly via the chief cells in the parathyroid gland[186,187] and indirectly via increasing intestinal calcium absorption[188]. In addition to systemic interaction, PTH and 1,25-$(OH)_2D_3$ act at target level in an interrelated manner[189]. 1,25-$(OH)_2D_3$ reduces PTH-stimulated cAMP production, while activation of the cAMP pathway results in enhancement of the 1,25-$(OH)_2D_3$ action in osteoblasts[178]. The protein kinase C pathway has been shown to attenuate the 1,25-$(OH)_2D_3$ action in osteoblasts[189]. Whereas PTH is the principal hormone involved in the rapid (within minutes) response to changes in serum calcium levels, 1,25-$(OH)_2D_3$ plays a key role in longer-term control of calcium homeostasis.

1.4.2d Vitamin D and kidney

Another target for 1,25-$(OH)_2D_3$ in the regulation of calcium homeostasis is the kidney. In the kidneys calcium reabsorption takes place via a saturable transcellular process and via a nonsaturable paracellular process[146,190]. In the proximal tubules calcium reabsorption is predominantly paracellular in a Na-dependent manner, while only a small amount is reabsorbed via a transcellular pathway, of which the mechanism still has to be elucidated. 1,25-$(OH)_2D_3$ has no detectable effect on calcium transport[191] and enhances phosphate reabsorption[192,193] by the proximal tubule. In the distal tubule 1,25-$(OH)_2D_3$ may stimulate active calcium reabsorption[128,141] by increasing epithelial calcium channels[194,195], inducing CaBP-D_{28K} synthesis[190,196] and/or CaBP-D_{9k} synthesis[197] or by stimulating basolateral membrane Ca^{2+}-ATPase activity[191]. Finally, 1,25-$(OH)_2D_3$ may modulate the PTH-dependent calcium reabsorption[146].

1.5 Introduction on genetic factors of osteoporosis

1.5.1 Genetic factors and bone mass

Factors that determine fracture risk are skeletal factors (see Chapter 1.2) and other factors, like nutrional factors (calcium and alcohol intake), life style factors (smoking and physical activity), body weight, age, and diseases and medications affecting bone metabolism, such as diabetes mellitus and chronic glucocorticoid use[198-201]. In the last decades twin- and family studies have shown that also genetic factors play an important role in the pathogenesis of osteoporosis. These studies showed that as much as 60-80% of the variation in age-specific BMD at various sites is genetically determined[202-204]. Also other skeletal factors as measured by ultrasound, hip axis length and bone turnover parameters have been demonstrated to be under genetic control[25-207].

Osteoporosis can be described as a 'complex trait'. This refers to a common disease that is polygenetic (multiple genes are involved), has a multifactorial nature (environmental and genetic conditions may interact) and usually has a late onset with variable clinical manifestations. To identify the genes determining osteoporosis, two major genetic approaches have been taken, namely the candidate gene approach and genome searches. Candidate genes can be selected on the basis of their involvement in a particular biochemical pathway in bone metabolism. Examples are the genes for the VDR, ER, collagen type I, TGFβ, and interleukin-6. The involvement of certain genes in bone biology can be established when the gene product is specifically expressed and/or functional in bone tissue or when the gene-of-interest is knocked-out in mice and a bone-phenotype occurs. Furthermore, candidate genes can be identified by studying the transmission of a mutation in pedigrees suffering from monogenetic disorders that lead to distorted bone metabolism. Drawbacks of this approach are the detailed molecular genetic knowledge on the gene needed before it can be studied, and the biased view it may provide, because only one selected gene is studied.

A more objective approach is the genome search. Genome searches are based on the assumption that individuals with the same disease phenotype, e.g. low BMD, share one or more chromosomal regions containing a sequence variant of one or more osteoporosis genes derived from a common ancestor. Osteoporosis is a polygenic disease with partial contributions of different genes. Therefore, a major drawback of this approach is that samples from hundreds to thousands of well characterized patients and their relatives have to be collected.

Crucial in any genetic analysis is detection of variations in the basepair sequence (polymorphisms) of the genomic or mitochondrial DNA and detection of variations in polymorphisms between individuals with and without the disease of interest. Sequence variants are termed 'polymorphisms' if they occur at population frequency of one percent or more and 'mutations' if they occur at frequencies of less than one percent. There are two main types of DNA sequence polymorphisms: single nucleotide polymorphism (SNP) and tandem repeats of short sequence motifs. A common method to detect SNPs is by studying restriction fragment length polymorphism (RFLP) within a PCR fragment generated from the locus and detect them by electrophoretic separation. Examples include the *Bsm*I, *Apa*I, and *Taq*I polymorphisms in the VDR gene and the *Pvu*II and *Xba*I polymorphisms in the ERα gene. A combination of alleles of adjacent loci is called a haplotype.

RFLPs can be divided into anonymous and functional polymorphisms. Anonymous RFLPs are used as markers for a functional sequence variation elsewhere in the gene or a nearby gene. The use of anonymous polymorphisms as markers in osteoporosis makes testing much more feasible since they are abundant in the human genome. If one of the marker alleles is positively associated with the phenotype of interest, one assumes that the marker allele is in linkage disequilibrium (LD) with a functional polymorphism elsewhere in the gene or in another gene. Such LD is not constant among different populations and can contribute to controversial results. Functional polymorphisms can include sequence variations leading to alterations in the amino acid composition of the protein, changes in the 5' promotor region leading to differences in expression, and/or polymorphisms in the 3' region leading to differences in mRNA degradation. In general, haplotypes are the most desirable definition of DNA sequence variation in a certain region, across functional parts of a gene (such as the promotor and the 3'UTR) and across the complete gene. Haplotypes describe most accurately the combination of SNPs that act at a functional level, e.g. as combinations of alleles with functional SNPs in the promotor region of a gene. While two subjects can have identical genotypes for a specific SNP, they can have substantial differences at a functional level because of different haplotypes. Therefore, it is hazardous in association studies to select a single SNP because this can easily miss true associations. Direct haplotyping of several polymorphic sites in one gene is a method to increase the genetic resolution, but requires a concomitant increase in sample size to achieve stable and precise point estimates.

Controversial results may also be related to allelic heterogeneity, i.e. the candidate gene may be implicated in BMD, but different gene variants are associated with BMD in different

populations. The lack of association can then be explained by the fact that a given polymorphism is not testing for the candidate gene variant in a particular population and by using less informative genetic markers.

In the following paragraph two candidate genes for osteoporosis, the ERα and VDR, will be discussed.

1.5.1 Estrogen receptor polymorphisms

For the study described in Chapter 8 we identified three anonymous polymorphisms in the ERα gene by direct molecular haplotyping methods (see Chapter 8.4): a TA-repeat polymorphism in the promoter region 1174 bp upstream from the first exon and two polymorphisms (*PVU*II and *Xba*I) in the first intron, i.e. a T to C substitution 397 bp upstream from exon 2 (e2-397)[208] and an A to G substitution 351 bp upstream from exon 2 (e2-351)[209,210] (Figures 1.4 and 8.2). The variation in TA-repeats in the promotor region is in strong disequilibrium with the T to C substitution in the first intron[211,212,213]. These ERα gene polymorphisms have been found to be associated with osteoporotic phenotypes and response to hormonal replacement therapy[211,212,214-224], natural and surgical menopause[225], spontaneous abortion[226,227], breast cancer[210,228,229] and primary hyperparathyroidism[230]. Recently, an association between a polymorphism in the ERβ gene with bone mineral density was also described[231]. These supposedly anonymous ERα and ERβ gene polymorphisms are assumed to be in linkage disequilibrium with truly functional sequence variation(s) to explain the described associations. So far, no functional studies have been reported which provide insight in how these ER gene polymorphisms could influence the effects of E_2 on cells.

1.5.2 Vitamin D receptor polymorphisms

Sequence variation in the 3'-untranslated region of the VDR gene has been the primary site in the VDR polymorphism studies. This segment contains polymorphic restriction sites for the endonucleases *Bsm*I and *Apa*I in intron 8 and a third polymorphic site for the endonuclease *Taq*I in exon 9 (Figures 1.5 and 8.1). Haplotypes of the *Bsm*I, *Apa*I and *Taq*I RFLPs can be

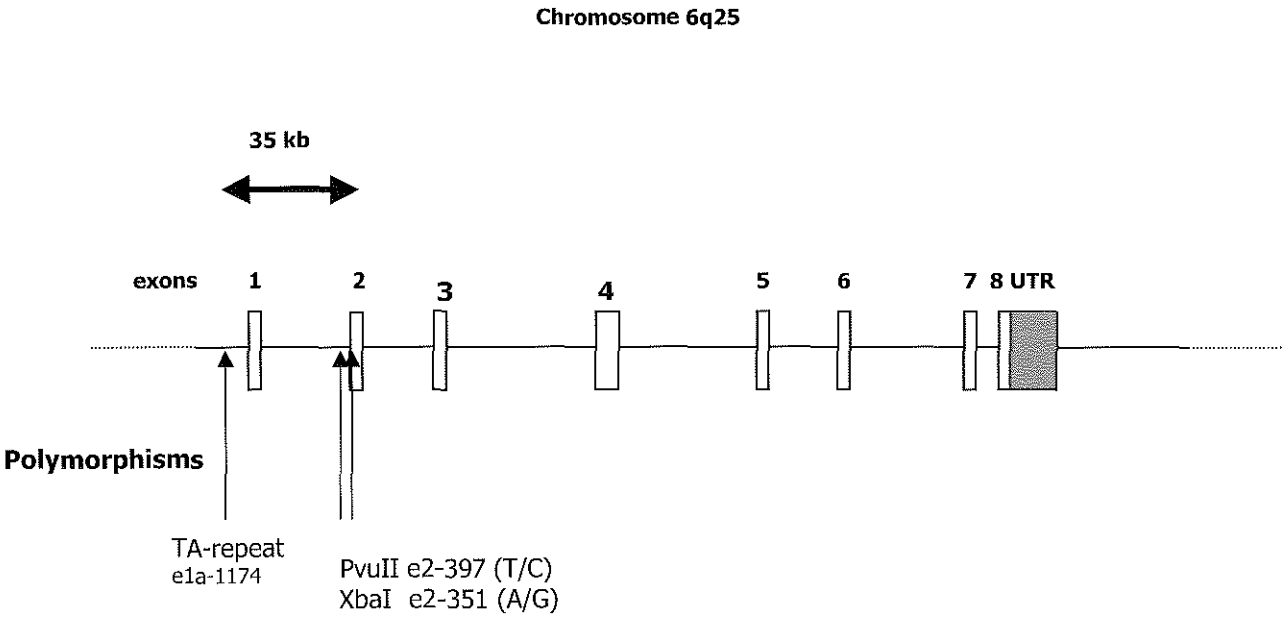

Figure 1.4 Polymorphisms in the estrogen receptor α gene

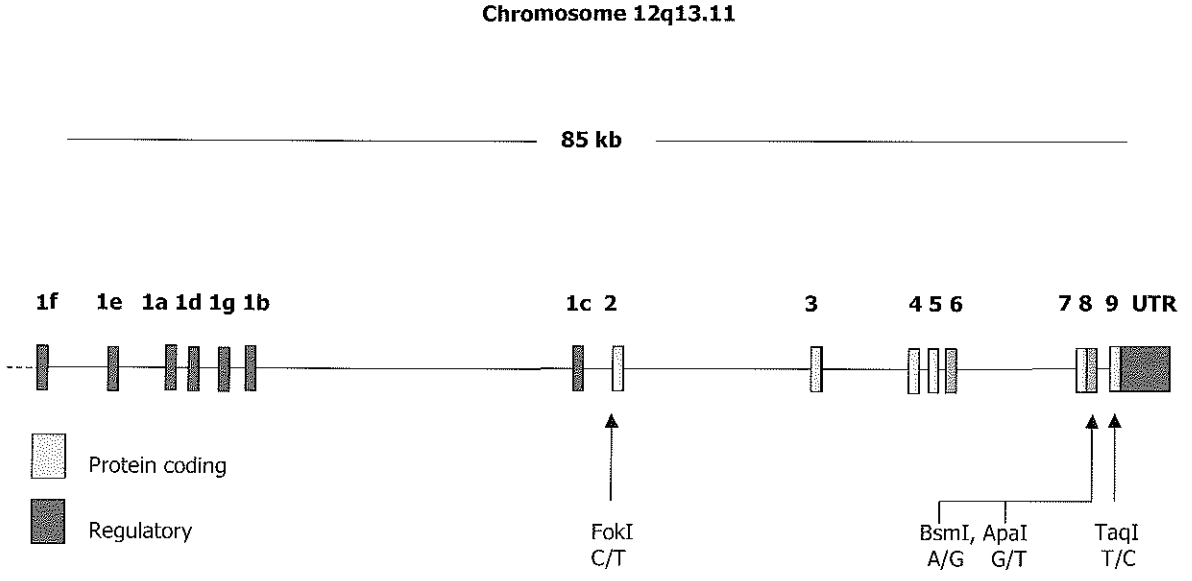

Figure 1.5 Polymorphisms in the vitamin D receptor gene

determined by direct molecular haplotyping methods as described previously[232]. Numerous studies have focused on the association between these anonymous VDR polymorphisms and BMD. Uitterlinden et al. found in 1782 Dutch elderly men and women from the Rotterdam Study, the largest study published so far, no association between single RFLP's and BMD[232]. However, a small but significant association with BMD was found using haplotypes of the three adjacent 3' RFLP's. In line with this observation, two meta-analyses showed a small effect of VDR genotype on BMD[233,234]. Only a few studies have focused on fracture risk. In two studies VDR genotypes have been associated with fracture risk[235,236], but this could not be confirmed in another study[237]. Some studies have suggested that the '*baT* ' allele (VDR haplotype 1) is associated with aberrant expression levels of VDR mRNA, possibly through changes in mRNA stability[238-241]. However, no consistent association between one allele and biological responses to treatment with vitamin D, calcium, hormone replacement therapy and bisphosphonates has been found by means of *in vitro* cell biological and molecular studies and using *in vivo* approaches[242-253].

Also at the 5'end of the gene a polymorphism has been identified[254,255]. This so-called *FokI* polymorphism is characterized by the presence of either two ATG start codons separated by 6 nucleotides or only the presence of the most 3' ATG codon due to a T to C substitution in the 5' ATG site. Thus, two variant forms of the VDR protein of 427 and 424 amino acids in length are translated. The shorter form was found to give the greatest transcriptional activity[254,256]. The longer form has been associated with low BMD by some authors[254,257-261], but others could not confirm this[262,263].

Recent investigations into the portion of the VDR gene encoding the 5' untranslated region have revealed a complex of at least seven exons with evidence for alternative splicing. It is possible that undiscovered common polymorphic sites exist in this newly described complex of multiple exons at the 5' end of the gene. Such polymorphisms could alter VDR protein structure[264] or might affect the activity of one of the three proposed VDR promotors, leading to the expression of altered quantities of VDR proteins under physiologic conditions[265].

1.6 Interactions between estrogen and vitamin D endocrine system

Several interactions between the estrogen and vitamin D endocrine system have been described. Firstly, E_2 and 1,25-$(OH)_2D_3$ may mutually affect each other's biosynthesis. Several studies have shown that E_2 stimulates 1,25-$(OH)_2D_3$ synthesis in the kidney[94,95,98,100]. On the other hand, 1,25-$(OH)_2D_3$ may play an important role in estrogen biosynthesis in both female and male gonads[15]. This is suggested by the fact that serum E_2 levels are decreased in VDR

knock-out mice as compared with wild-type mice. Furthermore, several studies have suggested that E_2 and 1,25-$(OH)_2D_3$ may regulate each other's receptor expression[17,80,266,267]. E_2 may up-regulate VDR expression in rat duodenal mucosa[80] and in osteoblastic cells[55] and concurrently enhance the effect of 1,25-$(OH)_2D_3$ on intestinal calcium transport[69,78,80,81] and alkaline phosphatase activity[54], respectively. On the other hand, cell-specific heterologous regulation of the ER by 1,25-$(OH)_2D_3$, has been demonstrated[267-269]. 1,25-$(OH)_2D_3$ increases ER expression in osteoblast-like cells[268], while 1,25-$(OH)_2D_3$ exerts a negative effect on ER expression in MCF-7 human breast cancer cells[267,269]. Also some genetic studies have focused on the interaction between the estrogen and vitamin D endocrine system. Recently, interactions between ERα and VDR genotypes with respect to BMD[217,270,271] and growth of infants[272] have been reported.

1.7 Scope of the thesis

Estrogen deficiency and vitamin D deficiency play key roles in the pathogenesis of postmenopausal osteoporosis. Aim of the studies in this thesis is to extend our knowledge on the interaction between E_2 and 1,25-$(OH)_2D_3$ and thereby to provide more insight into the significance of E_2 for 1,25-$(OH)_2D_3$ mediated processes in calcium and bone metabolism. Furthermore the significance of VDR genotypes for the biological response to 1,25-$(OH)_2D_3$ and the interaction between ERα and VDR genotypes in relation to BMD and fracture risk are studied.

The first part of the thesis focuses on the effect of E_2 on 1,25-$(OH)_2D_3$ mediated processes. In chapters 2 and 3 the effect of E_2 deficiency and E_2 repletion on 1,25-$(OH)_2D_3$ synthesis and 1,25-$(OH)_2D_3$ mediated intestinal calcium absorption in a rat model for postmenopausal osteoporosis is described. An important bone anabolic factor is IGF-I. Both 1,25-$(OH)_2D_3$ as well as E_2 have been shown to regulate IGF-I expression *in vitro*. Chapter 4 describes the effect of E_2 deficiency and E_2 repletion on IGF-I levels in bone *in vivo* in relation to bone metabolism. The effect of E_2 on bone mineralization is discussed in Chapter 5.

The second part of the thesis considers the significance of VDR genotypes for the biological response to 1,25-$(OH)_2D_3$ and the interaction between ERα and VDR gene polymorphisms in relation to BMD and fracture risk. Chapter 6 describes a pilot study addressed to whether differences in rates of bone turnover between women with either extremely low or extremely high BMD can be ascribed to genetic variations of the VDR. Furthermore, the biochemical response to short-term substitution of 1,25-$(OH)_2D_3$ in both BMD groups was related to VDR gene polymorphisms. In chapter 7 functional consequences of VDR gene polymorphisms *in*

vitro are studied. Chapter 8 discusses the association between ERα gene polymorphism and fracture risk in postmenopausal women. Moreover, the interaction between ERα gene and VDR gene polymorphisms on fracture risk is described. In chapter 9 the results are discussed and suggestions for future research are made. Finally, the main findings are summarized in Chapter 10.

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Part One

Interactions between estrogens and 1,25-dihydroxy-vitamin D_3 in relation to calcium and bone metabolism

Chapter 2

Calcium and bone metabolism in the rat: interactions between 17ß-estradiol and the calciotropic hormones

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2.1 ABSTRACT

This study investigated the regulation of calcium and bone metabolism during estrogen supplementation in relation to the calciotropic hormones 1,25-$(OH)_2D_3$ and PTH. Three months old female Brown Norway rats were sham operated (Sham) or ovariectomized. In ovariectomized rats either a placebo pellet (OVX) or a 0.05 mg 17ß-estradiol (E_2) 60 day-release pellet was subcutaneously implanted (n=12 per group). Rats were sacrificed after four weeks. Serum E_2 level in the E_2 group was at estrous level and in OVX rats very low. Uterus and spleen weights were as expected for ovariectomy and estrogen treatment.

Calcium metabolism in a broader perspective was examined by measuring various serum and urine markers. Serum PTH concentration and serum calcium, phosphate and alkaline phosphatase levels were similar in all groups. Urinary calcium excretion was significantly increased in the E_2-supplemented rats, while it was unchanged in the ovariectomized rats compared to the control rats. A strong decrease of about 70% in serum 1,25-$(OH)_2D_3$ concentration was observed in the E_2-supplemented group compared to sham and ovariectomized rats. No difference in vitamin D binding protein levels between these groups was detected. As a consequence the estimated free 1,25-$(OH)_2D_3$ levels were also significantly decreased in the E_2-supplemented group compared to the Sham and OVX groups. In contrast to the strong reduction in serum 1,25-$(OH)_2D_3$ levels, the serum 25-$(OH)D_3$ was only reduced by about 15% ($p < 0.01$) in the E_2-supplemented group. Together, concerning calcium homeostasis the following observations were made: E_2 supplementation resulted in 1) no change in serum calcium, 2) rise in bone mineral density, 3) increased urinary calcium excretion, while 4) 1,25-$(OH)_2D_3$ decreased. In conclusion, these changes can only be explained by a E_2-enhanced intestinal calcium absorption either directly or via an effect on 1,25-$(OH)_2D_3$-induced calcium absorption. In general, the present study in rats demonstrates a close interaction between the sex-steroid E_2 and 1,25-$(OH)_2D_3$ in the regulation of calcium homeostasis. These data thereby support the significance of E_2 in the regulation of calcium and bone metabolism.

2.2 INTRODUCTION

Calcium homeostasis in mammals is mainly regulated by two classical calciotropic hormones 1,25-$(OH)_2D_3$ and PTH. However, estrogens also interact with calcium and bone metabolism. It has been shown that the decrease in calcium absorption and bone mineral density in postmenopausal osteoporosis can be corrected by estrogen therapy[1-5].

Several mechanisms for the calciotropic action of estrogens in man have been proposed. Firstly, estrogens may influence the renal synthesis of 1,25-$(OH)_2D_3$ by potentiating PTH-mediated

stimulation of renal 1,25-$(OH)_2D_3$ formation[2]. Secondly, estrogens may directly affect the action of 1,25-$(OH)_2D_3$ as shown by the observation that the decreased 1,25-$(OH)_2D_3$-induced intestinal calcium absorption in ovariectomized women can be corrected by estrogen supplementation[4]. Thirdly, estrogens may change the setpoint for PTH secretion[6,7] and inhibit bone cell responsiveness to PTH[8-10]. Furthermore, estrogens are believed to directly affect bone metabolism by suppressing cancellous bone remodeling and maintaining remodeling balance between osteoblastic and osteoclastic activity[11,12]. Finally, estrogens might directly stimulate intestinal calcium absorption[13] via estrogen receptors, which have recently been shown in rat intestinal epithelial cells[14].

It can therefore be supposed that the classical calciotropic hormones and estrogens do not act independently but that there is a close interaction between them with respect to calcium and bone metabolism. In the present study we focused on this relationship between estrogens and the major calciotropic hormones 1,25-$(OH)_2D_3$ and PTH in an *in vivo* rat model. The rat is an established model to study bone metabolism under estrogen-deficient and estrogen-repleted conditions[15]. Calcium and bone metabolism parameters in relation to changes in the 1,25-$(OH)_2D_3$ and PTH were examined under these conditions.

2.3 MATERIALS AND METHODS

Animals

Thirty-six 13 weeks old female Brown-Norway rats were divided into three equal groups. In sham-operated (sham) and bilaterally ovariectomized (OVX) groups a placebo-pellet was subcutaneously implanted in the neck. In the E_2-repleted group (OVX+E_2) a 0.05 mg slow-release E_2 pellet (Innovative Research of America, Toledo, OH, U.S.A.) was implanted. These pellets are formulated to release a constant amount of E_2 for up to 60 days when implanted subcutaneously. Deionized water and a standard diet containing 0.7% calcium, 0.5% phosphorus and 1,5 U vitamin D_3/gram (Hopefarm, Woerden, The Netherlands) were available ad libitum to the sham-operated and E_2-repleted group. The OVX rats were fed the same amount of food per gram body weight as the sham group to minimize the increase in body-weight associated with ovariectomy.

After three weeks the rats were put in metabolic cages and overnight non-fasting urine was collected. On day 28 the animals were bled from the abdominal aorta and blood was collected and samples were stored at -20°C. Uterus and spleen were dissected and weighed. The femura were dissected. The study was approved by the Committee for Animal Use of the Erasmus University Rotterdam.

Analytical procedures

The BMD of the total bone (T-BMD) and subregions of the right femur were measured *ex vivo* using dual energy x-ray absorptiometry (DPX-L, Lunar Corp., Madison, WI, U.S.A.). Femora were placed under 2.5 cm of water using the high resolution mode as recommended by the manufacturer. The Lunar Small Animal Software Package Ic was used for analysis. Furthermore, two subregions of interest, corresponding to the metaphyseal (M) zone (rich in cancellous bone) and diaphyseal zone (D) (rich in cortical bone) were measured. In each femur scan the two subregions were positioned at the same distance from the distal femur extremity (2.0 mm for M and 5.4 mm for D) and with the same height (3.4 mm for M and 9.2 mm for D) for all the femora. The coefficient of variation evaluated by scanning the same femur 10 times over a short period of time was 1.1% for T-BMD, 2.5% for D-BMD and 2.4% for M-BMD measurements.

Total calcium, anorganic phosphate, alkaline phosphatase, creatinine and total protein were analyzed with standard methods. Intact PTH was measured using a rat-specific immunoradiometric method (Nichols Institute, San Clemente, CA, U.S.A.). Osteocalcin was determined by a rat-specific radioimmunoassay[16]. 1,25-$(OH)_2D_3$ was measured by immunoextraction followed by quantification by ^{125}I-RIA (IDS, Boldon, UK). Concentrations of 25-$(OH)D_3$) were measured by radioimmunoassay (Incstar Corp., Stillwater, MN, U.S.A.). Serum concentrations of vitamin D-binding protein (DBP) were measured by radial immunodiffusion[17]. The ratio ($x10^3$) between the molar concentrations of 1,25-$(OH)_2D_3$ and DBP was used as an estimate of free hormone concentration[18]. Serum E_2 was measured by an extracture procedure using diethyl ether followed by RIA (DPC, Los Angeles, CA, U.S.A.).

Statistical analysis

Data analysis involved estimation of means, standard deviations, standard errors and one-way analysis of variance. Linear regression analysis shows the correlation between the serum levels of E_2, 1,25-$(OH)_2D_3$ and osteocalcin irrespective of the experimental group. $P < 0.05$ was considered statistically significant.

2.4 RESULTS

General measurements

Table 2.1 shows that ovariectomized rats had very low serum E_2 levels, which resulted in reduced uterus weights and increased spleen and body weights in spite of pair-feeding. E_2

Table 2.1 Effects of ovariectomy (OVX) and 17β-estradiol (OVX+E_2) in rats four weeks after operation

	Sham	OVX	OVX + E_2
	n = 12	*n = 12*	*n = 12*
Body weight			
Beginning (g)	151 ± 2.6	151 ± 2.5	152 ± 2.4
Final (g)	161 ± 1.8	169 ± 3.0[c]	155 ± 1.9
Organ weight			
Uterus (g)	0.297 ± 0.020	0.082 ± 0.003[a]	0.818 ± 0.114[a]
Spleen (g)	0.358 ± 0.005	0.407 ± 0.014[a]	0.309 ± 0.007[a]
Serum E_2 (pmol/l)	16.0 ± 3.6	7.1 ± 0.8[c]	23.7 ± 2.6

Data are presented as means ± SEM [a]$p < 0.001$ vs. sham [b]$p < 0.01$ vs. sham [c]$p < 0.05$ vs. sham

release from the pellets resulted after 1 week in high serum E_2 levels (data not shown). However, from week 2 onwards the E_2 levels were not significantly different from those of sham rats. E_2 treatment resulted in significantly increased uterus weight and significantly decreased spleen weight while body weight was not significantly different from the sham group.

Bone metabolism

As shown in Figure 2.1 BMD as measured by DEXA decreased by 5% following ovariectomy. E_2 suppletion resulted in an increased BMD by 6.3% compared to sham rats. Differences were more pronounced in the distal metaphysis which mainly consists of trabecular bone. Metaphyseal BMD decreased with 9.8% in the ovariectomized group and increased with 13.1% in the E_2-repleted group. In the diaphysis, which mainly consists of cortical bone, differences were smaller. The effects of ovariectomy and E_2 treatment on bone turnover are reflected by changes in serum osteocalcin levels. In OVX rats serum osteocalcin levels were elevated while estrogen treatment resulted in lower levels (Table 2.2). Irrespective of the experimental groups serum E_2 and osteocalcin levels were negatively correlated (Figure 2.2a, r = -0.502, p = 0.002) while 1,25-$(OH)_2D_3$ and osteocalcin levels were positively correlated (Figure 2.2b, r = 0.612, $p < 0.001$).

Calcium metabolism

No differences between the groups in serum calcium, inorganic phosphate and alkaline

Table 2.2 Effects of ovariectomy (OVX) and 17β-estradiol (OVX+E_2) on calcium and bone metabolism parameters in serum and urine four weeks after operation

	Sham *n = 12*	OVX *n = 12*	OVX + E_2 *n = 12*
Serum			
Phosphate (mmol/l)	1.96 ± 0.12	2.17 ± 0.11	1.70 ± 0.13
Calcium (mmol/l)	2.38 ± 0.02	2.34 ± 0.01	2.36 ± 0.02
Alkaline Phosphatase (units/ml)	79.8 ± 4.85	72.9 ± 3.90	71.3 ± 5.20
Osteocalcin (µg/l)	324.9 ± 25.6	437.5 ± 28.2[b]	203.0 ± 19.0[a]
Urine			
Calcium/creatinine	0.08 ± 0.02	0.11 ± 0.02	0.24 ± 0.06[b]

Data are presented as means ± SEM [a]$p < 0.001$ vs. sham [b]$p < 0.01$ vs. sham

phosphatase levels were detected (Table 2.2). In contrast to the normal serum calcium levels nocturnal non-fasting urinary calcium excretion was significantly increased in the E_2-repleted group, while it was unchanged in the ovariectomized rats (Table 2.2).

Vitamin D_3 and PTH endocrine system

Serum total 1,25-$(OH)_2D_3$ levels were only slightly elevated in the ovariectomized rats, but decreased by almost 70% in the E_2-repleted group in comparison with the sham-operated rats (Table 2.3). Serum vitamin D binding protein levels were not influenced by 17β-E_2 deficiency or suppletion (Table 2.3) and therefore free 1,25-$(OH)_2D_3$ index showed similar changes as total 1,25-$(OH)_2D_3$ levels. Serum 25-$(OH)D_3$ levels were only reduced by 15% in E_2-repleted rats (Table 2.3).

There were no significant differences in serum PTH levels between the groups (Table 2.3) and no correlation was observed between serum PTH and serum E_2 levels (data not shown).

2.5 DISCUSSION

The present study demonstrates a close coupling between estrogen and the vitamin D and PTH endocrine system with respect to calcium and bone metabolism. Moreover, our data support a direct effect of E_2 on intestinal calcium absorption. This was established in a rat model existing

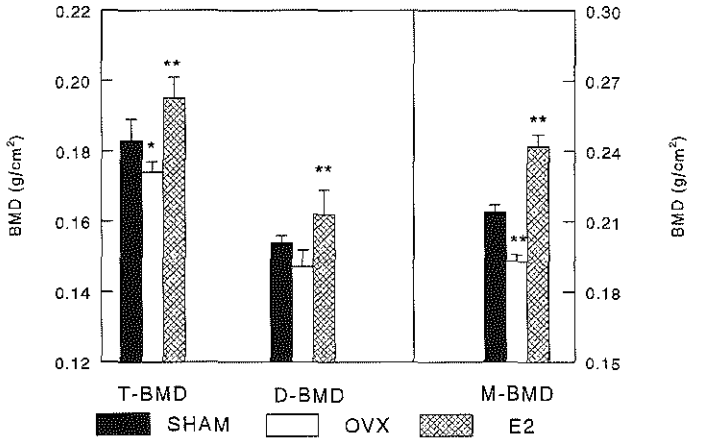

Figure 2.1 Effects of ovariectomy (OVX) and 17β-estradiol (E_2) on bone mineral density of the total femur (T-BMD), diaphysis (D-BMD) and metaphysis (M-BMD). Data are presented as means ± SEM. *$p < 0.05$ v. sham **$p < 0.01$ vs. sham.

of three different experimental groups, i.e. sham-operated, OVX and OVX with E_2 supplementation. The groups displayed the various phenotypes at uterus, spleen and bone as expected for ovariectomy and ovariectomy with E_2 supplementation[19-22]. Although the serum E_2 concentration in the E_2 supplemented group was not significantly different from that of the sham group the uterus weight and BMD were significantly higher and the spleen weight was significantly lower than in the sham group. This can be explained by the fact that the E_2 levels in the E_2 supplemented group were constantly at estrous level in contrast to the cycling sham rats or by the peak serum E_2 levels in the first week.

Serum total 1,25-$(OH)_2D_3$ levels were not significantly elevated following ovariectomy. This is in agreement with earlier observations in rats[15,23]. Nyomba et al. found increased total serum 1,25-$(OH)_2D_3$ levels but normal free serum 1,25-$(OH)_2D_3$ levels following ovariectomy as vitamin D binding protein levels were also elevated[24]. Postmenopausal women also have normal serum 1,25-$(OH)_2D_3$ levels[4,25-27]. Major changes in serum 1,25-$(OH)_2D_3$ levels were found with E_2 supplementation. This resulted in substantially reduced total serum 1,25-$(OH)_2D_3$ levels as has also been shown by Kalu et al.[15]. As E_2 supplementation did not alter serum vitamin D binding protein production, estimated free 1,25-$(OH)_2D_3$ levels showed a similar reduction as total 1,25-$(OH)_2D_3$ levels. These observations appear to be in contrast to the human situation. In women transdermal estrogen administration has no effect on either serum vitamin D binding protein or serum 1,25-$(OH)_2D_3$ levels[28-30].

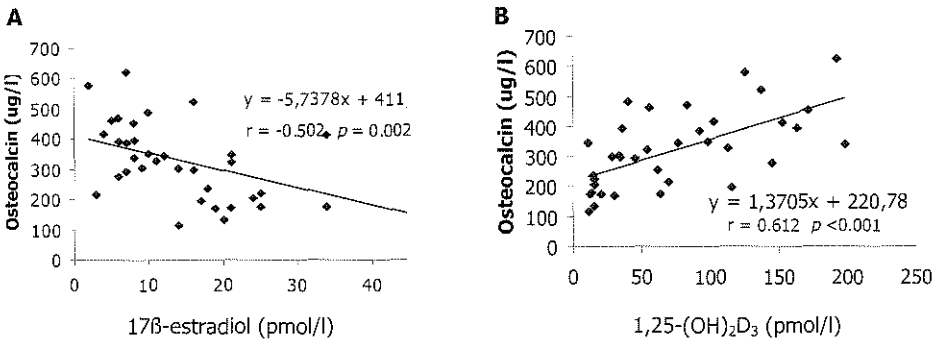

Figure 2.2 Regression analysis of 17ß-estradiol and osteocalcin values (A) and regression analysis of total serum 1,25-$(OH)_2D_3$ levels and serum osteocalcin in rats. Data of all rats irrespective of the experimental group showed a significant correlation between serum 17ß-estradiol and osteocalcin levels and between serum 1,25-$(OH)_2D_3$ levels and serum osteocalcin levels, respectively.

The changes in 1,25-$(OH)_2D_3$ levels can only be partially explained by the relatively small decrease in serum 25-$(OH)D_3$ levels in the E_2 repleted group. Therefore, renal conversion of 25-$(OH)D_3$ into 1,25-$(OH)_2D_3$ by 1α-hydroxylase should have been reduced and/or catabolism of 1,25-$(OH)_2D_3$ should have been increased. This is in agreement with observations of Kalu et al. who found slightly elevated serum 25-$(OH)D_3$ levels but decreased serum 1,25-$(OH)_2D_3$ levels in E_2-repleted rats[15]. It is unlikely that the initial peak in serum E_2 level during the first week has such a dramatic effect on a rapidly regulated enzyme as 1α-hydroxylase. However, the non-cycling character of E_2 at estrus level may form an explanation for the strong reduction in serum 1,25-$(OH)_2D_3$ concentration.

Urinary calcium excretion was not affected by ovariectomy. To the contrary, in women estrogen deficiency results in an increased urinary calcium excretion[31,32]. This has only been measured in ovariectomized rats if they were withheld food for 24 hours prior to and during the period of urine collection[33] or if rats were given a low calcium diet[23]. In contrast to the ovariectomized group, in the E_2 supplemented group urinary calcium excretion was increased. This may be due to increased intestinal calcium absorption under the influence of E_2 because the rats were pair-fed during nocturnal collection of urine and/or a reduced calcium reabsorption[2,4,13]. Stimulation of calcium reabsorption is, like 1α-hydroxylase activity, a renal PTH response. Therefore the

Table 2.3 Effects of ovariectomy (OVX) and 17β-estradiol (OVX+17β-E_2) on vitamin D and PTH endocrine system in rats four weeks after operation

	Sham *n = 12*	OVX *n = 12*	OVX + E_2 *n = 12*
Serum			
PTH (ngl/l)	11.9 ± 1.15	14.5 ± 1.79	10.2 ± 1.15
25-$(OH)D_3$ (nmol/l)	91.7 ± 3.9	88.8 ± 3.3	77.7 ± 2.1[a]
1,25-$(OH)_2D_3$ (pmol/l)	87.6 ± 15.8	111.7 ± 13.9	28.9 ± 7.9[a]
Vitamin D binding protein (µmol/l)	7.3 ± 0.1	7.7 ± 0.1	7.3 ± 0.1
Free 1,25-$(OH)_2D_3$ (index x 10^{-3})	12.3 ± 2.3	14.6 ± 1.9	4.0 ± 1.2[a]

Data are presented as means ± SEM [a]$p < 0.01$ vs. sham

effects of E_2 could have been exerted via PTH. These effects were not a result of reduction in PTH levels by E_2 supplementation as these were not different between the groups. Furthermore regression analysis did not show any correlation between serum E_2 and PTH levels. The estrogen receptor has been demonstrated to be present in the kidney[34,35] and therefore E_2 may have acted directly at the kidney to inhibit PTH-stimulated 1,25-$(OH)_2D_3$ synthesis and calcium reabsorption. In literature conflicting data are presented concerning the effect of E_2 on serum PTH levels and 1α-hydroxylase activity. Stock et al. have shown that E_2 inhibits the PTH-stimulated intracellular cAMP accumulation in opossum kidney cells[36]. In laying hen E_2 reduces PTH receptor binding affinity in calvaria and kidney[37]. However, estrogen substitution in postmenopausal women may result in either unchanged[3,29,38] or increased renal 1α-hydroxylase activity either due to increased serum PTH levels[2], an increased renal responsiveness to PTH[39] or due to a direct stimulating effect on 1α-hydroxylase activity[39]. Furthermore Ash et al. have shown in a rat model that estrogens promote PTH-induced synthesis of 1,25-$(OH)_2D_3$[40]. Besides interference with PTH action in the kidney, E_2 could also have been acting directly on 1α-hydroxylase activity and renal calcium absorption. Previously, with cultured opossum kidney cells a direct effect of E_2 on 1α-hydroxylase activity has been shown[41]. E_2 suppletion resulted in an increased bone mineral density[42]. The effects on BMD are parallelled, although in opposite direction, by effects on osteocalcin levels, demonstrating effects of E_2 on bone turnover[38,43,44]. In the E_2-repleted group the decreased osteocalcin levels may also result from decreased

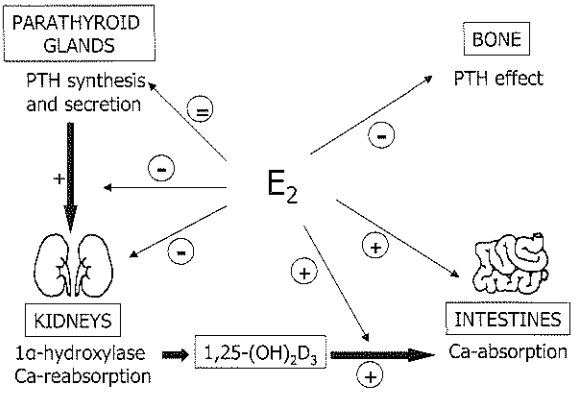

Figure 2.3 Diagram of the possible interactions between estrogens and the calciotropic hormones in rats. 17β-Estradiol (E_2) does not affect parathyroid hormone (PTH) synthesis and/or secretion by the parathyroid glands. Estrogens are believed to directly suppress cancellous bone remodeling[11,12] or indirectly by reducing bone cell responsiveness to PTH[9,10,48-50]. The estrogen receptor has been demonstrated in the kidney[34,35]. Therefore E_2 may act directly at the kidney to inhibit 1,25-dihydroxyvitamin D_3 ($1,25\text{-}(OH)_2D_3$) synthesis and calcium reabsorption[41] or indirectly by inhibition of renal responsiveness to PTH[36]. Also serum $1,25\text{-}(OH)_2D_3$ can be reduced as a consequence of E_2-stimulated catabolism of $1,25\text{-}(OH)_2D_3$. E_2 repleted rats have normal serum calcium levels in spite of an increased bone mineral density, increased urinary calcium excretion and decreased serum $1,25\text{-}(OH)_2D_3$ levels. This can only be explained by a E_2-enhanced intestinal calcium absorption either directly or indirectly via an effect on $1,25\text{-}(OH)_2D_3$-induced calcium absorption.

$1,25\text{-}(OH)_2D_3$ levels[45-47]. The suppression of bone turnover by estrogens in spite of normal PTH levels suggest that not only kidney cells but also bone cells are less responsive to PTH under E_2 repleted conditions[9,10,48-50].

So, E_2-repleted rats had very low serum $1,25\text{-}(OH)_2D_3$ levels, while more calcium was accreted in bone and excreted in the urine. Still E_2 repleted rats had normal serum calcium levels. Therefore E_2 repleted rats should have been kept in calcium homeostasis through a stimulatory action of estrogens on intestinal calcium absorption. This is supported by the following observation. In the OVX group calcium is released from bone while serum calcium and urinary calcium excretion are unchanged. This would imply that in the E_2 deficient state, calcium absorption in the intestines is reduced. In postmenopausal women estrogen supplementation has been shown to normalize intestinal calcium transport, but these women had normal serum $1,25\text{-}(OH)_2D_3$ levels[2,4]. The ER has been shown in rat small intestinal epithelial cells[14]. Like $1,25\text{-}(OH)_2D_3$, estrogens might increase paracellular calcium permeability by changing the

phospholipid composition of the brush border membrane[51] or by changing the chemical structure of the junctional complex[52]. Furthermore estrogens might directly elevate the active transport by influencing the calbindin expression, which has been shown to play a central role in the active intestinal and renal calcium transport in the rat[53]. However, Delorme et al. have reported that the duodenal calbindin protein content does not change in estrogen deficient and repleted states but is influenced by the serum 1,25-$(OH)_2D_3$ level[54]. Estrogens might also potentiate the calcium absorptive action of 1,25-$(OH)_2D_3$ on intestines, e.g. by affecting the VDR. In bone cells it has been shown that estrogens increase vitamin D binding capacity[55]. Studies to substantiate the role of E_2 in intestinal calcium absorption are in progress.

In summary E_2 does not affect PTH secretion from the parathyroid glands but may reduce the end-organ responsiveness to PTH in bone and kidneys. This results in reduced bone turnover, urinary calcium reabsorption and 1,25-$(OH)_2D_3$ synthesis. However, the present data suggest that E_2 has a positive effect on calcium absorption in the intestine either independent of 1,25-$(OH)_2D_3$ or by enhancing the 1,25-$(OH)_2D_3$ effect. Figure 2.3 shows schematically the possible interactions between estrogens and the calciotropic hormones on basis of the present data and data from literature. Together it is clear that estrogens influence calcium and bone metabolism via complex interactions with the calciotropic hormones.

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Chapter 3

Evidence for involvement of 17ß-estradiol in intestinal calcium transport in the rat

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3.1 ABSTRACT

E_2 has a broad spectrum of actions, including effects on bone and calcium metabolism. In Chapter 2, we have shown that supplementation of E_2 to OVX rats resulted in a strong decrease in serum 1,25-$(OH)_2D_3$ while serum calcium concentrations remained normal. This could not be explained by release of calcium from bone or retention in the kidney. The present study was designed to analyze the effect of 17ß-E_2 on intestinal calcium absorption and to further investigate the interplay with 1,25-$(OH)_2D_3$. Three months old female Brown Norway rats were sham operated, OVX or OVX with implantation of either a 0.025 mg or 0.05 mg E_2 pellet. After four weeks in none of the groups serum calcium, phosphate or PTH was altered compared to the sham operated group. Osteocalcin was modified as can be expected for OVX and E_2 supplementation. Supplementation of OVX resulted in 80% reduction of 1,25-$(OH)_2D_3$ levels and only 20% reduction in 25-$(OH)D_3$ levels. As analyzed by the *in situ* intestinal loop technique, OVX resulted in a significant decrease in the calcium absorption in the duodenum, which could be partially restored by suppletion with E_2. In view of the large differences in 1,25-$(OH)_2D_3$ levels between the OVX group (143.5 ± 29 pmol/l) and the E_2 repleted groups (18.1 ± 2.1 and 16.4 ± 2.2 pmol/l), 1,25-$(OH)_2D_3$-regulated intestinal calcium absorption should have been more efficient in the E_2 repleted groups compared with the OVX and sham groups. None of the treatments led to significant changes in calcium handling in the jejunum. E_2 did not facilitate duodenal calcium absorption via an increase in the VDR number. Also, in the kidneys no differences in VDR levels between the groups were found. Taken together, the present study demonstrates that E_2 is positively involved in intestinal calcium absorption either directly and/or via sensitizing the action of 1,25-$(OH)_2D_3$. If E_2 exerts its actions via interplay with 1,25-$(OH)_2D_3$, then it is independent of changes in VDR number.

3.2 INTRODUCTION

Estrogens exert their genomic effects via the estrogen receptors. These receptors have been found in tissues not considered classic targets, including central nervous system, endocrine glands, skin, myocardium, aorta, kidney, intestine and bone[1-3]. Therefore estrogens are considered to be more than sex hormones. Recent studies have indicated that estrogens are also involved in regulation of calcium and bone metabolism. In bone E_2 maintains the balance between bone resorption and bone formation[4,5], thereby affecting bone mass and bone architecture[6-9]. These effects can be directly and/or indirectly by interaction with other factors influencing bone metabolism, e.g. PTH and insulin-like growth factor I[10-15].

In addition, estrogens may stabilize bone mass by providing calcium through a stimulating effect on intestinal calcium absorption[16]. Indeed, menopause is associated with decreased calcium absorption, which can be normalized by E_2 suppletion[18,19]. Recently, we found in our laboratory that E_2 suppletion in rats results in a strong decrease in serum 1,25-$(OH)_2D_3$ levels. At the same time BMD and urinary calcium excretion increased, while serum calcium levels were normal in these rats. These data indicated that E_2 affects intestinal calcium absorption.

1,25-$(OH)_2D_3$ is considered the classic stimulator of intestinal calcium absorption. Like other steroid hormones, 1,25-$(OH)_2D_3$ exerts its action via its nuclear receptor, the VDR. In intestinal epithelial cells the activated hormone receptor complex induces synthesis of calcium binding proteins, which are involved in active, transcellular calcium transport[20-22]. E_2 may affect intestinal calcium absorption via 1,25-$(OH)_2D_3$ and regulation of the VDR. Ebeling et al. reported that in humans a decline in intestinal VDR with age could account for the decrease in calcium absorption with aging, and thereby suggested that estrogen deficiency might result in decreased intestinal VDR levels[23]. In contrast, in another study intestinal VDR levels were similar in pre- and postmenopausal women, while calcium absorption was significantly reduced in the latter group[24]. This study was designed to address the involvement of E_2 in intestinal calcium absorption and to examine the effect on VDR content.

3.3 MATERIALS AND METHODS

Animals

1) Thirty female Brown Norway rats were divided into four groups to evaluate effects of E_2 on intestinal calcium transport and calcium and bone metabolism parameters in relation to serum PTH and 1,25-$(OH)_2D_3$ levels. Eight rats were sham-operated (sham) and 22 rats were ovariectomized (OVX). In 6 rats (OVX+E_2L) a 0.025 mg E_2 60-days release pellet and in 8 rats (OVX+E_2H) a 0.05 mg E_2 pellet (Innovative Research of America, Toledo, OH, U.S.A.) was subcutaneously implanted in the neck. These pellets are formulated to release a constant amount of E_2 for up to 60 days when implanted subcutaneously.

2) The thirty-six rats described in Chapter 2.3 were used to study effects of E_2 on intestinal and renal VDR and calbindin-D_{28K} content.

In both experiments deionized water and a standard diet containing 0.7% calcium, 0.5% phosphorus and 1,5 U vitamin D_3/gram (Hopefarm, Woerden, The Netherlands) were available ad libitum to the sham-operated group. The treated groups were fed the same amount of food per gram body weight as the sham group to minimize the increase in body weight associated with ovariectomy. After four weeks all rats were bled under ether anaesthesia from the

abdominal aorta and several parameters for calcium and bone metabolism were measured. In the second experiment intestines and kidneys were collected to quantitate calbindin-D_{28K} and/or VDR content. The study was approved by the committee for use of laboratory animals of the Erasmus University Rotterdam.

Analytical procedures

In serum calcium, inorganic phosphate, alkaline phosphatase, creatinine and total protein and in urine calcium, inorganic phosphate and creatinine were analyzed with standard methods. Osteocalcin was determined by a rat-specific radioimmunoassay[25]. Intact PTH was measured using a rat-specific immunoradiometric method (Nichols Institute, San Clemente, CA, U.S.A.). 1,25-$(OH)_2D_3$ was measured by immunoextraction followed by quantification by ^{125}I-RIA (IDS, Boldon, U.K.). Concentrations of 25-$(OH)D_3$ were measured by radioimmunoassay (Incstar Corp., Stillwater, MN, U.S.A.). Serum E_2 was measured by an extraction procedure using diethyl ether followed by RIA (DPC, Los Angeles, CA, U.S.A.).

Calcium transport measurement

$^{45}CaCl_2$ (1.61 mCi/mg Ca) was obtained from Amersham (Arlington Heights, IL, U.S.A.) and [1,2-^{3}H]polyethyleneglycol (PEG, MW 4000; 0.45 mCi/g) was obtained from DuPont-NEN (Boston, MA, U.S.A).

Four weeks after operation calcium transport measurements were performed under ether anaesthesia by *in situ* loop technique after overnight fasting[26]. Shortly, after a midline incision the bile duct and two intestinal segments were ligated. The first segment was from 1 cm distal to the pylorus to 2 cm distal to Treitz' ligament. The second part encompassed jejunum and was from 13 cm to 23 cm distal to the pylorus. Transport medium (750 µl), prewarmed to 37°C, was injected with a 25 gauge needle in each ligated segment. The transport medium contained 164 mM NaCl, 0.88 mM $^{45}CaCl_2$ (610-680 dpm/nmol) and [1,2-^{3}H]polyethyleneglycol (720 dpm/nmol). Following an equilibration period of 5 minutes a 50 µl sample from the luminal content was taken by means of a transmural puncture to calculate the initial intra-luminal solvent volume, ^{45}Ca and ^{40}Ca content. Two times 10 µl of the sample were dissolved in 5 ml scintillation fluid (Packard, Downers Grove, IL, U.S.A.) and ^{45}Ca and ^{3}H were determined in a Packard 2650 Tricarb scintillation counter. ^{40}Ca was measured by a calcium kit (Sigma Chemical Co., St. Louis, MO, U.S.A.). Duodenal and jejunal loops were removed after 25 and 30 minutes, respectively. The length was measured and two samples of 20 µl were taken from the luminal content for determination of ^{45}Ca and ^{3}H radioactivity. Also the ^{40}Ca concentration in two

samples of 10 µl was determined in the luminal fluid. The lumen was flushed with 500 µl NaCl 0.9% to determine the recovery of ^{3}H-PEG in order to assess whether transport medium had leaked from the intestinal lumen to the abdominal cavity. Intestinal calcium transport was calculated via the following formulae:

1) *Ca-influx* (nmol Ca per cm intestine per 30 min) is defined as the unidirectional movement of calcium from intestinal lumen into tissue (mean value over the period studied)

$$\text{Ca-influx} = \frac{V_i(^{45}Ca_i) - V_f(^{45}Ca_f)}{SA \times L}, \text{ whereby}$$

V_i (initial volume in µl): Refers to injected solvent volume corrected for dilution by residual endogenous luminal fluid at t_0 on basis of the change in PEG concentration in the previous 5 minutes.

V_f (final volume in µl): Refers to solvent volume left at the end of the absorption period (t_{30}). This parameter is calculated as the product of the ratio of PEG concentration at t_0 versus t_{30} and V_i.

$^{45}Ca_i$ and $^{45}Ca_f$ (dpm per µl intraluminal fluid): Refer to initial and final ^{45}Ca concentration at t_0 and t_{30}, respectively.

SA (dpm per nmol Ca): Refers to the specific activity of Ca.

L: Refers to the length of the ligated intestinal segment

2) *Net Ca absorption* (nmol Ca per cm intestine per 30 min) is defined as the total amount of Ca absorbed from the intestinal lumen

$$\text{Net Ca abs} = {}^{40}Ca_iV_i - {}^{40}Ca_fV_f$$

$^{40}Ca_i$ and $^{40}Ca_f$ (nmol per µl intraluminal fluid): Refer to the initial and final ^{40}Ca concentration.

3. *Ca-efflux* (nmol Ca per cm intestine per 30 min) is defined as unidirectional movement of calcium that enters the intestinal lumen from the tissue.

$$\text{Ca-efflux} = \text{Ca-influx} - \text{Net Ca abs.}$$

In the calculations is assumed that ^{45}Ca-efflux is negligible.

Vitamin D receptor analysis

Nuclear extracts were prepared as described previously[27]. Shortly, 20 cm of proximal small intestines was excised and flushed with 20 ml ice-cold TED-saline (50 mM Tris-HCl pH 7.4, 1.5 mM EDTA, 5 mM DTT, 0.2 mM phenylmethylsulfonyl fluoride (PMSF), 10 µg/ml TPCK, 1 µg/ml leupeptin and 1 µg/ml pepstatin). Scraped mucosa and kidneys were washed three times in TED-saline for ten minutes at 3000 x g. Mucosa and kidneys were homogenized in 10 vol (w/v) KTED-300 (TED, 300 mM KCL) in a glass-Teflon homogenizer and polytron, respectively. Homogenates were centrifuged for 1 hr at 100,000 x g and extracts were stored at -80°C. The concentration of VDR was measured by a VDR-specific ELISA[28].

Calbindin-D_{28K} quantification

Kidneys were homogenized (20% wt/vol) in PBS in presence of enzyme inhibitors (10^{-4}M PMSF, 10^{-4}M TPCK) using a polytron. Homogenates were centrifuged at 38,000 x g. Supernatant solution was stored at -80°C and analyzed as described previously[29].

Statistical analysis

Data analysis involved estimation of the means, standard deviations, standard errors and one-way analysis of variance. $P < 0.05$ was considered statistically significant.

3.4 RESULTS

General measurements

Table 3.1 shows that OVX and E_2 suppletion resulted in the anticipated phenotypes. OVX rats rats had reduced uterus weights. Although serum E_2 levels in OVX+E_2L rats were similar as in sham rats, uterus weights were significantly increased. Supplementation with the highest E_2 dose resulted in a similar increase in uterus weight as in OVX+E_2L group, while serum E_2 levels in OVX+E_2H rats were higher, i.e. at estrous level. Body weights in the treated groups were not significantly different from the sham rats, but in the E_2H group they were significantly lower compared with the OVX rats.

Calcium and bone metabolism

No significant differences between E_2 treated groups and sham rats in serum calcium and inorganic phosphate levels were detected, while serum inorganic phosphate levels were

Table 3.1 Effects of ovariectomy (OVX) and 0.025 mg 17β-estradiol (OVX+E_2L) or 0.05 mg 17β-estradiol (OVX+E_2H) in rats four weeks after operation

	Sham *n = 8*	OVX *n = 8*	OVX+E_2L *n = 6*	OVX+E_2H *n = 8*
Body weight				
Beginning (g)	139 ± 3.9	140 ± 3.4	142 ± 1.4	140 ± 2.1
Final (g)	138 ± 4.0	143 ± 3.3	135 ± 2.1	131 ± 2.7c
Uterus weight (g)	0.327 ± 0.036	0.082 ± 0.005[a]	0.601 ± 0.028[ab]	0.617 ± 0.027[a]
Serum E_2 (pmol/l)	16.1 ± 4.6	3.0 ± 1.5[a]	21.2 ± 4.0[c]	41.5 ± 4.6[ab]

Data are means ± SEM [a]$p < 0.001$ vs. sham [b]$p < 0.001$ vs. OVX [c]$p < 0.01$ vs. OVX

Table 3.2 Effects of ovariectomy (OVX) and 0.025 mg 17β-estradiol (OVX+E_2L) or 0.05 mg 17β-estradiol (OVX+E_2H) on calcium and bone metabolism parameters four weeks after operation. Urinary calcium excretion was measured three weeks after operation

	Sham *n = 8*	OVX *n = 8*	OVX+E_2L *n = 6*	OVX+E_2H *n = 8*
Serum				
Calcium (mmol/l)	2.11 ± 0.03	2.15 ± 0.07	2.17 ± 0.27	2.18 ± 0.02
Phosphate (mmol/l)	2.99 ± 0.07	3.18 ± 0.14	2.67 ± 0.13[f]	2.65 ± 0.06[f]
Alkaline Phosphatase (units/ml)	42.6 ± 1.4	47.7 ± 2.8	32.8 ± 1.7[ad]	33.4 ± 1.0[ad]
Osteocalcin (µg/l)	514.4 ± 37.8	739.8 ± 59.9[b]	473.7 ± 26.5[e]	392.3 ± 16.1[cd]
PTH (ngl/l)	41.9 ± 11.2	53.1 ± 12.2	58.5 ± 10.8	56.6 ± 6.9
25-$(OH)D_3$ (nmol/l)	72.7 ± 2.5	71.3 ± 1.9	56.0 ± 2.8[ad]	58.9 ± 6.9[ad]
1,25-$(OH)_2D_3$ (pmol/l)	105.4 ± 21.5	143.5 ± 29.0	18.1 ± 2.1[ad]	16.4 ± 2.2[ad]
Urine				
Calcium/creatinine	0.80 ± 0.15	0.48 ± 0.05[c]	0.73 ± 0.03[e]	0.98 ± 0.06[d]

Data are presented as means ± SEM [a]$p < 0.001$ vs. sham [b]$p < 0.01$ vs. sham [c]$p < 0.05$ vs. sham [d]$p < 0.001$ vs. OVX [e]$p < 0.01$ vs. OVX [f]$p < 0.05$ vs. OVX

significantly lower compared with OVX rats. Serum alkaline phosphatase levels were significantly reduced in both E_2 repleted groups compared to sham-operated as well as OVX rats (Table 3.2).

Effects of ovariectomy and E_2 treatment on bone turnover are also reflected by changes in serum osteocalcin levels. In OVX rats serum osteocalcin levels were elevated while treatment with E_2 significantly suppressed this increase (Table 3.2).

Serum total 1,25-$(OH)_2D_3$ levels were not significantly elevated in the OVX rats, but decreased by more than 80% in 17ß-E_2-repleted rats in comparison with sham-operated rats. Serum 25-$(OH)D_3$ levels were only reduced by about 20% in E_2-repleted rats while serum PTH levels were similar in all groups (Table 3.2).

Calcium transport

Despite normal or even somewhat raised serum 1,25-$(OH)_2D_3$ levels in OVX rats, calcium influx in the duodenum was significantly reduced by 45% compared with the sham-group (Figure 3.1a). Calcium efflux was increased, although not significantly, by 41% in the OVX group. These opposite changes in calcium influx and calcium efflux resulted in a significantly decreased net calcium absorption in duodenum following OVX (Figure 3.1a).

The calcium influx was significantly reduced in both E_2 repleted groups compared with sham-operated rats (Figure 3.1a). Although the E_2 repleted rats had an about 90% lower serum 1,25-$(OH)_2D_3$ level than OVX rats, calcium influx was even somewhat higher than in OVX rats. The calcium efflux was not changed in the E_2 repleted rats compared with the sham-group and therefore calcium efflux was less than in the OVX group. Taken together, E_2 treatment partially restored net calcium absorption in duodenum and was not significantly different from the sham group, despite a strongly reduced 1,25-$(OH)_2D_3$ concentration.

In the jejunum no significant differences between the groups were observed (Figure 3.1b). However, similar trends were observed as in the duodenum. Again in E_2 repleted rats, despite low serum 1,25-$(OH)_2D_3$ levels, calcium influx was higher and calcium efflux was lower, resulting in an apparently higher net calcium absorption compared with OVX rats.

Overnight non-fasting urinary calcium excretion, which indirectly reflects total intestinal calcium absorption, was significantly lower in OVX rats compared with sham-operated and E_2 repleted rats. No significant differences in urinary calcium excretion were found between E_2 repleted rats and sham-operated rats (Table 3.2).

Intestinal VDR and renal VDR and calbindin-D_{28K} content

A possible mechanism by which 17ß-E_2 might affect intestinal calcium absorption is by facilitating the 1,25-$(OH)_2D_3$ action via an increase in its receptor number, thereby potentially compensating for the reduction in 1,25-$(OH)_2D_3$ levels. Therefore, nuclear extracts of intestines of sham-operated, OVX and E_2 repleted rats were prepared and the VDR content was established. Neither OVX nor E_2 treatment resulted in a significant change in intestinal VDR number (Table 3.3). In addition, in the kidney, the VDR number was not different between sham, OVX and E_2 repleted rats (Table 3.3). In addition, we measured in the kidneys whether the alterations in renal calcium excretion could be related to changes in calbindin-D_{28K} levels. As shown in Table 3.3, neither OVX nor E_2 treatment modified the renal calbindin-D_{28K} levels.

3.5 DISCUSSION

This study shows that E_2 has important effects on intestinal calcium absorption. This conclusion is based on the following observations. First, in the present study calcium influx from the duodenum of OVX rats was strongly reduced, although serum 1,25-$(OH)_2D_3$ levels were even slightly (about 30%) higher than in the sham rats. Although not significant, calcium efflux was even higher in E_2 deficient rats and therefore net calcium absorption was significantly reduced in OVX rats. This supports previous findings that menopause is associated with decreased intestinal calcium absorption[17]. Furthermore, it is consistent with the observation that in postmenopausal women estrogen supplementation normalizes intestinal calcium transport[19,30]. Second, in both E_2-repleted groups serum 1,25-$(OH)_2D_3$ levels were reduced by about 90% compared with the OVX group, which would have implicated an even further decrease in intestinal calcium transport[31-33]. However, in contrast to the OVX group, in both E_2 repleted groups net calcium absorption in the duodenum was not significantly different from the sham group. These direct calcium movement measurements support the calcium absorption data from food-intake studies[16]. Finally, an indirect measurement, nocturnal non-fasting urinary calcium excretion, which can be considered to reflect intestinal calcium absorption in rats eating during the night, was significantly higher in the E_2 repleted groups compared with the OVX rats. If the calcium influx is assumed to result from an interplay between E_2 and 1,25-$(OH)_2D_3$ on calcium transport mechanisms, then one could calculate a ratio for calcium influx and serum 1,25-$(OH)_2D_3$ as an indicator of the effectiveness of 1,25-$(OH)_2D_3$ regulated calcium influx under E_2 repleted conditions. Interestingly, expressed as this ratio, calcium influx in the duodenum of rats repleted with the lowest E_2 dose appeared to be almost 7 times higher than in OVX rats.

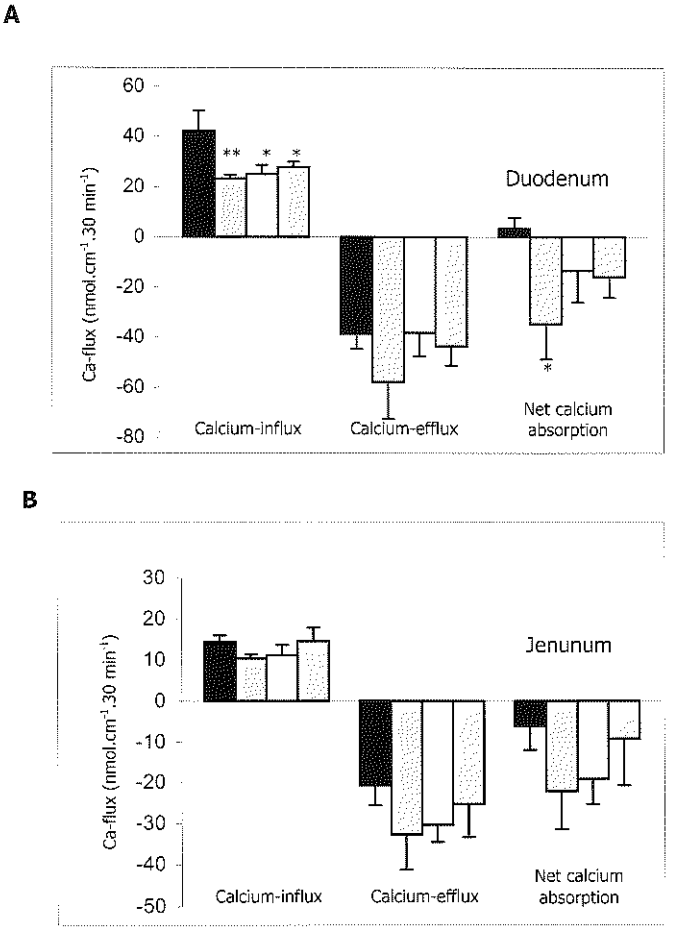

Fig. 3.1 Effects of OVX and treatment with E_2 on calcium absorption in (A) duodenum and (B) jejunum. Calcium movements were measured and calculated as described in the Materials and Methods. Calcium influx is movement of calcium from the intestinal lumen into the animal; calcium efflux is movement of calcium from the animal into the intestinal lumen; net calcium absorption is difference between calcium influx and calcium efflux. Solid bars = sham operated rats (n = 8); hatched bars = ovariectomized rats (n = 8); open bars = OVX rats treated with 0.025 mg E_2 (n = 6); grey bars = OVX rats treated with 0.05 mg E_2 (n = 8); Data are presented as means ± SEM. $^{*}p < 0.05$ versus sham; $^{**}p < 0.01$ versus sham.

Moreover, in OVX+E_2H group calcium influx was almost 11 times higher than in the OVX group. These data indicate that E_2 facilitates 1,25-$(OH)_2D_3$ mediated calcium absorption.

Table 3.3 Effects of ovariectomy (OVX) and 0.05 mg 17β-estradiol (OVX+E_2H) on intestinal VDR and renal VDR and calbindin-D_{28K} content four weeks after operation

	Sham	OVX	OVX+E_2H
Intestines			
VDR (fmol/mg protein)	190.9 ± 48.0	193.9 ± 32.1	217.3 ± 38.7
Kidneys			
VDR (fmol/mg protein)	18.2 ± 2.5	19.6 ± 2.2	22.2 ± 8.1
CaBP-D_{28K} (μg/mg protein)	5.16 ± 0.25	5.07 ± 0.21	5.53 ± 0.19

Data are presented as means ± SEM

E_2 could exert this facilitated calcium transport by affecting intestinal VDR, which plays a central role in the action of 1,25-$(OH)_2D_3$. Ebeling et al. have reported that the decreased intestinal calcium absorption of postmenopausal women could be attributed to a decline in intestinal VDR number[23]. However, our data did not show any change in intestinal VDR level under the various conditions. This is in line with recent data from Kinyamu et al. who found no change in intestinal VDR level, while intestinal calcium absorption was decreased in postmenopausal women compared to premenopausal women[24]. The present data are not conclusive whether E_2 specifically sensitizes 1,25-$(OH)_2D_3$ action in intestine, but the current data show that if so, then it is not exerted via an effect on VDR level.

Another process that is regulated by 1,25-$(OH)_2D_3$ is the renal calcium reabsorption in the distal tubuli[33]. 1,25-$(OH)_2D_3$ has been shown to regulate the expression of calbindin-D_{28K}[34]. This protein is thought to play a role in the calcium uptake from the lumen by renal epithelial cells[35]. In our study calbindin-D_{28K} content was not affected by E_2 repletion, although serum 1,25-$(OH)_2D_3$ levels were 80 to 90% decreased as compared with sham and OVX rats. This may be explained by the observation that the regulation of calbindin-D_{28K} gene expression is more sensitive to changes in serum calcium levels than for changes in serum 1,25-$(OH)_2D_3$ levels[36]. Serum calcium levels were similar in all groups and may therefore account for the lack of difference in calbindin-D_{28K} expression. Another possibility is that E_2 also facilitates this 1,25-$(OH)_2D_3$ mediated response, thereby compensating for the reduction in serum 1,25-$(OH)_2D_3$ levels.

E_2 may facilitate intestinal calcium transport via several other mechanisms than by affecting the VDR. These mechanisms might encompass direct stimulation of calcium influx at the apical site

of the epithelial cells, calcium extrusion at the basal site and the intracellular calcium transport from the apical to the basal site of the intestinal epithelial cells. E_2 may stimulate calcium influx by changing the phospholipid structure of the brush border membrane[37] or the chemical structure of the junctional complex[38]. One of the proteins involved in intestinal calcium transport is calbindin-D_{9K}. This protein is thought to serve as a calcium translocator and as a cytosolic calcium buffer in intestinal epithelial cells[39,40]. It is unlikely that E_2 exerts its action on intestinal calcium transport via a direct effect on calbindin-D_{9K} expression, because it is only regulated by 1,25-$(OH)_2D_3$ and not by E_2[41]. Another mechanism by which E_2 could facilitate calcium transport is by affecting the expression of the calcium membrane pump (PMCA). The PMCA is involved in the calcium transport across the basolateral membrane[42]. Estrogen deficiency does not appear to change the PMCA mRNA expression[36]. However, whether E_2 suppletion affects PMCA expression has yet to be resolved.

Although the present data demonstrate that E_2 facilitates calcium absorption, the reduction in calcium absorption after ovariectomy could not be completely overcome by E_2 supplementation. Various factors, including general anaesthesia[43], intraluminal pH[44] and glucose[45,46] and bile salt concentration[47] are known to affect intestinal calcium transport. Therefore, calcium influx may have been reduced, while calcium efflux remained unaffected, resulting in a negative calcium transport in the treated groups. However, these factors will have equally influenced calcium transport in the various groups. Therefore, it is unlikely that they account for the differences between the various groups. A more likely explanation is that E_2 also enhances calcium transport in other parts of the intestines. However, this seems not to be the case in the jejunum, because no significant differences in net calcium absorption between the various groups in this segment were found. Another possibility is the cecum which has been shown to play an important role in calcium absorption in rats[48,49], and which expresses the ER[3].

In conclusion, the present study demonstrates that E_2 is positively involved in intestinal calcium absorption either directly and/or via sensitizing the action of 1,25-$(OH)_2D_3$. If E_2 exerts its actions via 1,25-$(OH)_2D_3$ then it is independent of changes in VDR number.

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Chapter 4

In vivo studies on the estrogen effect on Insulin-like Growth Factor (IGF)-I and IGF Binding Protein (IGFBP) mRNA expression in bone cells: Dissociation with serum IGF-I levels

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4.1 ABSTRACT

Bone remodelling is regulated via a complex interplay between the sex-steroid E_2, the calciotropic hormone 1,25-$(OH)_2D_3$ and locally produced factors, like insulin-like growth factor (IGF)-I and insulin-like growth factor binding proteins (IGFBP). This study with 3-month old female Brown Norway rats was designed to investigate the effect of ovariectomy and E_2-replacement on serum IGF-I levels and local IGF-I and IGFBP mRNA expression in relation to several markers for bone turnover. Expression of IGF-I- and IGFBP-mRNA was investigated using non-radioactive *in situ* hybridisation. IGF-I mRNA was expressed in both osteoblasts and osteocytes. IGFBP-5 mRNA was only expressed in osteoblasts. A weaker signal was found for IGFBP-2, -3 and -4 mRNAs in the various groups. IGFBP-6 mRNA could not be detected. OVX resulted in a non-significant increase in serum 1,25-$(OH)_2D_3$ and IGF-I levels compared to sham-operated rats. In contrast, IGF-I mRNA expression in bone was depressed, while IGFBP-5 mRNA expression was not changed. Supplementation with E_2 resulted in an 80% reduction of 1,25-$(OH)_2D_3$ and a 17% reduction in serum total IGF-I levels. In bone, IGF-I mRNA expression was restored to sham-level, while IGFBP-5 mRNA expression was increased compared to sham-operated rats. These results indicate that presence of E_2 is important for osteoblastic IGF-I and IGFBP-5 mRNA expression in bone cells and might in this way influence bone mass. In addition, it was found that circulating IGF-I concentrations are not correlated with skeletal IGF-I mRNA expression. Therefore, the present data question the significance of serum measurement of IGF-I in relation to bone metabolism.

4.2 INTRODUCTION

Bone remodeling is regulated via a complex interplay between the calciotropic hormones, PTH and 1,25-$(OH)_2D_3$, E_2, and locally produced factors, like IGFs, bone morphogenetic proteins and cytokines[1-4].

IGF-I is produced by bone cells and is generally agreed to have anabolic effects on these cells[5,6]. It has been shown to have mitogenic and stimulative effects on bone cells, resulting in enhanced collagen production and bone matrix biosynthesis[7]. Aside IGF-I, IGFBPs are also produced by bone cells[8]. IGFBPs affect the half-life of the IGFs and the biological response of target tissues to IGFs[9]. The expression and activity of IGF-I are regulated by the calciotropic hormones and estrogens. PTH increases IGF-I synthesis in osteoblastic cells[10-12], while 1,25-$(OH)_2D_3$ has been shown to have controversial effects[13,14]. *In vitro* data show that E_2 upregulates IGF-I mRNA in osteoblasts, which could account for the bone preservative effect

of estrogens[15]. In E_2-treated rats bone matrix IGF-I is also increased[16]. In contrast to these observations, several studies have shown a negative relation between serum E_2 and serum IGF-I levels[17-19]. Furthermore bone matrix IGF-I but not serum IGF-I appeared to be associated with cancellous bone volume[20]. This dissociation may point to differences between local (in bone) and systemic (in the liver) regulation of IGF-I synthesis by E_2.
In this study we examined the effects of ovariectomy and ovariectomy with E_2 suppletion on serum IGF-I levels and local IGF-I and IGFBP mRNA expression in bone in relation to several bone turnover markers in an *in vivo* rat model.

4.3 MATERIALS AND METHODS

Animals

The studies were performed in the same thirty-six 13 weeks old female Brown-Norway rats (Harlan), divided into three equal groups, i.e. sham, OVX and OVX with E_2-suppletion, as described in Chapter 2.3. After four weeks these animals were bled from the abdominal aorta under ether anesthesia and blood was collected and samples were stored at -20°C. Uterus and spleen were dissected and weighed. The femura were dissected and used for analyses.

Analytical procedures

The BMD of the right femur was measured *ex vivo* as described in Chapter 2.3. Total calcium, anorganic phosphate, alkaline phosphatase, creatinine and total protein were analyzed with standard methods. Intact PTH, osteocalcin, $1,25\text{-}(OH)_2D_3$, $25\text{-}(OH)D_3$ and E_2 were measured in serum by methods described in Chapter 2.3. Total IGF-I was determined by radioimmunassay (Biosource, Fleurus, Belgium).

Tissue preparation

The left femura of nine rats of each group were fixed in 4% paraformaldehyde overnight at room temperature. For decalcification bones were incubated in a 5.5% solution of EDTA in 4% paraformaldehyde for at least 14 days at room temperature. Once a week the solution was refreshed. Finally, decalcified bones were dehydrated and embedded in paraffin according to standard procedures. Similar 4 µm sections from the region of the growth plate and metaphysis were mounted on 3-aminopropyltrioxysilane-coated slides and sections were dried at 37°C for 3 days.

Probe preparation

The IGFBP-2 to -6 cRNA probes were transcribed from templates described by Schuller et al.[21]. cDNA encoding mouse IGF-I was kindly provided by Dr. G.I. Bell (Howard Hughes Medical Institute, Chicago, IL, U.S.A.). Fragments were subcloned into pTZ18 and pTZ19 (EcoR1 for IGF-I). Digoxigenin-11-UTP labeled RNA probes were prepared according to manufacturer's prescription (Boehringer Mannheim GmbH, Biochemica, Mannheim, Germany) using T7 or SP6 RNA polymerase.

In situ hybridization

Sections of were dewaxed, hydrated and incubated in the following solutions: 0.2 N HCl, 0.3% Triton-X 100 in PBS, 15 µg/ml Proteinase K (37°C), 4% formalin in PBS and finally acetylated with acetic anhydride diluted in 0.1 M triethanolamine (750 µl/200 ml). Until hybridization sections were stored in a solution of 50% formamide in 2 x SSC at 37°C. For hybridization, probes were diluted in hybridization solution (50% deionized formamide, 10% dextran sulfate, 2 x SSC, 1 x Denhardt's solution, 1 µg/ml tRNA, 250 µg/ml herring sperm DNA) to a concentration of 100 ng/ml, incubated at 68°C for 15 min and layered onto the sections. Sections were hybridized overnight at 55°C in a humid chamber. Posthybridization washes were performed at 45°C using the following steps: 50% formamide in 2 x SSC. A 15 min incubation with RNAse T1 (2U/ml in 1 mM EDTA in 2 x SSC) at 37°C was followed by washes of 0.1 x SSC at 45°C and 2 x SSC at room temperature. The DIG-labeled hybrids were detected by antibody incubation performed according to the manufacturer's prescription (Boehringer Mannheim GmbH, Biochemica, Mannheim, Germany) with following modifications. A 1:2000 dilution of anti-digoxigenin (Fab) conjugated to alkaline phosphatase was used for a 2.5 h incubation at room temperature. Afterwards an extra washing step of 0.025% Tween in Tris buffered saline pH 7.5 was introduced. For staining, sections were layered with detection buffer (0.1 M Tris-HCl, 0.1 M NaCl, 0.05 M $MgCl_2$ pH 9.5) containing 0.33 mg/ml NBT (4-nitroblue tetrazolium chloride), 0.16 mg/ml BCIP (5-bromo-4-chloro-3-indolyl-phosphate), 7.5% PVA (polyvinylalcohol, m.w. 31000-50000 Aldrich Chemical Milwaukee, WI) and 1 mM levamisol (Sigma, St. Louis, MO, U.S.A.). The colour reaction was performed in the dark and was stopped when the desired intensity of the resulting blue precipitate was reached. Sections were washed in 10 mM Tris-HCl, 1 mM EDTA pH 8.0, counterstained with PAS and Nuclear red solution, dehydrated with ethanol gradients and mounted with the ethanol based mounting medium Euparal (Chroma-Gesellschaft, Stuttgart,

Germany). Control sections for morphological analysis were stained with hematoxylin and eosin.

Scoring of IGF-I and IGFBP expression

Each slide contained nine sections, i.e. three combinations of one sham, one OVX and one E_2-repleted rat. Two to four slides were made of each combination of sections. This means that of each rat at least two sections were hybridized and scored. All sections were hybridized together in one session to exclude variation in the hybridization procedure between the sections. First, all slides were inspected by three independent blinded observers to get an idea of the average of staining and staining intensity of the specific signal against the background. The score was based on comparison of the sections with respect to the extent of staining (number of positive cells) and staining intensity. The intensity of the nine sections per slide was scored twice by each observer. The score was an average of these two. The cumulative scores of the three observers per rat were calculated.

Statistical analysis

Data analysis involved estimation of the means, standard errors, one-way analysis of variance (ANOVA) and Student's t-test. $P < 0.05$ was considered statistically significant.

4.4 RESULTS

General measurements

Table 2.1 shows that OVX and E_2 suppletion resulted in the anticipated phenotypes. OVX rats had reduced uterus weights, while spleen and body weights were increased. Although supplementation with 0.05 mg E_2 resulted in similar serum E_2 levels as in sham rats, uterus weights were significantly increased and spleen weights were significantly decreased, while body weight was not significantly different from the sham-group.

Calcium and bone metabolism

As shown in Figure 2.1 BMD as measured by DEXA was significantly decreased following ovariectomy. E_2 suppletion restored BMD and even resulted in an increased BMD by 6.6% compared to sham-operated rats. The effects of ovariectomy and E_2 treatment on bone turnover are also reflected by changes in serum osteocalcin levels. In OVX rats serum osteocalcin levels were elevated while E_2 treatment resulted in lower levels. No differences between the groups in serum calcium, inorganic phosphate and alkaline phosphatase levels

were detected (Table 2.2).

Serum total 1,25-$(OH)_2D_3$ levels were not significantly changed in OVX rats (Table 2.3) [12]. In E_2 repleted rats serum total 1,25-$(OH)_2D_3$ levels were decreased significantly by almost 70% in comparison with the sham-operated rats. Serum 25-$(OH)D_3$ levels were reduced by 15% in E_2 repleted rats while serum PTH levels were similar in all groups.

IGF-I and IGFBPs

Serum total IGF-I level in OVX rats (66.6 ± 4.7 nmol/l) was not significantly changed compared to sham-operated rats (63.3 ± 2.1 nmol/l) while E_2 repleted rats had a significantly lower serum IGF-I level (52.4 ± 2.2 nmol/l; $p < 0.01$) than sham and OVX rats. Linear regression demonstrated that serum IGF-I level was negatively correlated to serum E_2 level ($y = 72.59 - 0.907x$, $r = -0.516$, $P=0.004$) and femoral BMD ($y = 0.270 - 0.0009x$, $r = -0.427$, $p = 0.01$). Serum IGF-I was positively correlated with serum osteocalcin ($y = -125.73 + 7.376x$, $r = 0.711$, $p < 0.0001$), serum alkaline phosphatase ($y = 37.12 + 0.623x$, $r = 0.464$, $p = 0.006$) and serum 1,25-$(OH)_2D_3$ level ($y = -63.41 + 2.320x$, $r = 0.539$, $p = 0.001$). There was no relation between serum IGF-I level and serum PTH level ($y = 56.88 + 0.277x$, $r = 0.096$, $p = 0.6$).

IGF-I mRNA was expressed predominantly in osteocytes (Figure 4.1A) but also occasionally osteoblasts were found to be positive. Figures 4.2 A-C show the expression of IGF-I mRNA in sections of sham-operated, OVX and E_2-repleted rats. In order to semiquantify the IGF-I mRNA expression the sections were examined as described in Materials and Methods. Ovariectomy reduced expression of IGF-I mRNA compared to sham rats. E_2-repletion restored IGF-I mRNA expression above sham levels and IGF-I mRNA expression was increased compared to E_2-deficient rats (Table 4.1). IGF-I mRNA expression was not associated with serum 1,25-$(OH)_2D_3$ level ($y = 5.718 - 0.006x$, $r = -0.182$, $p = 0.6$).

In bone also IGFBP-5 mRNA expression could be clearly detected and seemed to be confined to the osteoblasts (Figure 4.1B). As shown in Figures 4.2D-F and summarised in Table 4.2 ovariectomy did not change IGFBP-5 mRNA expression while in E_2-repleted rats IGFBP-5 mRNA expression was increased compared to both sham-operated and OVX-rats . IGFBP-5 mRNA expression was not correlated with serum 1,25-$(OH)_2D_3$ level ($y = 5.929 - 0.0039x$, $r = 0.090$, $p = 0.7$).

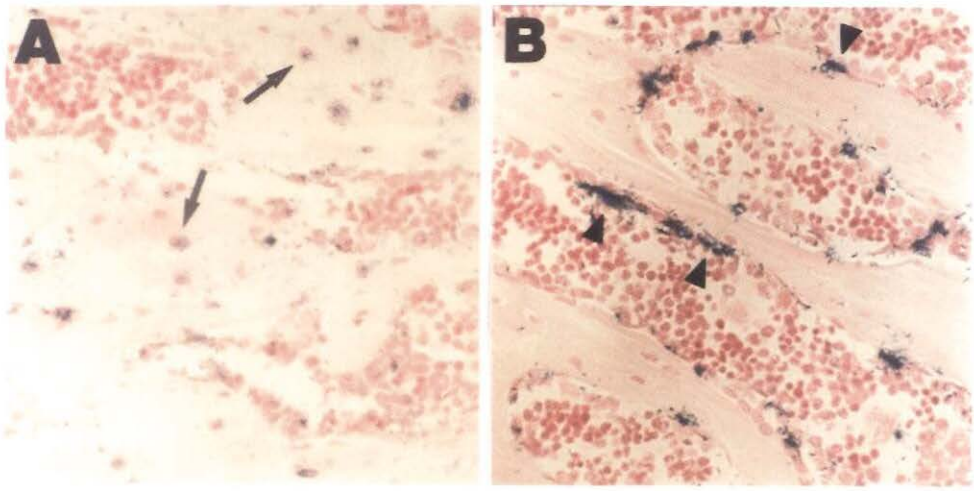

Figure 4.1 Expression of IGF-I mRNA in osteocytes (A) and IGFBP-5 (B) in osteoblasts. Arrow indicates an example of IGF-I expression in the osteocyte and the arrowhead IGFBP-5 expression in osteoblasts (Magnification 20x; bar = 50 μm).

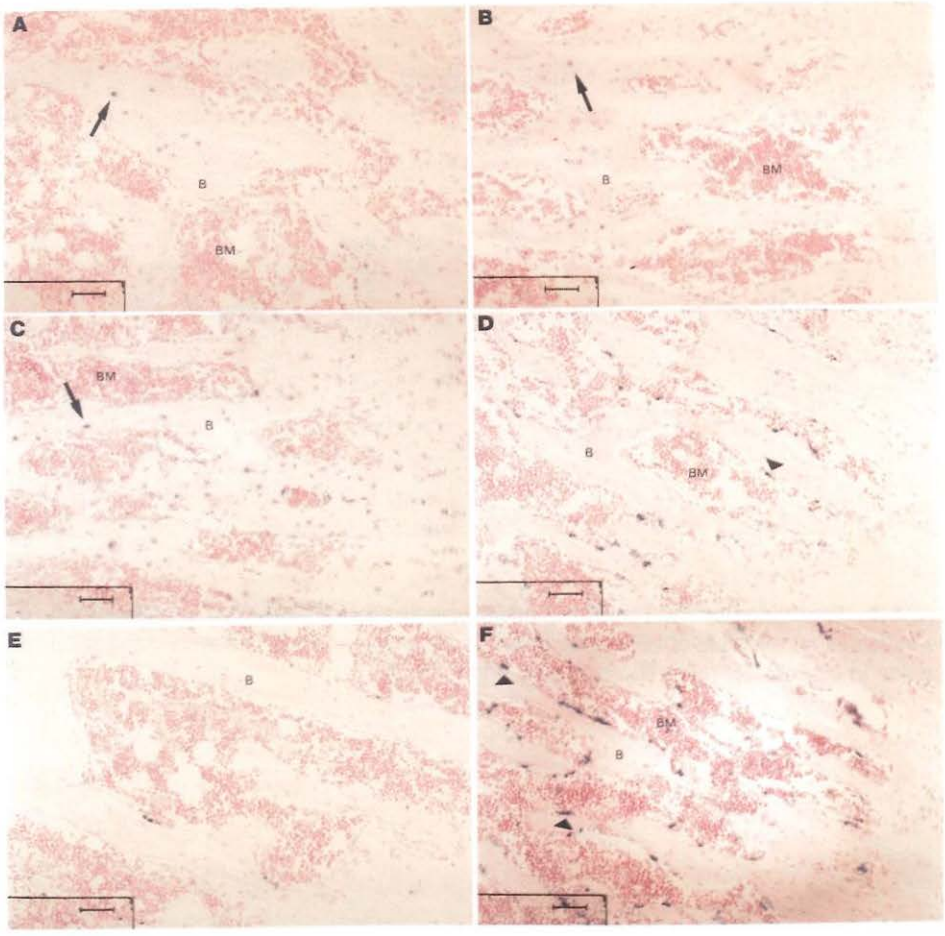

Figure 4.2 Effects of OVX and OVX + E_2 on IGF-I mRNA expression (A-C) and IGFBP-5 mRNA expression (D-F). Expression was detected by *in situ* hybridization as described in Materials and Methods in Sham (A and D), OVX (B and E) and OVX + E_2 (C and F). Arrow and arrowhead indicate example of expression in osteocyte and osteoblast, respectively. B = Bone matrix; BM = Bone marrow (Magnification 20x; bar = 50 μm).

Table 4.1 Effects of ovariectomy (OVX) and 0.05 mg 17β-estradiol (OVX + E_2) on IGF-I mRNA expression in bone four weeks after operation

Sham		OVX		OVX + E_2	
Ratno	IGF-I	Ratno	IGF-I	Ratno	IGF-I
1	6	2	3	4	6
17	6	14	3.5	13	6
29	3.5	25	3	27	6
32	6	34	5	28	9
19	6	14	4.5	9	6
29	6	20	3	11	9
31	4	25	3	27	5
23	4	15	2	21	4
18	9	16	6	24	5
SUM	50.5		33		56
MEAN	5.61		3.67		6.22
SEM	0.55		0.42		0.57

Data are cumulative scores (number of +) of the three observers and based on the following scoring: + low just above background; ++ intermediate; +++ high; ++++ very high.

In comparison with IGF-I and IGFBP-5 there was a weaker signal for IGFBP-2 and IGFBP-4 mRNAs in the various groups. Therefore, no clear effects of E_2 on IGFBP-2 and IGFBP-4 mRNAs could be established (data not shown). IGFBP-3 could be detected in some capillaries whereas IGFBP-6 could not be detected by *in situ* hybridization.

4.5 DISCUSSION

The present study shows coupling between E_2 and the IGF system with respect to bone metabolism. Relations were found both at systemic level and at local level in bone, although the relationships were different for serum IGF-I and IGF-I mRNA expression in bone cells. Serum IGF-I level was slightly but not significantly elevated following ovariectomy, while E_2 repletion resulted in significantly decreased serum IGF-I levels. Previous studies have also shown reduced serum IGF-I level in rats subcutaneously given E_2[22-24]. The increase in serum IGF-I level following ovariectomy[23,25,26] was shown to be time-dependent[24]. This might explain why serum IGF-I levels in our study (after 28 days) were not significantly elevated in OVX rats compared to sham-operated rats, although there was a trend. In women conflicting data have been reported on the relation between estrogen status and serum IGF-I level. The

Table 4.2 Effects of ovariectomy (OVX) and 0.05 mg 17β-estradiol (OVX + E_2) on IGFBP-5 mRNA expression in bone four weeks after operation

Sham		OVX		OVX + E_2	
Ratno	IGFBP-5	Ratno	IGFBP-5	Ratno	IGFBP-5
1	4	2	6	4	9
17	10	14	3.5	13	6
29	5	25	3	27	9
32	6	34	3	28	8.5
19	3	14	4.5	9	5
29	6	20	3	11	6
31	5	25	3	27	9
23	6	15	6	21	11
18	4	16	11	24	8
SUM	49		43		71.5
MEAN	5.44		4.78		7.94
SEM	0.67		0.88		0.64

Data are cumulative scores (number of +) of the three observers and based on the following scoring: + low just above background; ++ intermediate; + + high; ++++ very high.

decline in serum IGF-I level and bone matrix IGF-I with aging have been suggested to be one of the causes of skeletal aging[20,27]. Serum IGF-I levels were 30% lower in postmenopausal women as compared to premenopausal women[5]. Janssen et al. found a positive (age-adjusted) relationship between free E_2 serum levels and total serum IGF-I levels but not with free serum IGF-I levels[28]. Cosman et al. did not find a difference in total serum IGF-I levels between estrogen-treated and untreated postmenopausal women[29]. Others showed that orally administered E_2 suppresses serum IGF-I level in postmenopausal women[30-32] whereas intranasal and transdermally administered E_2 resulted in unchanged or increased serum IGF-I levels[18,30,32]. However, the increase in serum IGF-I levels in women following surgical menopause was prevented by transdermal estrogen administration[17] and is in line with the present data. These contradicting effects concerning E_2 effects on serum IGF-I levels may be ascribed to the first pass effect in the liver following oral administration, individual differences in the metabolism of the steroid, different schemes of treatment and differences in the predominant source of circulating IGF-I in the diverse studies[17]. Therefore, at this moment it is difficult to draw a final conclusion regarding the relationship between serum E_2 and serum IGF-I.

In the present study we examined the effect of OVX and E_2 on IGF-I and IGFBP-5 mRNA expression in bone cells by *in situ* hybridization. OVX rats had reduced skeletal IGF-I mRNA expression compared to sham-operated rats. Suppletion with E_2 did prevent the OVX induced reduction in IGF-I mRNA expression. IGF-I mRNA was found to be expressed by osteocytes and osteoblasts. *In vitro* studies have also shown that IGF-I is synthesized in osteocytes and osteoblastic cells[6,33,34] and that IGF-I synthesis is promoted by E_2[33-35]. *In situ* hybridization studies revealed that IGF-I mRNA is expressed predominantly in osteoblasts in trabeculae of tibiae in growing rats[36]. *In vivo* studies provide some conflicting data on the effects of E_2 on bone matrix IGF-I. Previous studies showed that E_2 replacement decreased IGF-I mRNA expression in femura and calvaria, while ovariectomy increased IGF-I mRNA expression[25,37]. This was assessed by Northern blotting of RNA extracts from whole femura one week following ovariectomy. This method does not discriminate in site-specific expression of IGF-I mRNA. In line with our data, Erdman et al. found that E_2 significantly increased bone matrix IGF-I protein concentration and calcium content in femur shafts of rats although ovariectomy had no consistent effects[16]. In another study E_2 decreased bone matrix IGF-I concentration[23]. These differences may be related to differences in methods of extraction and quantification of IGF-I[16]. Furthermore changes in IGF-I mRNA levels may not directly reflect changes in IGF-I protein secretion. In contrast with animal studies, a study by Seck et al. did not show an association between bone matrix IGF-I and menopause[20]. Several factors may contribute to this discrepancy between animal and human studies. Bone matrixIGF-I is accumulated in many years and may therefore not significantly change shortly after menopause. However, the synthesis of proteins as reflected by mRNA expression, can still be significantly changed. In rats, bone turnover after ovariectomy is very rapidly and significantly increased. So, in a relatively short period new bone matrix may be synthesized with a lower IGF-I content. Although differences have been observed between various studies it can be concluded that there is a close interaction between E_2 and IGF-I in bone. In view of the anabolic effects of IGF-I on bone[5] it is suggestive that the interplay between E_2 and IGF-I in bone could be part of the process leading to the reduced bone mass after ovariectomy. In addition, the present study shows dissociation between effects on circulating IGF-I level and IGF-I mRNA expression in bone cells. OVX rats had normal or slightly increased serum IGF-I levels while skeletal IGF-I mRNA expression was reduced compared to sham-operated rats. E_2 repletion resulted in a reduction in circulating IGF-I whereas skeletal IGF-I mRNA expression was restored to sham-operated rats. This is suggestive for the fact

that local IGF-I and not circulating IGF-I is more important for regulation of bone formation, which is in line with other observations[20,25].

Another interesting observation is that osteocytes highly express IGF-I mRNA, which supports the observation by others[6,33,34]. On basis of the anticipated role of osteocytes in bone it is tempting to speculate that IGF-I is a mediator between the mechanical loading and regulation of bone metabolism. However, Cheng et al. reported that strain-related proliferation of osteoblasts is mediated by IGF-II and not IGF-I[28]. Whether this is specific for osteoblasts and whether in osteocytes strain does act via IGF-I is unknown.

The biological action of IGF-I is modulated by IGFBPs[39]. *In vitro*, IGFBP-2 mostly inhibits the biological action of IGF-I, while IGFBP-5 promotes its action[1,40]. In bone cell cultures IGFBP-5 enhances the IGF binding to its receptor[9] and permits the attachment of IGF-I to the newly formed matrix[39]. Furthermore it promotes osteoblast mitogenesis by enhancement of the IGF-I effect[9] as well as in an IGF-I- independent manner[41]. The expression and secretion of IGFBP-2 and IGFBP-5 are, just as for the other IGFBPs, dependent on the developmental stage of the osteoblast and they have the same secretion pattern[42]. It has been suggested that IGFBP expression is hormonally regulated[42]. *In vitro* studies with ovarian cancer cells have shown that E_2 increases IGFBP-5 mRNA levels, while IGFBP-2 mRNA levels are only minimally depressed[43]. In our study estrogen suppletion increased osteoblastic IGFBP-5 mRNA expression, while ovariectomy did not change its expression. IGFBP-2 mRNA expression was only weakly expressed and no clear effects of estrogen status on IGFBP-2 mRNA expression could be detected.

Other factors that may mediate the effect of E_2 on IGF-I and IGFBP-5 mRNA expression in bone are the calciotropic hormones, PTH and 1,25-$(OH)_2D_3$. Exogenously administered PTH has been described to promote IGF-I synthesis by bone cells[11,44]. In rat osteosarcoma cells PTH has been shown to increase IGFBP-5[45]. However, in our study no differences in PTH levels between the various experimental groups were observed. It is therefore unlikely that the differences in IGF-I mRNA expression can be attributed to PTH.

Several studies have focussed on 1,25-$(OH)_2D_3$ mediated regulation of IGF-I in bone cells and cultured bone tissue. 1,25-$(OH)_2D_3$ inhibited production of IGF-I in mouse osteoblasts and mouse calvaria[44,46]. In rat osteoblast-like cells 1,25-$(OH)_2D_3$ slightly increased IGF-I production[13]. The current study did not show a significant correlation between serum 1,25-$(OH)_2D_3$ levels and IGF-I mRNA expression in bone. The failure of this study to detect a negative association between serum 1,25-$(OH)_2D_3$ levels and IGF-I mRNA expression might be due to the fact that in this study changes in serum 1,25-$(OH)_2D_3$ levels were accompanied

by changes in serum E_2 levels, potentially resulting in opposing effects on IGF-I expression in bone. Besides interactions at the level of IGF-I synthesis in bone cells also a functional interplay of 1,25-$(OH)_2D_3$ and IGF-I in the regulation of bone and bone cell metabolism has been described[47-49]. As for the relationship of E_2, also for 1,25-$(OH)_2D_3$ a difference between serum IGF-I and bone IGF-I mRNA expression was observed. In contrast to the absence at the IGF-I mRNA expression level in bone, there is a positive correlation between serum 1,25-$(OH)_2D_3$ and serum IGF-I levels. This might be related to a mutual positive stimulation of 1,25-$(OH)_2D_3$ and IGF-I synthesis in the kidney and liver, respectively[50-52].

In conclusion, the major finding of the present study is the close interaction between E_2 and the IGF-I system at bone level. Changes in serum E_2 levels result in an altered expression of IGF-I and IGFBP-5 mRNA in osteoblasts and osteocytes, and in osteoblasts, respectively. E_2 has a positive effect on expression of both IGF-I and IGFBP-5 mRNA in bone cells. These data indicate that the IGF-I system in bone may be part of the E_2 control of bone turnover. In addition, this study demonstrates dissociation between E_2 induced changes in serum IGF-I levels and the effects on expression in bone. Therefore, it is questionable whether serum IGF-I level is a good estimate for changes in IGF-I in target tissue and thereby whether it is a good marker to assess the relation between IGF-I and bone turnover.

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Chapter 5

Reduced *in vitro* mineralization by human osteoblasts by sera from women with high bone mineral density: A potential role of 17β-estradiol

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5.1 ABSTRACT

The risk for osteoporotic fractures is determined by both quantity and quality of bone. The elasticity of bone and its resistance to fracture is related to its degree of mineralization. In the present study we analyzed whether there are differences in effects on human osteoblast's proliferation and mineralization between sera from postmenopausal women with high and low bone mineral densities.

For this purpose sera of 21 women with a femoral neck BMD within the lowest age-adjusted quintile (<0.75 g/cm^2), and sera of 29 women with a femoral neck BMD within the highest age-adjusted quintile (≥0.92 g/cm^2) were tested. Serum and urine parameters for bone turnover were measured. Human osteoblast-like (MG-63) cells were cultured in medium supplemented with the sera for 7 days and DNA content was measured. Human fetal osteoblast (SV-HFO) cells, which proceed through different stages of differentiation in culture, including extracellular matrix formation (days 7-14) and mineralization (days 14-21), were cultured in medium supplemented with the sera for 21 days. DNA content, mineralization, as assessed by calcium deposition in the extracellular matrix, and alkaline phosphatase (Alp) activity were measured after 21 days of culture.

Postmenopausal women with a low BMD had higher serum Alp and serum osteocalcin levels than women with a high BMD. Serum 1,25-$(OH)_2D_3$ and serum PTH levels were similar in the two groups. Interestingly, serum E_2 levels were significantly lower in women with a low BMD compared to women with a high BMD (17.0 ±4.6 pmol/l vs. 45.4 ± 6.9 pmol/l, $p < 0.01$).

There was no difference in proliferation of MG-63 cells and SV-HFO cells, cultured in serum from women with either a low BMD or high BMD. AP induction in SV-HFO cells was also similar by sera of both groups. *In vitro* induction of calcium deposition in SV-HFO cells was significantly increased by sera of the low BMD group compared to sera of the high BMD group (0.18 ± 0.009 nmol/µg DNA vs. 0.15 ± 0.009 nmol/µg DNA, $p = 0.02$).

In conclusion, serum from postmenopausal women with low BMD and low serum E_2 levels results in increased mineralization as tested in our human *in vitro* system. Based on these data we hypothesize a new role for E_2 in bone homeostasis: i.e. control of the extent or set point of mineralization, and thereby the elasticity of bone, which potentially contributes to the quality of bone.

5.2 INTRODUCTION

Osteoporosis is a systemic skeletal disease, characterized by low bone mass and microarchitectural detoriation of bone tissue with a consequent increase in bone fragility and susceptibility to fracture[1]. The risk for osteoporotic fractures is determined by both quantity and quality of bone. Both structural and material properties determine the elastic and plastic stiffness of bone and the strength of bone to withstand mechanical forces. The elasticity of bone is related to its degree of mineralization[2].

After menopause, when serum E_2 levels fall, bone turnover, i.e. bone resorption and formation, is increased. During this process, bone resorption exceeds bone formation resulting in net bone loss[3,4]. E_2 is thought to modulate bone remodeling by its effects on osteoclastic and osteoblastic number and activity. E_2 inhibits bone resorption by regulation of several osteoclast-activating factors, like Il-1, and Il-6 and RANK[5,6]. Furthermore E_2 may have anabolic effects on bone. These effects can be via direct effects on bone cells, but also indirectly via interplay with other factors, like the calciotropic hormones, 1,25-$(OH)_2D_3$ and PTH, and insulin-like growth factor-I[7-11]. Differences in serum E_2 levels have been related to differences in effects of these factors and might thus influence bone metabolism and result in decreased BMD[12].

Investigation of the precise action of hormones and factors on bone formation *in vivo* is impeded by the close coupling between bone formation and resorption. In this respect, several studies have shown that rat serum contains factors that modulate bone formation[13-16]. Klein et al. showed differences in induction of bone mineralization by rat bone marrow cells when cultured in sera from perimenopausal women with either a low or high BMD[14]. We hypothesized that there are differences in *in vitro* response of human osteoblasts to sera from postmenopausal women with either a low or high BMD that are related to divergent actions of hormones and factors, involved in bone remodeling, between these two groups. This hypothesis was tested in an *in vitro* culture system with the human osteoblast-like cell line MG-63 and with the *in vitro* mineralizing human fetal SV-HFO osteoblasts.

5.3 MATERIALS AND METHODS

Subjects

Postmenopausal women aged 55-69 years (mean age ± SD, 66.1 ± 0.6 years) were selected from independently living subjects of the Rotterdam Study, from whom subjects using hormonal replacement therapy, cytostatics, vitamin D, thyroid hormone or known to have diabetes were excluded[17]. 21 Women who had a femoral neck BMD in the lowest age-adjusted quintile ($\leq$

0.75 g/cm^2) and 29 women who had a BMD in the highest age-adjusted quintile ($\geq$0.92 g/cm^2) were included. This substudy within the Rotterdam study was approved by the medical ethics committee of the Erasmus MC Rotterdam.

Biochemical markers

Fasting blood samples and overnight urine samples were stored frozen at –80°C until all samples were collected and were then analyzed. Osteocalcin, 1,25-$(OH)_2D_3$, 25-$(OH)D_3$, PTH, E_2, and cross-linked N-telopeptide of type I collagen (NTX) were measured. Serum osteocalcin was measured with a radioimmuno assay (Incstar Corp., Stillwater, MN, U.S.A.). 1,25-$(OH)_2D_3$ was measured by immunoextraction followed by quantification by ^{125}I-RIA (IDS, Boldon, U.K.). Concentrations of 25-$(OH)D_3$ were measured by radioimmunoassay (Incstar Corp., Stillwater, MN, U.S.A.). Serum PTH was measured by using a radio-isotopic-assay of the intact parathyroid hormone (Nichols Institute, San Juan Capistrano, CA, U.S.A.). Serum E_2 was measured by an extracting procedure using diethyl ether followed by RIA (DPC, Los Angeles, CA, U.S.A.). An enzyme-linked immunosorbent assay was used for the measurement of urinary NTX. Total serum calcium, anorganic phosphate, alkaline phosphatase (Alp), and serum and urinary creatinine were analyzed with standard methods.

Cell culture

MG-63 cells were suspended in phenol red free RPMI 1640 medium (Sigma Chemical Co, St. Louis, MO, U.S.A.) supplemented with 10% charcoal-treated fetal calf serum (FCS), 2 mM glutamine, 20 mM HEPES (Sigma, St. Louis, MI, U.S.A.), 100 units/ml penicillin, 100 µg/ml streptomycin (Life Technologies, Breda, The Netherlands) and 24 mM sodium bicarbonate. Cells were seeded in a density of 3,3 x 10^3 cells per cm^2 in 6-wells plates. After one day, medium was replaced by phenol red free RPMI 1640 medium (Sigma Chemical Co, St. Louis, MO, U.S.A.) supplemented with 5% human serum, 2 mM glutamine, 20 mM HEPES (Sigma, St. Louis, MI, U.S.A.), 100 units/ml penicillin, 100 µg/ml streptomycin (Life Technologies, Breda, The Netherlands) and 24 mM sodium bicarbonate. Cells were tested in separate cultures with sera from 26 individual women for 7 days (11 women with low BMD and 15 women with high BMD). Medium was replaced after 3 days and 1,25-$(OH)_2D_3$ (10^{-9} to 10^{-7} M) was added to the cells. At the end of culture cells were scraped in PBS containing 0.1% triton X-100 and stored at –80°C.
SV-HFO cells were seeded in a density of 5.5 x 10^3 vital cells per cm^2 in phenol-red free α-Minimal Essential Medium (α-MEM Gibco BRL, Paisley, U.K.) supplemented with 20 mM HEPES (Sigma, St. Louis, MI, U.S.A.), streptavidin/penicillin, 1.8 mM $CaCl_2{\cdot}2H_2O$ (Sigma, St. Louis, MI,

U.S.A.), 10% heat-inactivated FCS at 37°C and 5% CO_2 in a humidified atmosphere. Medium was replaced every 2-3 days and after one week cells were trypsinized for further culture under experimental conditions. For the cell culture experiments cells were seeded in a density of 1 x 10^4 vital cells per cm^2 and cultured in phenol-red free α-MEM (Gibco BRL, Paisley, U.K.) supplemented with 20 mM HEPES (Sigma, St. Louis, MI, U.S.A.), streptavidin/penicillin, 1.8 mM $CaCl_2 \cdot 2H_2O$ (Sigma, St. Louis, MI, U.S.A.), 2% human serum at 37°C and 5% CO_2 in a humidified atmosphere for 23 days. Cells were tested in separate cultures with sera from 50 individual volunteers (21 women with low BMD and 29 women with high BMD). Medium was replaced every 2-3 days supplemented with freshly diluted 1 μM dexamethasone (Sigma, St. Louis, MI, U.S.A.) and 10 mM β-glycerophosphate (Sigma, St. Louis, MI, U.S.A.). SV-HFO cells and culture supernatant from duplicate wells were collected at day 21 of culture. Medium was stored at –20°C and cells were scraped in PBS containing 0.1% triton X-100 and stored at –80°C. Prior to use, MG-63 and SV-HFO cell lysates were sonificated on ice in a sonifier cell disruptor for 2 x 15 seconds.

For the cell culture experiments serum from women with low BMD will be referred to as low BMD serum while that of women with high BMD will be called high BMD serum.

DNA content

MG-63 and SV-HFO cell lysates were treated with heparin and RNAse A (50 μg/ml in PBS) for 30 minutes at 37°C. DNA content was measured according to the ethidium bromide method of Karsten and Wollenberger[18].

Alkaline phosphatase activity

Alp activity was assayed by determining the release of paranitrophenol from paranitrophenylphosphate (20 mM in 1 M diethanolamin buffer supplemented with 1 mM $MgCl_2$ at pH 9.8) in the SV-HFO cell lysates for 10 minutes at 37°C. The reaction was stopped by adding 0.1 M NaOH and absorption was measured at 405 nm using a Packard Spectra Count. Results were adjusted for DNA content of the corresponding cell lysates.

Calcium deposition

SV-HFO cell lysates were incubated overnight with 0.25 M HCl at 4°C. Calcium content was colorimetrically determined with a calcium assay kit (Sigma, St. Louis, MI, U.S.A.) according to

the manufacturer's description at 595 nm (Packard Spectra Count). Results were adjusted for DNA content of the corresponding cell lysates.

Statistical analysis

We performed all analyses separately for all women with low and high bone mass. Analysis of variance was performed and for comparison student's t-test was used.

5.3 RESULTS

Characteristics of study population

As shown in Table 5.1 there were no significant differences in age, age at menopause, years since menopause, and BMI between the two groups.

Serum Alp and osteocalcin levels, markers for bone formation, were significantly increased in the lower BMD group compared to the higher BMD group. Bone resorption, reflected by urinary NTX/creatinine levels, also tended to be higher in women with a low BMD.

Serum levels of the calciotropic hormones were determined. There were no differences in serum 25-$(OH)D_3$, 1,25-$(OH)_2D_3$ and PTH levels between the two groups. However, serum E_2 levels were significantly lower in postmenopausal women with a low BMD compared to those with a high BMD.

In vitro response of human osteoblasts to sera from women with either a low or high BMD

First, the effect on proliferation of the human osteoblast-like cell line MG-63 was examined. Cells were cultured for 7 days in medium containing 5% of either low or high BMD serum.

After 7 days there was no significant difference in total amount of DNA between cells cultured in either low or high BMD serum (43.9 ± 2.1 µg DNA vs. 49.5 ± 2.1 µg DNA, respectively; $p = 0.18$).

To further examine the effects of low and high BMD serum on human osteoblast activity a second osteoblast culture model, the SV-HFO cell-line, was used. SV-40 virus immortalized human SV-HFO cells proceed in about three weeks of culture through a characteristic differentiation process (Figure 5.1). There is an initial phase of rapid proliferation followed by a slowing down of proliferation, although throughout the culture there is a gradual increase in the amount of DNA. During the first week of culture the cells start to synthesize collagen type I and to produce an extracellular matrix. This is followed in the second week of culture by an increase in Alp activity and decrease in osteocalcin production.

Table 5.1 Characteristics of study population (mean ± SEM) according to BMD group

	Bone mineral density groups		*p* -value
	Low (≤ 0.75 g/cm^2)	High (≥0.92 g/cm^2)	
General characteristics			
Number	21	29	
Femoral neck BMD (g/cm^2)	0.67 ± 0.01	1.01 ± 0.01	
Lumbar spine BMD (g/cm^2)	0.89 ± 0.02	1.14 ± 0.03	
Age (yrs)	66.4 ± 1.0	65.9 ± 1.0	NS
Age of menopause (yrs)	47.1 ± 1.0	48.5 ± 1.0	NS
Years since menopause	20.4 ± 1.9	17.8 ± 1.5	NS
BMI (kg/m^2)	24.1 ± 0.6	25.4 ± 0.6	NS
Biochemical markers of bone turnover			
Serum			
Alkaline phosphatase (units/ml)	55.0 ± 2.5	48.6 ± 1.7	<0.05
Osteocalcin (µg/l)	5.8 ± 0.4	4.7 ± 0.3	<0.05
Urine			
NTX/creatinine	84.4 ± 9.9	73.2 ± 13.2	NS
Hormones			
25-(OH)D_3 (nmol/l)	86.0 ± 7.0	81.8 ± 5.1	NS
1,25-$(OH)_2D_3$ (pmol/l)	106.6 ± 6.5	102.0 ± 6.1	NS
PTH (ng/l)	34.2 ± 3.9	32.9 ± 2.6	NS
E_2 (pmol/l)	17.0 ± 4.6	45.4 ± 6.9	<0.01

Finally, in the third week of culture the extracellular matrix formed starts to be mineralized and after 21 days a strong mineralization can be detected. As the various osteoblast parameters peak at various time-points during culture, ideally measurements should be made at multiple time-points. However, due to limited availability of serum this was not possible and measurements had to be restricted to a single time point, i.e. to day 21 when mineralization can readily be detected. Comparable to the MG-63 cells, also in the SV-HFO

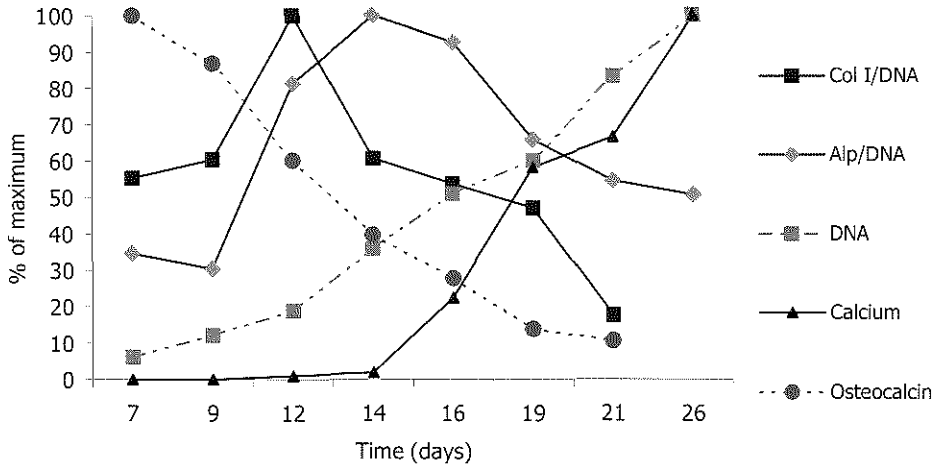

Figure 5.1 Temporal expression of phenotypic characteristics during osteoblast differentiation. See Chapter 5.3 for details.

cells no difference in effect on total DNA between low and high BMD serum was detected (Figure 5.2).

In SV-HFO cells no difference in induction of AP activity between sera from women with either low or high bone mineral density was found. However, mineralization was significantly higher in cells cultured in low BMD serum compared to high BMD (Figure 5.2).

5.5 DISCUSSION

In the present study we observed that sera of postmenopausal women with high BMD induce less *in vitro* mineralization by human osteoblasts compared to sera of women with low BMD.

Several studies have shown that the state of bone metabolism can be reflected by the response of bone cells in culture to serum[14,16,19]. All these studies have been performed with rat bone cells. In the present study, performed with two human osteoblast cell lines cultured with serum from women with either low or high BMD, no difference was found in cell numbers between the two groups at day 7 of culture in MG-63 cells and at day 21 in SV-HFO cells. These data are in line with a study of Klein et al., who found no difference in induction

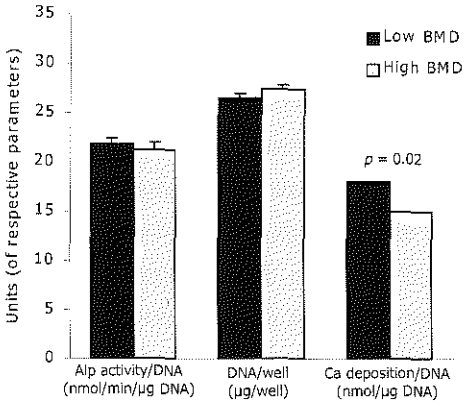

Figure 5.2 Effect of sera from women with either a low or high bone mineral density (BMD) on induction of alkaline phosphatase (Alp) activity, cell number and mineralization in human fetal osteoblasts. Cells were cultured and analyses were performed as described in Materials and Methods. Data are presented as means ± SEM.

of proliferation of rat osteoprogenitor cells by sera from women with a low or high BMD at day 11 of culture[14]. However, others showed a relation between serum E_2 levels and proliferation of osteoblasts in culture[16,19]. Sera from climacteric women after hormonal replacement therapy (HRT) decreased rat osteoprogenitor cell counts as compared with sera before HRT[19]. In another study there appeared to be a time-dependent proliferative response of rat osteoblast-like cells to sera from sham or ovariectomized (OVX) rats[16]. Yokose et al. reported that the DNA content in cells cultured in sera from OVX rats was higher than the DNA content in cells cultured with sera from sham-operated rats on days 5 and 10 of culture, but they became almost identical on days 15 and 20. These differences in effects may be related to differences in characteristics of proliferation between human and rat cell lines, and between mature osteoblasts and osteoblast progenitors cells, and to the use of either human or rat serum. In the present study differences in DNA content in SV-HFO cells, cultured in either low or high BMD, might have fainted, because proliferation of SV-HFO cells is maximal at 10 days of proliferation and then subsides[20]. However, the fact that we also found no difference in proliferation in the cultured MG-63 cells makes an effect of E_2 on proliferation of mature human osteoblasts questionable.

Osteoblast activity is reflected by Alp activity and serum osteocalcin levels. Although serum Alp and osteocalcin levels were elevated in the low BMD group, *in vitro* induction of Alp activity was not increased by low BMD sera compared with high BMD sera. Others also found no effect on Alp activity in rat marrow stroma cells, cultured for 11 days in sera from perimenopausal women with either a low or high BMD[14] and in sera from women before and after hormone replacement therapy[19]. Waters et al. demonstrated that E_2 increases Alp activity in ERα positive human fetal osteoblast (hFOB) cells but not in ERβ positive hFBO cells[21]. So specific receptor expression may play a role: different responses after activation of ERα or ERβ. Yokose et al. showed that rat osteoblast-like cells exposed to sera of ovariectomized rats exhibited higher levels of Alp activity than osteoblast-like cells exposed to sham sera[16]. These conflicting results may be ascribed to different osteoblast cell lines and different factors in sera of women and female rats. The fact that in the current study we do not see an effect on Alp activity may be ascribed to the fact that peak Alp activity in SV-HFO cells is in the late proliferation and early differentiation phase at 7–14 days of incubation. Therefore, after incubation for 21 days eventual differences in induction of AP activity between the two groups might have been missed. Unfortunately due to the limited availability of serum the analysis in this study had to be restricted to a single time point. This might also explain why the observed *in vivo* difference in serum AP is not reflected *in vitro*.

A key feature of osteoblasts and bone is mineralization of the extracellular protein matrix. The current data show that the mineralization by SV-HFO cells is higher in low BMD serum than in high BMD serum. Interestingly, postmenopausal women with low BMD had significantly lower serum E_2 levels than women with high BMD, while there were no differences in the other calciotropic hormones, $1,25\text{-}(OH)_2D_3$ and PTH. A role of E_2 in mineralization is supported by the observation that E_2 suppletion is inversely related with *in vitro* mineralization by SV-HFO cells [Unpublished data]. Our findings are in line with the study of Yokose et al., who showed that sera from ovariectomized rats induced higher calcium content in the rat osteoblast-like cell layer and a higher formation of bone-like nodules than in the cells exposed to sham sera[16]. In contrast, Klein et al. found in a rat osteoblast model a decreased cell-mediated mineralization after 21 days of culture with sera from women with low BMD compared to women with a high BMD[14]. This study was performed with sera from perimenopausal women who may have in general higher serum E_2 levels than our study population. Klein et al. added 15% fetal calf serum to phenol-red containing medium, which are both potential sources of E_2. Also differences in calcium concentration of the media may have attributed to the different results. All these aspects might have obscured the E_2 effect

on mineralization. Finally, Klein et al. used osteoblast progenitor cells, while in the present study mature osteoblasts were used. The current observation suggests that low BMD in postmenopausal women is predominantly caused by increased bone resorption, and not by decreased bone formation.

Although not directly expected in view of the relationship of BMD and osteoporosis, the increase in mineralization in the low BMD/low E_2 group may contribute to the risk of osteoporotic fractures. The degree of mineralization determines the elasticity of bone and thus its resistance to fracture. The optimal mineralization density value for bone strength is currently not known. If bone is highly mineralized, microfractures can readily propagate through bone, which subsequently becomes liable to fracture[22]. E_2 deficiency may thus result in overmineralization and increased susceptibility to fracture. Recently, E_2 has also been shown to regulate bone mineralization both *in vitro* and *in vivo*. E_2 inhibits mineralization in human osteoblast cells transfected with ERα[21], while short-term E_2 suppression in women results in the accumulation of bone with a higher mineralization density[2]. Our finding that sera from women with a high BMD and high serum E_2 level induce lower mineralization *in vitro* supports the idea that E_2 protects against overmineralization and thereby decreases fracture risk and in this way also contributes to an optimal bone quality. Thus, after menopause, when E_2 levels fall, bone mass decreases due to the fact that bone resorption exceeds bone formation. The newly formed bone may then be overmineralized. Both decreased bone mass, which is actually measured by DEXA, and increased bone mineralization may increase bone fragility.

In conclusion, we hypothesize that E_2 is one of the serum factors in postmenopausal women that determine bone mass and bone quality. The present data also suggest a new role for E_2 in bone homeostasis: i.e. control of the extent or set point of mineralization, and thereby the elasticity of bone, which potentially contributes to the quality of bone.

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Part Two

The significance of vitamin D receptor gene polymorphisms for 1,25-dihydroxyvitamin D_3 action and interaction between estrogen receptor α and vitamin D receptor gene polymorphisms

Chapter 6

Vitamin D receptor 3′ polymorphism is associated with rate of bone turnover in women with low bone mineral density

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6.1 ABSTRACT

A decreased BMD is a strong risk factor of osteoporotic fractures and is thought to result from changes in rate of bone turnover. Bone turnover rate has been shown to be under genetic control, but genes involved remain ill defined. 1,25-$(OH)_2D_3$ plays, via the VDR, a central role in bone turnover rate. Therefore, we investigated whether genetic variations of the VDR could explain differences in bone turnover rate among 88 postmenopausal women with either a low (n = 41) or high (n = 47) BMD. Furthermore, we examined whether VDR genotype was associated with a difference in biochemical response to a 7 days treatment with 2μg of 1,25-$(OH)_2D_3$ orally. Indices of bone turnover were measured in urine and blood samples collected after a overnight fast and VDR genotype was determined as haplotype of three clustered restriction fragment length polymorphism (*BsmI*, *ApaI* and *TaqI*) at the 3' end of the gene.

At baseline the osteocalcin serum levels (p = 0.09) and urinary NTX/creatinine ratio (p = 0.02) were increased in women having low BMD values compared to the high BMD group. Interestingly, in the low BMD group these biochemical markers were highest in women homozygous for VDR haplotype 2 ('BAt') alleles, intermediate for the heterozygotes and lowest for the reference group without haplotype 2. The response of any of the biochemical markers to short-term substitution of 2 μg 1,25-$(OH)_2D_3$ a day, did not differ significantly among the BMD groups and responses were similar among the VDR genotypes.

In this pilot study, we observed higher osteocalcin and NTX/creatinine levels in women having low BMD compared to women having high BMD. This increased bone turnover rate in women having low BMD was associated with VDR 3' haplotype polymorphism. This genotype-dependent bone turnover rate could not be explained by a divergent response to short term substitution with 1,25-$(OH)_2D_3$.

6.2 INTRODUCTION

Fractures, the clinically significant endpoint of osteoporosis, are a major cause of increasing morbidity affecting the elderly population. One of the strongest predictors of fractures is a decline of BMD independent of age[1]. A decrease in BMD with age results from both an increased bone turnover rate, i.e. the ratio of bone formation and bone resorption, and an imbalance in bone turnover, favouring bone resorption. Recently, twin studies showed that the rate of bone turnover is under strong genetic influence, but the genes involved remain ill defined[2,3].

One approach to investigate which genes are associated with bone turnover rate is the candidate gene approach. Such candidate genes can be selected on the basis of their involvement in a particular biochemical pathway in bone metabolism. An important candidate gene in this respect is the VDR gene. 1,25-$(OH)_2D_3$ plays a central role in bone metabolism and exerts its effect via the VDR. 1,25-$(OH)_2D_3$ regulates growth and differentiation of osteoblasts and osteoclasts, which are cells of bone formation and bone resorption, respectively[4,5]. In line with this, substitution of vitamin D reduces the incidence of fractures in the elderly, although conflicting results have been reported[6]. A possible explanation for at least part of these contrasting results might be that genetic variability of the VDR cancause variation in the regulating effect of 1,25-$(OH)_2D_3$ on bone turnover. This might result in VDR gene dependent variances in BMD, which ultimately lead to differences in fracture rate[7-11].
In light of the above we conducted a pilot study to investigate whether differences in rates of bone turnover among women with either a low or high BMD could be explained by genetic variations of the VDR. Furthermore, we examined whether VDR gene polymorphism influenced the biochemical response to short-term substitution of 1,25-$(OH)_2D_3$ in both BMD groups.

6.3 MATERIALS AND METHODS

Subjects

The postmenopausal women included in this analysis were part of the Rotterdam Study, a population-based cohort study of persons aged 55 years and over, living in a district of Rotterdam, the Netherlands. The objective of the Rotterdam Study is to investigate the occurrence of chronic disabling diseases in relation to several potential determinants. Rationale and design have been described previously[12]. All 10,275 inhabitants were invited for the study between August 1990 and June 1993. Of those, 7,983 participated bringing the overall response rate to 78 percent.
For the present study we included a sample of independently living women who were part of a large (n = 1782) genetic-epidemiological study on osteoporosis[10]. In that study subjects were excluded according to the following criteria: aged 80 years and over, use of thyroid hormone, use of cytostatics, use of diuretics and known diabetes mellitus type II. We re-invited, four years after the start of the initial study on osteoporosis, women (n=202) who had a femoral neck BMD at baseline in the lowest quintile ($\leq$ 0.75 g/cm^2) and those who had a femoral neck BMD in the highest quintile ($\geq$ 0.92 g/cm^2). Because of recent use of drugs known to influence the bone metabolism, 30 women could not be included and 84 women were not willing to participate. This brought the total number of participating women on 88

(44%) with n=47 in the highest and n=41 in the lowest BMD group. This substudy within the Rotterdam study was approved by the medical ethics committee of the Erasmus University Medical School, and renewed written informed consent was obtained from each woman.

Intervention

Each subject received two microgram (2-µg) 1,25-$(OH)_2D_3$ each morning during 7 consecutive days. Fastening blood and overnight urine samples were collected at three time points: baseline, day 4 and day 7. To assess compliance, capsules were counted at each visit.

Measurements

A. Bone mineral density

At baseline (start of the Rotterdam Study) measurement of femoral neck BMD (expressed in g/cm^2) was performed by dual energy X-ray absorptiometry (DEXA; DPX-L densitometer; Lunar Corp., Madison, WI, U.S.A.) as described before[13].

B. Biochemical markers

Fasting blood and overnight urine samples were stored frozen at –20° C until all samples were analyzed. Analysis was performed without prior knowledge of the BMD and genotype status. We measured 1,25-$(OH)_2D_3$, 25-$(OH)D_3$, osteocalcin, PTH and NTX. For the quantitative determination of serum 1,25-$(OH)_2D_3$ a ^{125}I-radioimmunoassay (RIA) was used (Nichols Institute Diagnostics, San Juan Capistrano, CA, U.S.A.). The 25-$(OH)D_3$ serum level was measured by using a ^{125}I-RIA kit (Incstar Corp., Stillwater, MN, U.S.A.). Serum osteocalcin was measured with a radioimmuno assay (Incstar Corp., Stillwater, MN, U.S.A.). Serum PTH was measured by using a radioisotopic-assay of the intact parathyroid hormone (Nichols Institute Diagnostics, San Juan Capistrano, CA, U.S.A.). An enzyme-linked immunosorbent assay was used for the measurement of urinary NTX and the NTX/creatinine ratio was calculated. Urinary excretion of creatinine was measured by autoanalyzer techniques. The inter-assay and intra-assay coefficient of variation of all these assays varied between the 3-18 % and 2-19 %, respectively.

C. Genotyping

Three anonymous polymorphic restriction enzyme recognition sites at the 3' end of the VDR gene, i.e., for *Bsm*I, *Apa*I, and *Taq*I were assessed in relation to each other by a direct

molecular haplotyping PCR procedure which we developed[10]. The alleles were named as previously described for alleles defined by individual RFLPs[14,15]; in genotypes such as "BAt-baT" capitals denote absence and lower case letters denote presence of the site for the restriction enzymes *Bsm*I (B/b), *Apa*I (A/a), and *Taq*I (T/t) on each of the alleles. The haplotype alleles were coded 1-5 in order of decreasing frequency in the population; the most frequent genotypes are presented as combinations of two alleles (1 = baT, 2 = BAt, and 3 = bAT). Detailed information on haplotype alleles and genotype frequencies in a larger sample from the Rotterdam Study, including this population, can be found elsewhere[10].

Statistical analysis

Data for each biochemical marker of bone turnover at each visit were first examined for deviation from normal distribution by using the Kolmogorov-Smirnov test. If deviation was statistically significant, ^{10}log transformations were performed.

We performed all analyses separately for women with high and low BMD. The comparison of continuous variables among groups was done with a two-sample t-test or ANOVA test. The categorical characteristics were compared by Pearson's chi-square analysis. To adjust for potential confounders at baseline we used multiple regression analysis. Differences in response to 1,25-$(OH)_2D_3$ between groups were analyzed by using repeated measurement models taking each visit into account.

To analyze the relation between VDR genotype and biochemical markers of bone metabolism we stratified the subjects by carrier status of the three most frequent alleles, i.e., haplotype alleles 1, 2 and 3 and made groups of subjects carrying 0, 1 or 2 copies of the test allele. For example, for haplotype 2, the reference group consisted of subjects with genotype 11 or 13, the heterozygote group consisted of subjects with genotype 12, 23 or 24, while the homozygote group consisted of subjects with genotype 22. Allele dose effects were analyzed by comparing individuals of the reference group to individuals being heterozygous and homozygous for the test allele.

6.4 RESULTS

BMD groups

Anthropometric characteristics according to BMD groups are show in Table 6.1. Compared to women with high BMD values BMI was lower ($p < 0.01$) in women having a low BMD. For other characteristics like age, dietary calcium- and vitamin D intake there was no significant difference between the BMD groups.

Table 6.1 Characteristics according to BMD group (Values are number (%) or means ± SD)

	Bone mineral density groups		*p*-value
	High	**Low**	
Number (%)	47	41	
Age (yrs)	65.3 (4.2)	66.5 (4.2)	0.2
BMI (kg/m^2)	25.9 (3.5)	23.9 (2.9)	<0.01
Calcium intake (mg/day)	1201 (331)	1123 (370)	0.3
Vitamin D intake (mg/day)	1.81 (1.06)	1.98 (1.40)	0.5
Femoral neck BMD (g/cm^2)	1.01 (0.08)	0.66 (0.05)	< 0.001
Haplotype 1 alleles (%)	46 (48.9)	37 (45.1)	0.7
Haplotype 2 alleles (%)	40 (42.6)	38 (46.3)	0.7
Haplotype 3 alleles (%)	7 (7.4)	7 (8.5)	0.7
Biochemical markers	**High**	**Low**	*p-value*
1,25-$(OH)_2D_3$ (pmol/l)	99.3 (1.3)	95.5 (1.3)	0.6
25-(OH) Vitamin D (nmol/l)	74.1 (1.5)	67.6 (1.5)	0.3
Osteocalcin (µg/l)	4.5 (2.1)	5.3 (2.4)	0.09
NTX/creatinine (nmol/µmol)	57.5 (1.9)	75.8 (1.7)	0.02
PTH (ng/l)	31.6 (1.5)	30.9 (1.6)	0.9

Biochemical markers

The distributions of values for biochemical markers of bone turnover were skewed at every visit, except for osteocalcin. Therefore we ^{10}log transformed those values to achieve normal distribution.

At baseline, increased osteocalcin serum levels (p = 0.09) and urinary NTX/creatinine (p = 0.02) was observed in women having a low BMD compared to the high BMD group (Table 6.1). This observation did not essentially change after adjustment for age and BMI. All other

levels of markers of bone metabolism were similar between women having either a low or high BMD.

Response to 1,25-$(OH)_2D_3$

One woman stopped taking her medication at day two, bringing the compliance rate to 98% in the low BMD group whereas this was 100% in the high BMD group. We excluded this woman for further analysis on the response to 1,25-$(OH)_2D_3$. Figure 6.1 shows the short-term response in biochemical markers of bone turnover to 1,25-$(OH)_2D_3$ for women having either a low or high BMD. At day 4, both BMD groups showed a significant increase in 1,25-$(OH)_2D_3$ levels ($p < 0.01$) and osteocalcin levels ($p < 0.01$), whereas the 25-$(OH)D_3$ level did not change essentially ($p = 0.2$). A significant decrease was found for the NTX/creatinine ratio ($p < 0.001$) and PTH levels ($p < 0.001$). At day 7, 1,25-$(OH)_2D_3$ and 25-$(OH)D_3$ levels returned to baseline level, whereas osteocalcin increased further and NTX/creatinine ratio and PTH remained low. Although the trend of higher levels of biochemical markers at baseline observed in the low BMD group became more obvious during follow-up, the absolute response to 1,25-$(OH)_2D_3$ was similar between the two BMD groups (Interaction terms for all biochemical markers $p > 0.5$).

VDR 3' haplotype

At baseline there were no significant differences among VDR 3' haplotype genotype groups concerning age, calcium- and vitamin D intake for both BMD groups measured (Table 6.2). A significant difference was observed in the high BMD group for femoral neck BMD among the VDR 3' haplotype 2 genotype, while BMI differed when they were grouped according to either VDR 3' haplotype 1 or haplotype 2 genotype. However, for both femoral neck BMD and BMI there was no evidence of a recessive, dominant or an allele-dose effect.

Biochemical markers

Table 6.3 shows the biochemical markers of bone metabolism at baseline according to VDR 3' haplotype genotypes stratified by BMD group. Interestingly, in women with low BMD the rate of bone turnover was observed to be genotype dependent in women carrying haplotype 2 with evidence of an allele dose effect. In the low BMD group osteocalcin and NTX/creatinine ratio were highest for the homozygote haplotype 2 group, intermediate for the heterozygote and lowest for the reference group. PTH, 1,25-$(OH)_2D_3$ and 25-$(OH)D_3$

Figure 6.1 Response in biochemical markers of bone turnover to 1,25-$(OH)_2D_3$ according to BMD group

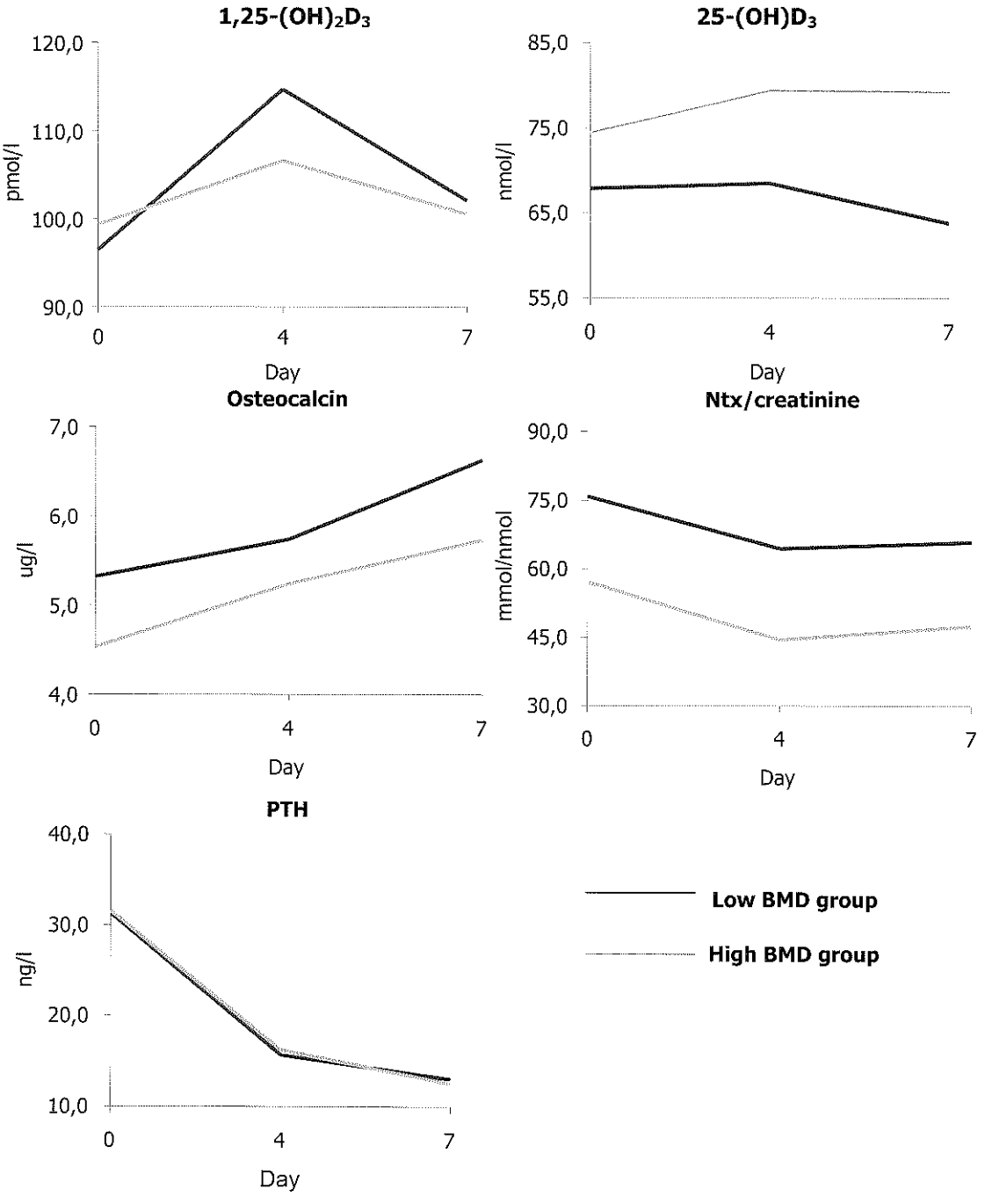


Table 6.2 Characteristics according to carrier status for each VDR 3′ Haplotype stratified by BMD group

	VDR 3′ Haplotype							
	Haplotype 1			***Haplotype 2***			***Haplotype 3***	
High BMD	**Reference**	**Heterozygote**	**Homozygote**	**Reference**	**Heterozygote**	**Homozygote**	**Reference**	**Heterozygote**
Number (%)	16 (34)	16 (34)	15 (32)	18 (38)	18 (38)	11 (24)	40 (85)	7 (15)
Age (yrs)	66.8 (3.6)	64.3 (4.0)	64.7 (4.9)	64.9 (4.8)	64.3 (3.8)	67.5 (3.3)	65.4 (4.3)	64.9 (4.1)
BMI (kg/m^2)	25.2 (3.5)	28.1 (3.9)*	24.4 (1.7)	24.5 (2.0)	28.1 (3.9)*	24.7 (3.3)	25.9 (3.5)	26.0 (3.9)
Calcium intake (mg/day)	1248 (348)	1286 (324)	1032 (279)	1067 (263)	1301 (360)	1232 (332)	1196 (333)	1229 (346)
Vitamin D intake (mg/day)	1.50 (0.76)	1.96 (0.97)	1.97 (1.44)	2.08 (1.32)	1.90 (0.98)	1.25 (0.49)	1.72 (1.07)	2.33 (0.94)
Femoral neck BMD (g/cm^2)	1.05 (0.10)	0.98 (0.05)	1.01 (0.06)	1.01 (0.06)	0.99 (0.06)	1.07 (0.11)*	1.02 (0.08)	0.99 (0.09)
Low BMD	**Reference**	**Heterozygote**	**Homozygote**	**Reference**	**Heterozygote**	**Homozygote**	**Reference**	**Heterozygote**
Number (%)	13 (32)	19 (46)	9 (22)	10 (24)	24 (59)	7 (17)	34 (83)	7 (17)
Age (yrs)	64.7 (3.9)	67.1 (4.3)	67.9 (4.2)	67.6 (4.2)	67.0 (4.2)	63.3 (2.5)	66.6 (4.3)	66.0 (3.7)
BMI (kg/m^2)	23.7 (2.6)	24.2 (3.1)	23.6 (3.4)	23.4 (3.2)	24.3 (2.9)	23.3 (2.7)	23.9 (3.0)	23.8 (2.6)
Calcium intake (mg/day)	1064 (372)	1145 (325)	1171 (467)	1191 (444)	1165 (348)	901 (270)	1090 (367)	1270 (373)
Vitamin D intake (mg/day)	2.43 (1.96)	1.82 (1.13)	1.61 (0.57)	1.56 (0.56)	2.01 (1.15)	2.50 (2.55)	1.94 (1.46)	2.17 (1.16)
Femoral neck BMD (g/cm^2)	0.65 (0.05)	0.68 (0.05)	0.66 (0.03)	0.66 (0.03)	0.67 (0.06)	0.66 (0.04)	0.67 (0.04)	0.65 (0.06)

Values are means (SD) or numbers (%)

*p-value ANOVA <0.05

Table 6.3 Baseline levels of biochemical markers of bone turnover (mean (SD) according to VDR 3' haplotype stratified by BMD group

	VDR 3' Haplotype							
	Haplotype 1			***Haplotype 2***			***Haplotype 3***	
High BMD	**Reference**	**Heterozygote**	**Homozygote**	**Reference**	**Heterozygote**	**Homozygote**	**Reference**	**Heterozygote**
Number (%)	16 (34)	16 (34)	15 (32)	18 (38)	18 (38)	11 (24)	40 (85)	7 (15)
1,25-$(OH)_2D_3$ (pmol/l)	93.3 (1.3)	100.0 (1.3)	104.7 (1.4)	104.7 (1.4)	100.0 (1.4)	89.1 (1.3)	97.7 (1.3)	104.7 (1.5)
25-$(OH)D_3$ (nmol/l)	81.3 (1.2)	63.1 (1.7)	79.4 (1.4)	77.6 (1.4)	70.8 (1.7)	74.1 (1.3)	72.4 (1.5)	85.1 (1.4)
Osteocalcin (µg/l)	4.56 (2.0)	4.51 (1.9)	4.51 (2.4)	4.44 (2.2)	4.74 (1.8)	4.31 (2.3)	4.52 (2.2)	4.60 (0.6)
NTX / creatinine (nmol/µmol)	50.1 (2.2)	64.6 (1.5)	57.5 (2.1)	57.5 (2.0)	67.6 (1.4)	41.7 (2.4)	56.2 (2.0)	64.6 (1.6)
PTH (ng/l)	28.2 (1.5)	35.5 (1.6)	30.9 (1.5)	24.0 (1.5)	35.5 (1.6)	29.5 (1.6)	31.6 (1.5)	30.2 (1.4)
Low BMD	**Reference**	**Heterozygote**	**Homozygote**	**Reference**	**Heterozygote**	**Homozygote**	**Reference**	**Heterozygote**
Number (%)	13 (32)	19 (46)	9 (22)	10 (24)	24 (59)	7 (17)	34 (83)	7 (17)
1,25-$(OH)_2D_3$ (pmol/l)	102.3 (1.3)	93.3 (1.3)	93.3 (1.2)	95.5 (1.2)	91.2 (1.3)	117.5 (1.3)	97.7 (1.3)	91.2 (1.3)
25-$(OH)D_3$ (nmol/l)	75.9 (1.6)	66.1 (1.6)	61.7 (1.0)	64.6 (1.3)	63.1 (1.6)	93.3 (1.5)	69.2 (1.5)	61.6 (1.5)
Osteocalcin (µg/l)	5.43 (2.2)	5.45 (2.6)	4.89 (2.1)	4.40 (2.5)	5.34 (2.4)	6.57 (1.6)*	5.69 (2.2)	3.53 (2.6)
NTX / creatinine (nmol/µmol)	89.1 (1.9)	70.8 (1.7)	74.1 (1.4)	69.2 (1.5)	72.4 (1.8)	109.6 (1.6)*	79.4 (1.6)	64.6 (2.0)
PTH (ng/l)	29.5 (1.5)	36.3 (1.6)	24.5 (1.6)	24.0 (1.5)	35.5 (1.5)	28.8 (1.7)	31.6 (1.6)	28.2 (1.4)

*p-value allele dose effect =0.06

levels did not differ significantly among the subjects grouped by VDR 3' haplotype 2. After adjustment for potential confounders like age and BMI, these differences did not essentially change. For haplotype 1 and haplotype 3 a less obvious but inverse relation was found. In women with high BMD we did not observe such a genotype-dependent association.

Response to 1,25-$(OH)_2D_3$

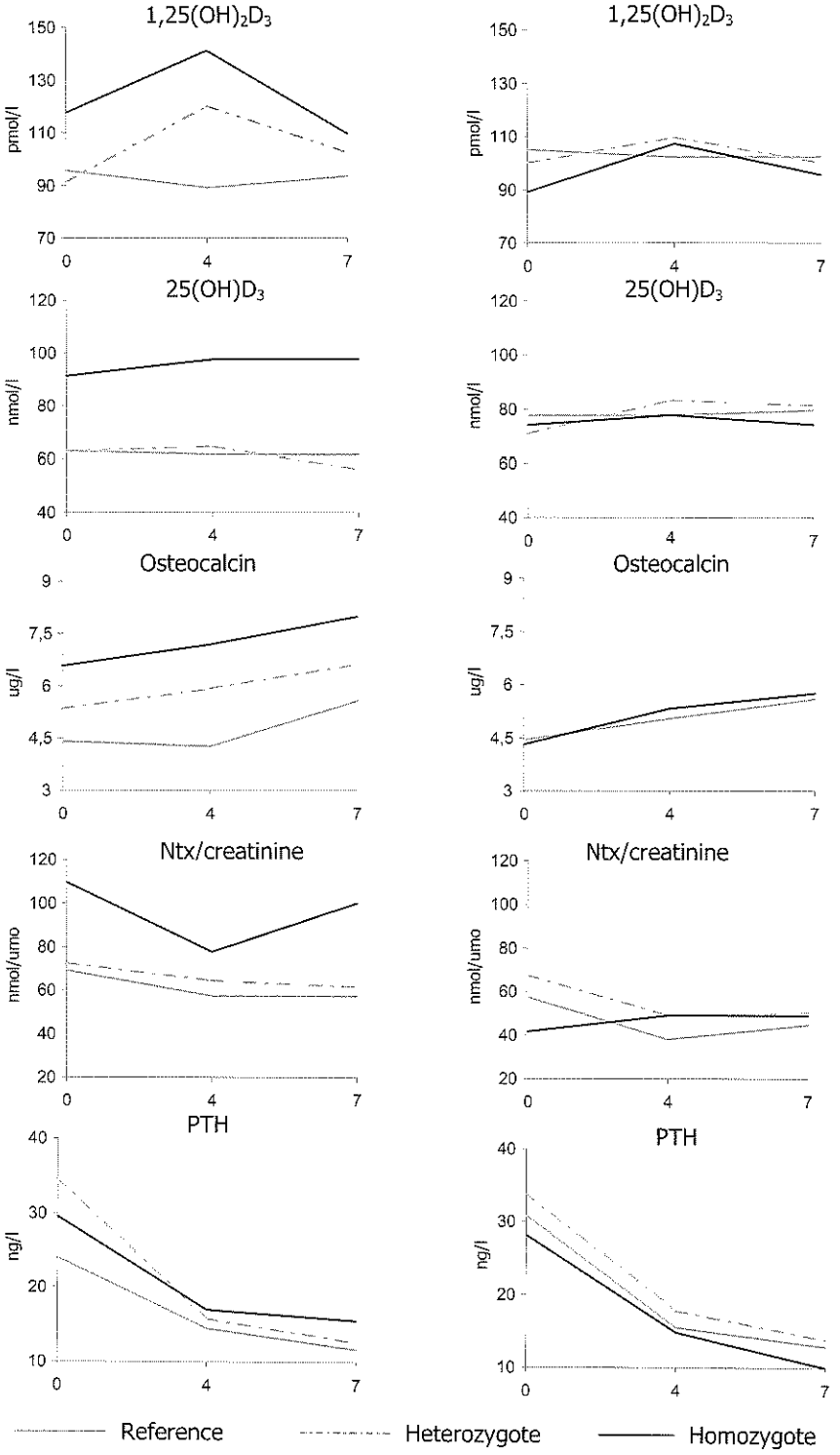
Figure 6.2 shows the response in biochemical markers of bone turnover to 1,25-$(OH)_2D_3$ among VDR haplotype 2 genotype groups. The observed VDR genotype dependent effect on the biochemical markers of bone metabolism in women having low BMD at baseline remained consistent during the follow-up visits. The absolute response of none of the biochemical markers to 1,25-$(OH)_2D_3$ differed significantly among the haplotypes. However, the homozygote haplotype 2 group showed maximum increase of 1,25-$(OH)_2D_3$ levels at day 4 while the levels remained similar in the reference group. For haplotype 1 and 3 no difference in response to 1,25-$(OH)_2D_3$ was found.

6.5 DISCUSSION

In this study performed in postmenopausal women, we observed higher osteocalcin and NTX/creatinine levels in women having low BMD compared to women having high BMD. Interestingly, this increased bone turnover rate in women having low BMD was associated with VDR polymorphism measured as haplotypes of 3 RLFP sites (*BsmI*, *ApaI* and *TaqI*) at the 3' end of the VDR gene. Furthermore, this VDR 3' genotype dependent bone turnover rate could not be explained by a difference in sensitivity for 1,25-$(OH)_2D_3$ of the different genotypes.

Before interpreting the results some elements have to be discussed. With respect to the design of the study, the number of participants was small resulting in insufficient power to detect small differences. For example, the estimated power within this study group of 88 women was 40% for the observed differences in osteocalcin levels between the BMD groups. To get sufficient power of 80% at least 240 participants had to be studied. It is therefore important to look for consistency in the effects. Several studies have shown that the fasting morning samples are most accurate for measuring bone turnover rate[16]. We used overnight fasting urine samples and therefore, circadian variance of bone markers is not likely to influence the results. Furthermore, since the inter- and intra assay coefficients of variation of the assays of bone markers are high, the observed effect on the regulation of the calcium and bone metabolism after stimulation of 1,25-$(OH)_2D_3$ may have been imprecise. The study population has a relatively high calcium- and vitamin D intake compared to other populations, which might lead to an underestimation of the treatment effect[17,18]. In our study

Figure 6.2 Response in biochemical markers of bone turnover to 1,25-$(OH)_2D_3$ according to VDR3' haplotype 2 stratified by high (left) and low (right) BMD group



the biochemical markers of bone turnover were determined four years after BMD was measured. It is unlikely however, that low BMD leads to a higher rate of bone turnover while having low BMD as a result of low bone turnover rate is more likely[19]. Furthermore, we do not expect that these shortcomings of the study are genotype dependent. Therefore it should not have influenced the true association between genotypes and biochemical markers of bone turnover.

Regarding the short-term response to 1,25-$(OH)_2D_3$ in general, our data confirm observations made by others who also showed an increase in osteocalcin levels and a decrease in PTH[20-24]. Interestingly, those studies observed an increase in bone resorption in contrast to the decreased NTX/creatinine ratio of the present study[23,24]. The decreased bone resorption rate can be explained by a decrease in PTH, which is the most prominent effect modifier of treatment with 1,25-$(OH)_2D_3$[25-27]. Furthermore, those studies used other bone resorption markers while the NTX/creatinine ratio is the most sensitive and specific marker of bone resorption[28].

In our study the association between VDR polymorphism measured as haplotypes of 3 RFLP sites (*Bsm*I, *Apa*I and *Taq*I) at the 3' end and bone turnover rate was only obvious in women having low BMD values. A possible explanation for this contradictory finding might be residual confounding like osteoarthritis for example that could be more frequent in the high BMD group. Individuals with osteoarthritis have increased growth hormone levels that subsequently lead to higher rates of bone turnover[29-31]. In contrast to this, individuals not carrying a VDR haplotype 2 allele are more at risk for osteoarthritis[32]. Therefore theoretically, the observed lower bone turnover rate in the reference group (indivuals not carrying haplotype 2 alleles) compared to individuals carrying a haplotype 2 allele, could have been neutralized by the growth hormone induced bone turnover rate in the reference group. Subsequently this would have diluted the difference among the VDR haplotype genotypes.

Several studies have analyzed the relation between VDR 3' polymorphism and bone turnover [7,9,14,33,34]. Our data are in line with observations by others who used only the *Bsm*I RFLP and showed that the baseline level of markers of bone turnover (in particular osteocalcin) is higher in the BB genotype[9,14,33]. The BB genotype group corresponds to the homozygous haplotype 2 carriers (see methods). Thus, putting all the data together we conclude that the haplotype 2 allele is associated with an increased rate of bone turnover favoring bone formation, which implies that other haplotypes, i.e. haplotype 1 and/or 3, are associated with lower bone formation levels. In view of the close relation between bone turnover and BMD this in turn might be reflected in lower BMD values in subjects carrying those haplotypes and/or in increased fracture risk. Indeed, in a much larger sample of this study population

we observed haplotype 3 to be associated with low BMD[10], while we have recently observed haplotype 1 to be associated with increased fracture risk[11].

Concerning the short-term response to 1,25-$(OH)_2D_3$ we did not see a significant genotype effect as observed by Howard et al. in Australian women[33]. A possible explanation might be that we studied postmenopausal women whereas they only included premenopausal women (including two pairs of dyzygotic twins). Premenopausal women are known to have substantial variance in bone turnover rates[35]. Therefore, the observed difference between the genotypes in the Australian study could have been confounded by age differences between the genotypes. Nevertheless, in the present study we showed a continuos increase in bone formation rate during short-term administration of active vitamin D whereas the rate of bone resorption remained stable after 4 days. This suggests that during long term administration of vitamin D, the relatively higher bone turnover rate in individuals carrying homozygote haplotype 2 alleles will be superimposed on a higher rate of bone formation that might lead to different BMD values in the long-term. This notion is supported by the observation by Graafmans et al. who showed a significant increase in BMD in postmenopausal women homozygous for the 'B' allele of the *Bsm*I RLFP (corresponding to haplotype 2) after a 2 years administration of vitamin D[6].

The precise molecular mechanism causing the observed effect of the VDR 3' gene polymorphism in the relation with bone turnover remains to be elucidated. The polymorphisms we analyzed are anonymous and, therefore, are not likely to explain the associations observed by themselves. Supposedly they are in linkage disequilibrium with a truly functional polymorphism elsewhere in the VDR gene. Recent studies from our laboratory indicate that the VDR 3' haplotypes accurately indicate linkage groups across at least 20 kb in this part of the gene (Fang et al., manuscript in preparation). They can therefore be considered to be helpful markers to find the functional sequence variants.

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Chapter 7

Consequences of vitamin D receptor gene polymorphisms for growth inhibition of cultured human peripheral blood mononuclear cells by 1,25-dihydroxyvitamin D_3

E.M. Colin, A.E.A.M. Weel, A.G. Uitterlinden, C.J. Buurman, H.A.P. Pols, J.P.T.M. van Leeuwen. Clinical Endocrinology 2000;52:211-216.

7.1 ABSTRACT

In the VDR gene a *Bsm*I restriction fragment length polymorphism (RFLP) in intron 8 and a translational start-site polymorphism, identified as a *Fok*I RFLP, have been described. Crucial for a proper interpretation of these polymorphisms in association studies is the knowledge whether they have direct consequences for 1,25-$(OH)_2D_3$ action at cellular level. The present study was designed to assess functional significance of the *Fok*I and *Bsm*I VDR gene polymorphisms in peripheral blood mononuclear cells (PBMC) with a natural occurring VDR genotype for cell growth inhibition by for 1,25-$(OH)_2D_3$.

For this purpose PBMC of women were isolated, VDR genotyped and *in vitro* inhibition by 1,25-$(OH)_2D_3$ of phytohemagglutinin (PHA)-stimulated growth of PBMC was examined in relation to VDR genotype. PHA-stimulated growth and maximal growth inhibition were independent of VDR genotype. However, the FF genotype had a significantly lower ED_{50} than the Ff genotype corresponding to an allele dose effect of 0.32 nM per f allele copy (p = 0.0036). For *Bsm*I genotypes no differences in ED_{50} were observed.

In conclusion, the present study demonstrates for the first time in cells with a natural VDR genotype a direct functional consequence of the VDR gene translational start-site polymorphism for the action of 1,25-$(OH)_2D_3$. Especially under conditions of vitamin D insufficiency these findings might have clinical implications.

7.2 INTRODUCTION

Over the last years the genetic basis of osteoporosis has been intensively studied. In relation to this, several polymorphisms in the VDR gene have been identified. Firstly, the *Bsm*I RFLP is located in intron 8 at the 3' end of the VDR gene. Several studies have shown an association between this VDR polymorphism and bone mineral density[1-4], but other studies have not found this relationship[5-7]. Putting all these data together, there seems to be a weak association with bone mass[8]. Also associations with other phenotypes, like primary hyperparathyroidism, prostate cancer, and radiographic osteoarthritis have been described[9-12].

Recently, a polymorphism of the translational start-site has been identified in the VDR gene[13-16]. This polymorphism is characterised by the presence of either two ATG start codons separated by 6 nucleotides or due to a T to C substitution in the 5'-ATG site only the presence of the most 3'-ATG codon. The presence of the 5'-ATG-site results in a recognition sequence for the *Fok*I restriction enzyme[17]. For this VDR polymorphism also both the presence and the lack of association with bone mineral density has been described[16-19]. In contrast to the *Bsm*I polymorphism, the *Fok*I polymorphism has distinct structural consequences for the VDR. The

absence of the *FokI* restriction site, indicated as F, predicts a VDR protein of 424 amino acids, whereas the presence of the *FokI* site (f) results in a VDR of 427 amino acids.

Crucial for proper interpretation of genetic association studies is the demonstration of functional consequences of these polymorphisms. For the *BsmI* polymorphism some *in vivo* studies have been performed showing potential associations with parameters related to bone turnover and bone mineral density[20-24]. However, these *in vivo* studies are complex and do not provide direct insights into the consequences for 1,25-$(OH)_2D_3$ action at the cellular level. A few *in vitro* studies have been performed to address functionality of VDR gene polymorphisms. In these studies cells were transfected with either one of the VDR genotypes[1,16]. A potential disadvantage of transfecting cells with VDR genotypes is the absence of control over subtle differences in expression of the gene of interest. Especially, these differences may be very important for the differential phenotypic effect of naturally occurring gene polymorphisms. The present study was designed to investigate the functional consequences of the *BsmI* and *FokI* RFLP of the VDR gene for the action of 1,25-$(OH)_2D_3$ in cells, PBMC expressing the natural VDR genotypes. PBMC form a readily accessible target of vitamin D which is used as a model for studying genotype-dependent 1,25-$(OH)_2D_3$ effects.

7.3 MATERIALS AND METHODS

Subjects

Fasting blood samples from 72 healthy postmenopausal women, aged 59 to 75 years (mean age ± SD = 65.7 ± 4.3 years) were taken. Women were randomly selected from independently living subjects of the Rotterdam Study[25]; women using hormonal replacement therapy, cytostatics, vitamin D, thyroid hormone or known to have diabetes were excluded. Four women were excluded from analysis because no genotype data were available. Written informed consent was obtained from each participant. The study has been approved by the Medical Ethics Committee of the Erasmus Medical Center Rotterdam.

Cell culture

PBMC were prepared from heparinized blood by Ficoll-Hypaque (Pharmacia, Sweden) density gradient centrifugation[26]. The cells were suspended in phenol red-free RPMI 1640 medium (Sigma Chemical Co, St. Louis, MO) supplemented with 10% charcoal-treated FCS, 2 mM glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin (Life Technologies, Breda, The Netherlands) and 24 mM sodium bicarbonate. Immediately after isolation the cells were used for the proliferation analysis. For this purpose cells (4,4 x 10^6 cells per ml) were cultured for 96 hrs in 24-wells plates (300 μl/well) in the presence of 3 μg/ml phytohemagglutinin (PHA) and

10^{-10} M to 10^{-7} M 1,25-$(OH)_2D_3$ (kindly donated by Dr. L. Binderup of LEO Pharmaceutical Products, Ballerup, Denmark). At the end of the incubation, DNA content of adherent and non-adherent cells was measured according to the ethidium bromide method of Karsten and Wollenberger[27]. For each individual two PBMC cultures were performed each consisting of two DNA measurements.

Due to ethical reasons it was not possible to perform both PBMC growth analyses and VDR measurements in one and the same individual. For the PBMC growth study 3-4 tubes of blood were needed. In addition to this 3-4 other tubes had to be taken for clinical analyses. This would mean that over 10 tubes of blood from volunteers were needed in order to have at least the possibility to measure VDR. For measurement of VDR in monocytes only we have calculated that even 21 tubes would be necessary.

Genotyping procedure

The *Bsm*I RFLP at the 3' end of the VDR gene was assessed by a direct haplotyping PCR procedure as previously described[4]. The *Fok*I RFLP was analysed by PCR with the primers described previously[17]. The amplification protocol consisted of 28 cycles of 94, 60 and 72°C for 1 minute each. Ten microliters of the PCR products were digested with 10 U *Fok*I and 1.2 µl of a 10x buffer (containing 150 mM Tris-HCl, pH 7.5, 250 mM NaCl and 35 mM $MgCl_2$) by incubating for 30 minutes at 37°C. Digestion products were analyzed on a 3% NuSieve agarose gel run in 0.5X TBE. Capital letters denote absence and lowercase letters the presence of the site for the restriction enzyme *Bsm*I (B/b) or *Fok*I (F/f).

Statistical analysis

For each individual a best fitted growth curve and maximal inhibition and ED_{50} values were calculated and analyzed using GraphPad Prism software (GraphPad Software, Inc., San Diego, U.S.A.). Next, per genotype the mean maximal inhibition and ED_{50} (± SEM) were calculated. Analysis of variance (ANOVA) was performed and for pairwise comparison student's *t*-test was used. The relation between allele dose and ED_{50} was quantified by linear regression analysis.

7.4 RESULTS

As shown in Table 7.1 incubation of PBMC with PHA resulted in an almost 2.3 times stimulation of growth compared to control cells. PHA-stimulated cell growth was strongly and dose-dependently inhibited by 1,25-$(OH)_2D_3$ (Figure 7.1A) with a maximal inhibition of about 75% at 10^{-8} - 10^{-7} M (Table 7.1). The half-maximal inhibition was achieved at 1.05 nM 1,25-$(OH)_2D_3$ (Figure 7.1B). Neither PHA-stimulated cell growth nor maximal inhibition by 1,25-$(OH)_2D_3$

Table 7.1: Growth stimulation by PHA and maximal growth inhibition by 1,25-$(OH)_2D_3$ of PBMC according to VDR genotype.

Genotype	n (%)*	PHA-stimulated growth§ (fold-stimulation)	Maximal 1,25-$(OH)_2D_3$ effect (% inhibition)
All#		2.25 ± 0.07	72.8 ± 1.46
FF	34 (50.0)	2.18 ± 0.10	71.0 ± 2.15
Ff	25 (36.8)	2.28 ± 0.12	71.6 ± 2.18
ff	9 (13.2)	2.31 ± 0.19	79.5 ± 3.73
BB	10 (15.2)	2.31 ± 0.14	72.4 ± 4.55
Bb	35 (53.0)	2.21 ± 0.11	73.7 ± 5.91
bb	21 (34.9)	2.30 ± 0.13	71.5 ± 2.33

n = the number of subjects.

§PHA growth is expressed as fold stimulation over cell growth in the absence of PHA.
All = Effect independent of genotypes. Data are presented as means ± SEM.

appeared to be VDR genotype dependent. However, the ED_{50} showed differences between *FokI* genotypes in a gene-dose dependent manner (Figures 7.1A and B). The PMBCs with the FF-genotype had the lowest ED_{50} value (0.82 ± 0.072) Ff heterozygotes had an intermediate ED_{50} value (1.24 ± 0.15), while ff homozygotes had the highest ED_{50} value (1.39 ± 0.3). Linear regression analysis demonstrated an allele dose effect of 0.32 nM per f allele copy (p = 0.004). No significant differences were observed when the 3'-end of the VDR was genotyped by the direct haplotyping procedure[4], which may be due to too low numbers per genotype group to allow analysis (data not shown). Also when the analysis was restricted to the single *BsmI* polymorphism no differences in ED_{50} (Figure 1B) and maximal inhibition were observed (Table 7.1). The *ApaI* and *TaqI* polymorphisms were also not associated with differences in maximal inhibition by 1,25-$(OH)_2D_3$ and ED_{50} (data not shown).

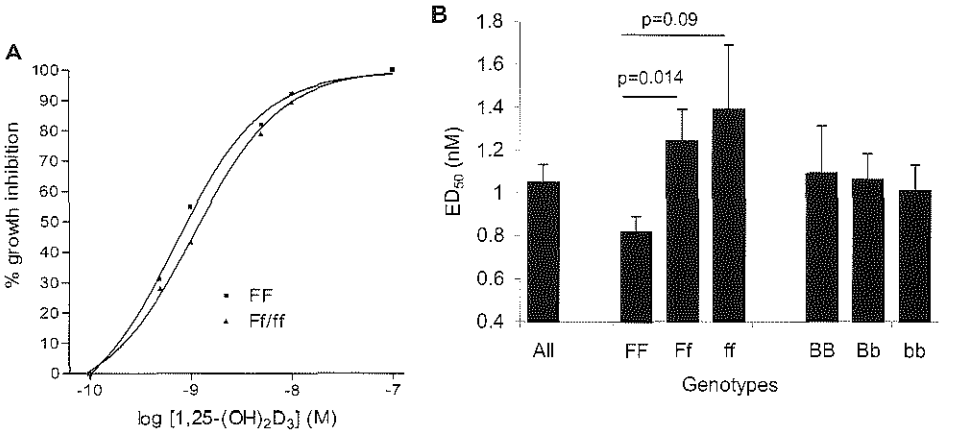

Figure 7.1 Dose-dependent inhibition of PHA-stimulated growth by 1,25-$(OH)_2D_3$ (A) and calculated ED_{50} of PMBC growth inhibition according to the *FokI* and *BsmI* VDR genotypes (B). For clarity reasons the dose-response curves of Ff and ff are combined. Data are transformed according to a maximal inhibition of 100 % (A). Data are presented as mean ± SEM (B). FF: number (n) = 34; Ff: n = 25; ff: n = 9; BB: n = 10; Bb: n = 35; bb: n = 21. All = ED_{50} independent of genotypes.

7.5 DISCUSSION

The current study demonstrates direct functional consequences of naturally occurring VDR gene polymorphisms for the cellular action of 1,25-$(OH)_2D_3$ in PBMC of healthy postmenopausal women. In most previous studies the functional consequences of VDR genotypes at the cellular level were only studied in cells transfected with different VDR alleles[1,16,28]. Our study demonstrates that the half maximal concentration for 1,25-$(OH)_2D_3$ inhibition of PHA-stimulated growth is significantly different between PBMC characterized by different alleles of the VDR translational start-site while the maximal inhibition is similar for all genotypes. The ED_{50} for cells homozygous for the 424 amino acids long VDR, i.e. the FF genotype, is lower than the ED_{50} for cells containing the 427 amino acids long VDR, i.e. the Ff and ff genotypes. A significant allele dose effect was observed which means that PBMC containing both forms of the VDR, the heterozygotes, have an intermediate ED_{50}. Arai et al. have also shown a more potent effect by the short VDR using HeLa cells transfected with a VDR *FokI* genotype and a 24-hydroxylase VDRE-reporter construct. They only tested one concentration of 1,25-$(OH)_2D_3$ and it is not clear whether this reflects the maximal

concentration or approaches the half-maximal effective dose for the response analyzed by Arai et al.[16]. Also others showed a more active 424 amino acids VDR[29,30] while one other study did not observe differences between VDR *Fok*I genotypes[28]. Together, our data support the hypothesis that the 424 amino acids-long VDR is more efficient in exerting 1,25-$(OH)_2D_3$ effects than the 427 amino acids-long VDR.

Although we were not able to measure VDR levels in the same PMBC of the individuals used in the growth study (see Materials and Methods), it is unlikely that the differences in effect are due to increased VDR expression by the FF genotypes[16]. Arai et al.[16] even reported that the FF genotype appears to have a somewhat (± 20 %) reduced VDR expression. Maybe the numbers of ff are too low to show significance, but the somewhat higher maximal inhibition by the ff genotypes may reflect an increased VDR level to compensate for the lower affinity.

In view of the localization of the polymorphism in the N-terminal A/B region of the VDR it is unlikely that it will have an effect on 1,25-$(OH)_2D_3$ binding to the ligand binding domain in the C-terminal E/F region. It is tempting to speculate that the difference in amino terminal sequence between the VDR genotypes directly affects binding of the VDR to its target genes. In this respect it is noteworthy that the VDR has a very short A/B region and, therefore, the sequence variation is located close to the DNA binding domain (C-region). Interesting in this respect are the observations by Jurutka et al. that the 424 amino acids VDR interacts more efficiently with the transcription co-factor TFIIB and possesses elevated transcriptional activity compared to the 427 amino acids VDR[29].

The VDR *Fok*I genotype effect on ED_{50} and not on maximal inhibition has potential clinical significance. In this way it is conceivable that dependent on the vitamin D status a VDR genotype effect is present or absent. So, especially under conditions of vitamin D insufficiency, for instance in elderly subjects, the biological consequences may become apparent, while at sufficient 1,25-$(OH)_2D_3$ levels the genotype consequences will be absent.

In contrast to the *Fok*I RFLP, no relationship was observed between the VDR *Bsm*I RFLP and 1,25-$(OH)_2D_3$ action which is in line with earlier findings[31]. An explanation for the dissociation with some *in vivo* data showing an association between *Bsm*I VDR genotypes and response to 1,25-$(OH)_2D_3$[19-23] is unknown and yet purely speculative. Considering the localization of the *Bsm*I polymorphism it is unlikely that it will have a direct structural effect on the VDR. The *Bsm*I RFLP can be in linkage with polymorphisms in the 3' UTR[1,11] and in this way be related to stability of VDR mRNA and VDR expression[32]. Maybe the present experimental system is not sufficiently sensitive to observe effects of potential differences in VDR mRNA stability between *Bsm*I genotypes. Some data showed that the *Bsm*I RFLP does not affect the abundance of the VDR mRNA[33], while other studies indicated a difference in abundance

of VDR mRNA between *TaqI* genotypes without an effect on VDR mRNA stability[34]. Unfortunately as mentioned above, it was not possible to relate our present observations directly to VDR levels in PBMCs of the individuals in this study. Although the 3' polymorphisms are not in linkage with the 5'-start site polymorphism analysis of combinations VDR genotypes may reveal further diversity in VDR activity[30]. Preliminary analysis of the various combinations of *FokI* and *BsmI* genotypes in the current study didn't provide additional information over the *FokI* analysis alone. It must, however, be noted that the number of individuals per genotype combination group is low and limits statistical analysis.

In conclusion, the current study provides evidence for direct functional consequences of the VDR translational start-site polymorphism for the cellular action of 1,25-$(OH)_2D_3$ and provides insights into the VDR genotype-phenotype association studies. The relationship with 1,25-$(OH)_2D_3$ concentrations is interesting and potentially important under conditions related to low 1,25-$(OH)_2D_3$ levels like vitamin D deficiency and renal impairment. More general, the present data demonstrate that on basis of naturally occurring variants of a single gene differences in responsiveness between individuals may occur. Finally, it should be noted that in the present study one specific response of one specific target cell of 1,25-$(OH)_2D_3$ has been examined. For a full 1,25-$(OH)_2D_3$ response interaction of the VDR with co-factors (e.g. TFIIB, see above) is essential and these co-factors may vary between responses and cell types. Therefore, additional analyses of other responses and cell types are needed to further assess the applicability of the concept that the 424 amino acids VDR is the more active form.

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Chapter 8

Interaction between vitamin D receptor genotype and estrogen receptor α genotype influences vertebral fracture risk

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8.1 ABSTRACT

In view of the interactions of the vitamin D and estrogen endocrine system we studied the combined influence of polymorphisms in the ER α gene and the VDR gene on the susceptibility to osteoporotic vertebral fractures in 634 women aged 55 years and older. Three VDR haplotypes (1, 2 and 3) of the *Bsm*I, *Apa*I, and *Taq*I RFLPs and three ERα haplotypes (1, 2 and 3) of the *Pvu*II- and *Xba*I RFLPs were identified. ERα haplotype 1 was dose-dependently associated with increased vertebral fracture risk ($p < 0.001$) corresponding to an odds ratio of 1.9 (95% CI, 0.9-4.1) per copy of the risk allele. VDR haplotype 1 was overrepresented in vertebral fracture cases. There was a significant interaction ($p = 0.01$) between ERα haplotype 1 and VDR haplotype 1 in determining vertebral fracture risk. The association of ERα haplotype 1 with vertebral fracture risk was only present in homozygous carriers of VDR haplotype 1. The risk of fracture was 2.5 (95% CI, 0.6-9.9) for heterozygous and 10.3 (95% CI, 2.7-40) for homozygous carriers of ERα haplotype 1. These associations were independent of BMD. In conclusion, interaction between ERα and VDR gene polymorphisms leads to increased risk of osteoporotic vertebral fractures in women, largely independent of BMD.

8.2 INTRODUCTION

Osteoporosis is characterized by low BMD, deterioration of the microarchitecture of bone and subsequent increased numbers of fractures[1,2]. Twin and family studies have suggested that BMD has a strong genetic component, besides being influenced by nutritional and life-style factors[3-6]. Osteoporosis is regarded as a complex genetic trait, which means that variants of several genes underlie the variability of the phenotype. Among the candidate genes in relation to BMD are the genes for collagen type Iα1, the VDR and the ERα[7-11]. Polymorphisms in the genes for the VDR and the ERα have been examined in relation to BMD. Although controversial reports have been published, two meta-analyses have shown a weak relation between the VDR gene and bone mineral density[12,13]. We and others have found a significant association between VDR polymorphisms and fracture risk[11,14] while other studies could not confirm such an association[15,16]. Also for ERα gene polymorphisms associations with BMD were reported[17-20] although others could not confirm these results[21-24].

VDR and ERα are interesting genes because several interactions between the vitamin D and estrogen endocrine system have been described. 1,25-$(OH)_2D_3$ and E_2 have a mutual effect on each other's biosynthesis[25-27] and receptor expression[28,29]. Also some genetic studies found an interaction between ERα and VDR genotypes with respect to BMD[30-32]. Suarez et al. found an interactive effect of ERα and VDR gene polymorphisms on growth in infants[33].

So far most genetic studies on osteoporosis have focused on BMD as the primary endpoint and not on the clinically more relevant endpoint of fractures. In the current study we focus on the interaction between ERα and VDR genotypes in relation to the most typical osteoporotic fracture, the vertebral fracture.

8.3 MATERIALS AND METHODS

Study subjects

All women included in this study were part of a population-based cohort study of subjects aged 55 years or more, independently living in the Ommoord district of the city of Rotterdam in The Netherlands. The objective of the study is to document the occurrence of disease in the elderly in relation to several potential determinants[34]. A total of 10,275 persons were invited for baseline examination in 1990. Of those 7,983 (61.1% women) participated, bringing the overall response rate to 78%. The baseline assessments included the measurement of anthropometric characteristics, and of femoral and lumbar spine BMD. Subjects were excluded according to the following criteria: age 80 years and over, use of a walking aid, use hormone replacement therapy, diuretics, thyroid hormone or cytostatics, or known diabetes mellitus. After genotyping women with the rare VDR haplotypes 4 and 5 (n = 16) were excluded. Anthropometric data, DNA samples and genotype data for both loci were finally available in a sample of 1062 women. Data on incident vertebral fractures were available for a subgroup of 634 women. The follow-up time was on average 6.5 (SD 0.4) years.

Measurements

At baseline height and weight were measured. BMD (in g/cm^2) was measured at the femoral neck and lumbar spine by dual energy X-ray absorptiometry (Lunar DPX-L densitometer), as reported earlier[35]. Body mass index (BMI) was computed as weight in kilograms divided by height in square meters (kg/m^2). Age at menopause was assessed by questionnaire. Dietary intakes for calcium (mg/day) and vitamin D (mg/day) were assessed by food frequency questionnaire and adjusted for energy intake. Both at baseline, between 1990 and 1993 and at the follow-up visit, between 1997-1999, radiographs of the spine from the fourth thoracic to the fifth lumbar vertebrae were taken. All photos were analyzed for the presence of vertebral fractures by the McCloskey/Kanis method[36].

Determination of VDR and ERα genotypes

For genotyping we determined haplotypes of the *Bsm*I, *Apa*I, and *Taq*I restriction fragment length polymorphisms (RFLPs) at the 3′ end of the VDR gene and haplotypes of the *Pvu*II- and *Xba*I RFLPs in the first intron of the ERα gene by direct molecular haplotyping methods as described previously[9] (Figure 8.1). Three frequent VDR haplotypes are discerned, encoded 1 (baT), 2 (BAt), and 3 (bAT). The less frequent haplotypes 4 and 5 were excluded from the analysis (n=16); women carrying these genotypes represent 1.5% of the population. For direct molecular haplotyping of the *Pvu*II and *Xba*I RFLPs a 346 bp PCR fragment was generated by a forward primer (ER-F: 5′-GATATCCAGGGT TATGTGGCA-3′) and a reverse primer (ER-R:5′-AGGTGTTGCCTATTATATTAACCTTGA-3′) in a reaction mixture of 10 μL containing 20 ng of genomic DNA, 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 0.2 mM deoxy-NTP, 2 pM of each primer, and 0.2 U Super Taq polymerase (HT Biotechnology Ltd., Cambridge, UK). The reactions were performed in 384-well format in a thermocycler (MJ-tetrad) with a cycling protocol of 94, 60 and 72 C for 45 seconds each for 30 cycles. Ten microliters of PCR product were digested by simultaneous addition of 5 μl of digestion mixture containing 5 U PvuII, 7 U XbaI restriction enzyme (MBI Fermentas) and 1.5 μL of ReactBuffer 2 (Life Technologies, Breda, The Netherlands) and incubating for 90 minutes at 37°C. The digestion products were analyzed by electrophoresis in a 3% agarose gel in 0.5xTBE (1xTBE = 89 mM Tris, 89 mM boric acid, 2 mM Na_2EDTA) for 80 minutes at 125 Volts. Separation patterns were documented with a digital camera (DC120, Kodak Company, Rochester, NY, U.S.A.) under UV illumination (302 nm). Three ERα haplotype alleles are identified, encoded 1 (px/T-A), 2 (PX/C-G) and 3 (Px/C-A) combining to six genotypes 11, 12, 13, 22, 23 and 33 (Figure 8.2). We did not observe the fourth possible haplotype (pX; -397int1T and –351int1G) in our population.

Statistical analysis.

Differences in mean age at baseline between the study group and the Rotterdam Study were evaluated by means of analysis of variance (ANOVA). All other differences in baseline characteristics were compared by analysis of covariance (ANCOVA) testing with age to adjust for possible confounding effects. Differences in baseline characteristics between the different genotype groups of the ERα gene were compared as follows. We grouped subjects by allele copy number (0,1,2) for the haplotype alleles of interest. We allowed for three possible models to explain differences between groups, i.e., an allele dose effect, a dominant effect or a recessive effect. Allele dose was defined as the number of copies of a certain allele in the genotype. In case of a consistent trend reflected as an allele dose effect, we performed

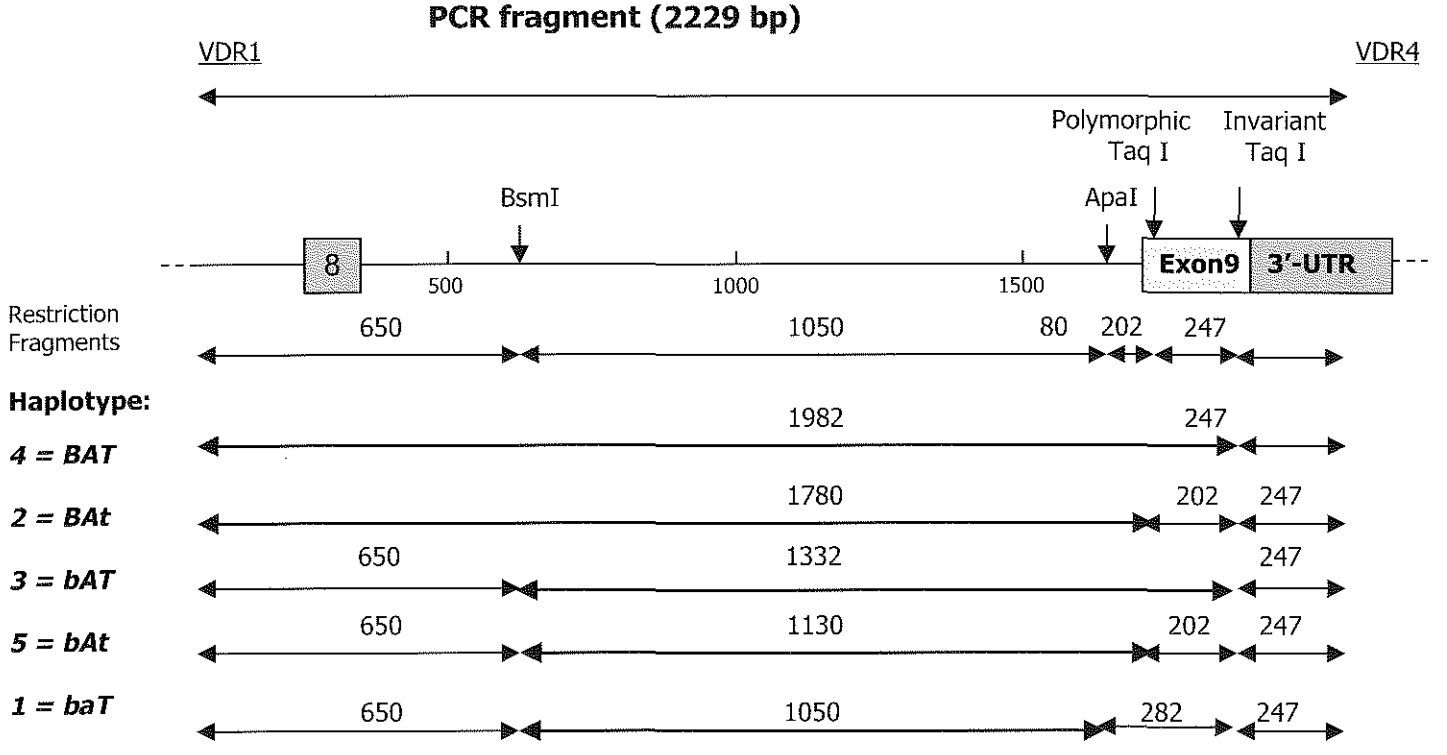

Figure 8.1 Vitamin D receptor gene: direct molecular haplotyping. For details see Materials and Methods.

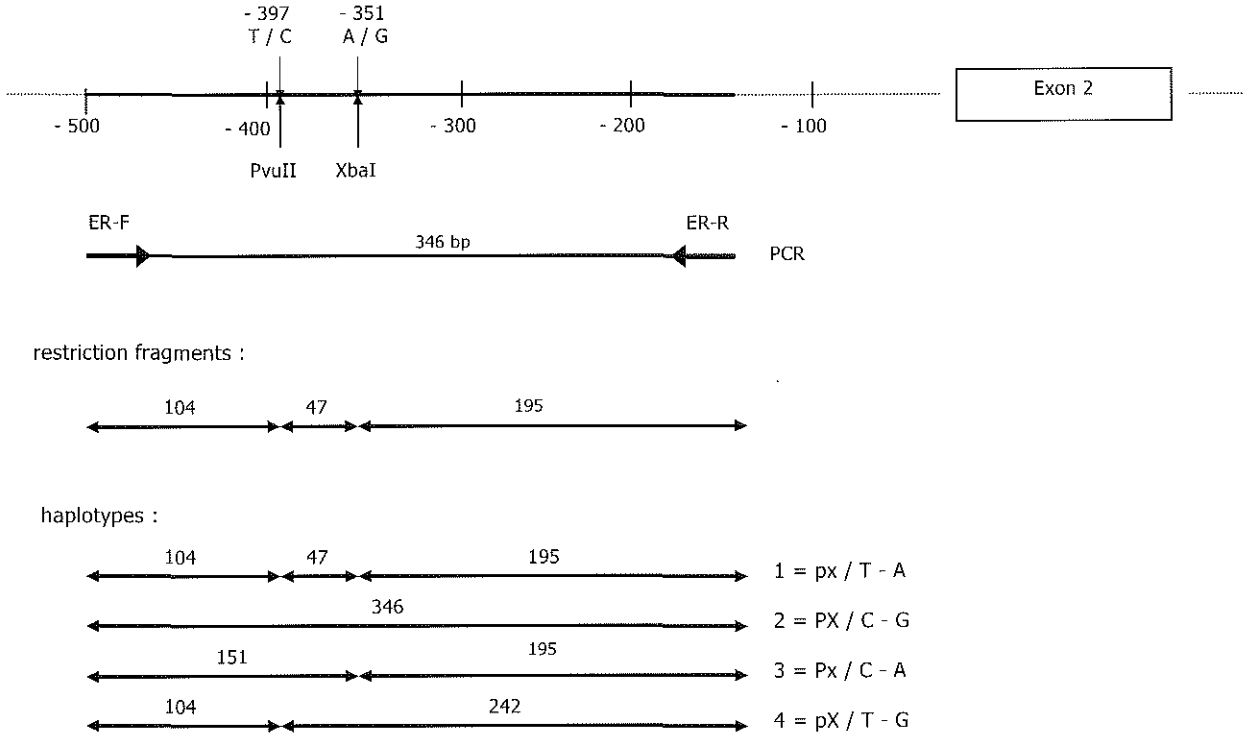

Figure 8.2 Estrogen receptor α gene: direct molecular haplotyping. For details see Materials and Methods.

a (multiple) linear or logistic regression analysis to quantify the association. In case of a dominant or recessive effect of the test-allele, ANOVA and ANCOVA tests were performed. For dominant effects we compared test-allele carriers versus non-carriers while for recessive effects, subjects homozygous for the test allele were compared to heterozygous carriers and non-carriers.

Odds ratios (ORs) with 95% confidence intervals (95% CI) were calculated by (multiple) logistic regression analyses to estimate the relative risk of fractures at baseline by genotypes of the risk allele, with no copies of the risk allele as the reference group. First, we calculated crude odds ratios, and, secondly, we adjusted for potentially confounding factors (age, BMI, BMD, and age at menopause). We used SPSS version 9.0 (SPSS Inc., Chicago, USA) for all our analyses.

8.4 RESULTS

Baseline characteristics

The study population (n=1062) was on average 67.0 (SD 6.9) years old, had a BMI of 26.1 (SD 3.7) kg/m^2, and age at menopause at 48.7 (SD 4.9) years. Dietary calcium and vitamin D intake were on average 1093 (SD 326) mg/day and 1.96 (SD 1.15) mg/day, respectively. Lumbar spine BMD was on average 1.01 (SD 0.17) g/cm^2 and femoral neck BMD was on average 0.81 (SD 0.12) g/cm^2, respectively.

Table 8.1 shows allele and genotype frequencies for ERα and VDR polymorphisms. The genotype distribution was found to be in Hardy Weinberg equilibrium. When we analyzed for known risk factors for osteoporosis by ERα and VDR genotypes, no differences were shown apart from ERα haplotype 1 that appeared to be dose-dependently associated with later onset of menopause, as we have published earlier[37] (Table 8.2). Similar data were found for the subgroup of 634 women participating in the analysis for vertebral fractures (data not shown).

Association of ERα and VDR with BMD

In Table 8.3 women are grouped according to carrier status for the ERα and VDR haplotypes as homozygous carriers (consisting of genotype 11) and heterozygous carriers (including the genotypes 12 and 13) of the ERα haplotype 1 and VDR haplotype 1, respectively, and women not carrying these haplotypes (reference group, including genotypes 22, 23 and 33). ERα haplotype 1 was dose-dependently associated with decreased lumbar spine BMD corresponding with 0.1 SD per copy ERα haplotype 1 (Table 8.3A, first data column). No

Table 8.1 Genotype and allele frequencies of ERα and VDR polymorphisms in the study population

ERα genotype	Number (%)	VDR genotype	Number (%)
11	297 (28.0)	11	271 (25.5)
12	409 (38.5)	12	401 (37.8)
13	124 (11.7)	13	105 (9.9)
22	138 (13.0)	22	183 (17.2)
23	82 (7.7)	23	89 (8.4)
33	12 (1.1)	33	13 (1.2)
Total	1062 (100)	Total	1062 (100)
p value HWE	0.81	*p* value HWE	0.13
ERα haplotype	Number (%)	VDR haplotype	Number (%)
1	1127 (53.1)	1	1048 (49.3)
2	767 (36.1)	2	856 (40.3)
3	230 (10.8)	3	220 (10.4)
Total	2124 (100)	Total	2124 (100)

association was found with femoral neck BMD (Table 8.3B, first data column). ERα haplotype 2 was associated with increased lumbar BMD, corresponding with 0.1 SD per copy ERα haplotype 2 (data not shown). These associations did not change after adjustment for potential confounders such as age, BMI, and age at menopause. No associations were found between ERα haplotype 3 and lumbar spine or femoral neck BMD (data not shown). Based on these data ERα haplotype 1 was considered as risk allele. In the sample of 634 women, in whom data on incident vertebral fractures were available, the association between ERα haplotype 1 and lumbar spine BMD showed a similar trend ($p = 0.11$).

Based on our previous analyses[11] we selected VDR haplotype 1 as risk allele. In the present study no association between VDR haplotype 1 and lumbar spine or femoral neck BMD was observed (Table 8.3A and B, first data rows).

Interaction of ERα and VDR genotype with respect to BMD

When the association of ERα haplotype 1 with BMD was analyzed according to the carrier status for VDR haplotype 1, there was a significant allele-dose effect of ERα haplotype 1 being associated with decreased lumbar spine BMD only for women homozygous for VDR

Table 8.2 Characteristics of 1062 postmenopausal women according to ERα haplotype 1 (A) and VDR (B) haplotype 1

A.

	ERα haplotype 1[b]		
	Reference	*Heterozygotes*	*Homozygotes*
Characteristic[a]	*(n = 232)*	*(n = 533)*	*(n = 297)*
Age (year)	67.6 ± 7.1	66.9 ± 6.9	66.8 ± 6.9
BMI (kg/m^2)	26.2 ± 3.8	26.1 ± 3.5	26.1 ± 4.0
Age at menopause (year)	47.9 ± 5.1	48.7 ± 5.0	49.2 ± 4.6[c]
Dietary calcium intake (mg/day)	1098 ± 364	1097 ± 319	1081 ± 306
Dietary vitamin D intake (mg/day)	2.04 ± 1.32	1.96 ± 1.08	1.89 ± 1.12

B.

	VDR haplotype 1[b]		
	Reference	*Heterozygotes*	*Homozygotes*
Characteristic[a]	*(n = 285)*	*(n = 506)*	*(n = 271)*
Age (year)	66.6 ± 6.6	67.3 ± 7.0	67.0 ± 6.8
BMI (kg/m^2)	26.0 ± 3.4	26.2 ± 3.7	26.3 ± 4.1
Age at menopause (year)	48.9 ± 4.8	48.3 ± 5.1	49.0 ± 4.7
Dietary calcium intake (mg/day)	1078 ± 334	1104 ± 331	1086 ± 306
Dietary vitamin D intake (mg/day)	2.03 ± 1.26	1.91 ± 1.08	1.97 ± 1.17

[a] Data shown are means ± SD.
[b] Reference includes ERα or VDR genotypes 22, 23, and 33; heterozygotes include 12 and 13; homozygotes include 11
[c] $p = 0.02$ (allele-dose association, tested by linear regression analysis)

haplotype 1 (Table 8.3A, fourth data column; $p < 0.001$). This association was not influenced by age, BMI, and age at menopause. When age, BMI, ERα genotype and VDR genotype were taken together in a multivariate regression model, there appeared to be a borderline significant interaction between ERα haplotype 1 and VDR haplotype 1 ($p = 0.09$ for the interaction term). In the subgroup of 634 women, in which data on incident vertebral fractures were available, similar associations were found (data not shown). No interaction between ERα

Table 8.3 Lumbar spine (A) BMD and femoral neck (B) BMD (mean ± SD) according to combined ERα haplotype 1 genotype and VDR haplotype 1 genotype

A

		VDR haplotype 1[a]			
ERα haplotype 1[a]	Total	Reference	Heterozygotes	Homozygotes	*p*-value
Total	1.01 ± 0.29 (1062)[c]	1.00 ± 0.17 (285)	1.02 ± 0.16 (506)	1.02 ± 0.16 (271)	NS
Reference	1.04 ± 0.17 (232)	1.01 ± 0.16 (59)	1.04 ± 0.16 (111)	1.05 ± 0.16 (62)	NS
Heterozygotes	1.02 ± 0.16 (533)	1.00 ± 0.16 (134)	1.02 ± 0.16 (260)	1.03 ± 0.17 (139)	NS
Homozygotes	0.99 ± 0.16 (297)	0.99 ± 0.16 (92)	1.01 ± 0.16 (135)	0.95 ± 0.16 (70)	0.05[d]
p value	0.003[e]	NS	NS	<0.001[f]	0.09[g]

B

		VDR haplotype 1[b]			
ERα haplotype 1[a]	Total	Reference	Heterozygotes	Homozygotes	*p*-value
Total	0.81 ± 0.23 (1062)	0.80 ± 0.12 (285)	0.81 ± 0.11 (506)	0.81 ± 0.12 (271)	NS
Reference	0.81 ± 0.11 (232)	0.79 ± 0.12 (59)	0.82 ± 0.12 (111)	0.81 ± 0.11 (62)	NS
Heterozygotes	0.81 ± 0.12 (533)	0.80 ± 0.12 (134)	0.80 ± 0.11 (260)	0.82 ± 0.12 (139)	NS
Homozygotes	0.80 ± 0.12 (297)	0.81 ± 0.12 (92)	0.80 ± 0.12 (135)	0.78 ± 0.12 (70)	NS
p value	NS	NS	NS	NS	0.13[g]

Values are adjusted for age, BMI; *p* values as tested by ANCOVA

[a] Reference includes ERα genotypes 22, 23, and 33; heterozygotes include 12 and 13; homozygote includes 11.

[b] Reference includes VDR genotypes 22, 23, and 33; heterozygotes include 12 and 13; homozygote includes 11.

[c] number of women

[d] $p = 0.02$ for recessive association as analysed by ANCOVA .

[e] $p = 0.001$ for allele dose association as analysed by linear regression analysis

[f] $p < 0.001$ for allele dose association as analysed by linear regression analysis

[g] *p*-value for the interaction term ERα haplotype 1*VDR haplotype 1

Table 8.4 Number of women with vertebral fractures according to ERα genotype

ERα genotype	No. with fracture/total no.(%)
11	40/187 (21.4)
12	26/236 (11.0)
13	10/71 (14.1)
22	3/79 (3.8)
23	6/53 (11.3)
33	0/8 (0)
X^2	19.2
p value	0.002

and VDR genotypes was found for femoral neck BMD (Table 8.3B; $p = 0.13$ for the interaction term).

Association of ERα and VDR with fracture

When we analyzed the distribution of fractures in women according to the ERα genotype, we observed an overrepresentation of vertebral fractures in women carrying the ERα haplotype 1 (Table 8.4). Figure 8.3 shows separately the distribution of vertebral fractures according to the ERα haplotype 1 and VDR haplotype 1 status. Vertebral fractures were overrepresented in women carrying ERα haplotype 1. This association appeared to be dose dependent with 6.4% in non-carriers of ERα haplotype 1, 12% vertebral fractures (OR 1.9, 95% CI 0.9-4.1) in women heterozygous for ERα haplotype 1 and 21 % vertebral fractures (OR 3.9, 95% CI 1.7-8.2) in women homozygous for ERα haplotype1. For women carrying ERα haplotype 2 there was an allele dose association with decreased vertebral fracture risk ($p < 0.001$) while for ERα haplotype 3 no differences were observed ($p = 0.53$) (data not shown).

In a previous study VDR haplotype 1 was found to be associated with increased fracture risk[11]. When women were grouped by VDR haplotype 1 genotype also an allele dose association was observed (Figure 8.3). Non-carriers of VDR haplotype 1 had 11% vertebral fractures, women heterozygous for VDR haplotype 1 had 13% vertebral fractures (OR 1.3, 95% CI 0.7-2.3), while women homozygous for VDR haplotype 1 had 18% fractures (OR 1.9, 95% CI 1.0-3.7). VDR haplotypes 2 and 3 were not associated with vertebral fracture risk (data not shown).

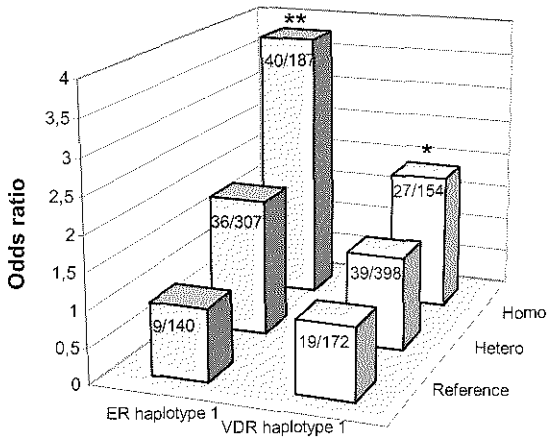

Figure 8.3 Odds ratios and numbers of vertebral fractures according to ERα haplotype 1 and VDR haplotype 1 genotype. 'Reference' includes ERα and VDR genotypes 22, 23, and 33; 'hetero' includes 12 and 13 genotypes; 'homo' includes 11 genotype. ***p*-value < 0.001 for allele dose association of ERα haplotype 1. **p*-value = 0.06 for allele dose association of VDR haplotype 1.

Interaction of ERα and VDR with respect to fracture risk

When we further stratified by VDR haplotype 1 genotype we observed the ERα haplotype 1 association to be modified by VDR haplotype 1 genotype (Figure 8.4). Significant ERα haplotype 1 genotype dependent differences were only observed in women homozygous for VDR haplotype 1. Logistic regression analysis showed that, compared to the 'doublereference' group, within the group of VDR genotype [1,1] women have a 2-fold (95% CI 0.5-7.9) and 10-fold (95% CI 2.7-38) increased risk for vertebral fractures when being heterozygous or homozygous for ERα haplotype 1, respectively. In non-carriers and heterozygous carriers of VDR haplotype 1 no significant ERα haplotype 1 genotype dependent differences were observed. When age, BMI, ERα genotype, and VDR genotype were taken together in a multivariate regression model, there appeared to be a significant interaction between ERα haplotype 1 and VDR haplotype 1 (p = 0.01 for the interaction term). After adjustment for lumbar spine BMD and for age at menopause the results did not change (data not shown).

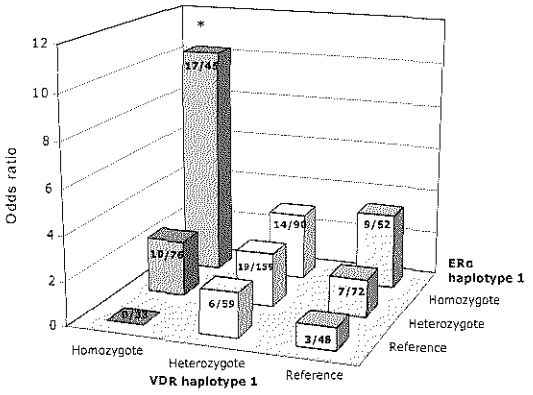

Figure 8.4 Odds ratios compared to the 'double reference' group and numbers of vertebral fractures according to combined ERα and VDR genotypes. 'Reference' includes ERα and VDR genotypes 22, 23, and 33; 'heterozygotes' include 12 and 13; 'homozygote' includes 11. *p-value < 0.001 for allele dose association of ERα haplotype 1 in VDR haplotype 1 homozygous carriers. p = 0.01 for the interaction term.

8.5 DISCUSSION

The current study in postmenopausal women demonstrates for the first time interaction of polymorphisms in the VDR and ERα gene in relation to the risk of incident vertebral fracture risk. Women homozygous for both the VDR haplotype 1 and ERα haplotype 1 had a 10-times higher vertebral fracture risk than non-carriers and a 3-4 times higher risk than carriers of either one of the risk haplotypes.

So far most association studies focused on single genes. Two meta-analyses showed a weak association of VDR genotypes with BMD, which supported our own findings in a sample of 2000 men and women from the Rotterdam study[9,12,13]. Also for ERα gene polymorphisms associations with BMD were reported[17-20] although others could not confirm these results[21-24]. Most genetic association studies for osteoporosis have been performed with BMD as endpoint while the clinically more relevant endpoint of osteoporosis is fracture. A limited number of studies have so far able to address the association of specific gene polymorphisms with fractures. Previously we have shown that VDR haplotype 1 is the risk allele for osteoarthritis, vertebral and nonvertebral fractures[11,38]. In the present study an association of ERα with lumbar spine BMD and vertebral fracture risk was found. Also at the lumbar spine a synergistic interaction between ERα and VDR genotype for BMD and fractureswas detected. No interaction effect between ERα and VDR genotypes was found for femoral neck BMD. This is in line with previous data, which show a higher response to

hormonal replacement therapy at the lumbar spine in contrast to the femoral neck[39-42]. The ERα effect may be more pronounced in the spine, which contains more trabecular bone resulting in a higher rate of bone turnover compared to cortical bone, as present for example in the femoral neck.

We and others previously observed that ERα genotype is associated with differences in age at menarche[43] and age at menopause[37]. However, in our current analyses age of menopause did not influence the interaction we observed. This suggests that differences in the age of menopause are small and do not explain the interaction. However, because of the relatively small effect, such influences might only be observed in studies of sufficient power.

An interesting observation was that the association of both ERα and VDR genotypes with vertebral fracture incidence was independent of BMD. This indicates the significance of other bone characteristics for the risk of fracture. But it also points to the involvement of ERα and VDR genes in pathways (e.g. bone matrix synthesis and bone turnover) other than those directly reflected in BMD, and which also determine strength of bone and thereby fracture risk[44,45].

A limitation of the present study may be health selection bias. Health seems not to be genotype dependent and therefore we do not expect this to influence the results. Furthermore, potential selection bias was avoided by deriving cases and non-cases from the same source population. In addition, power of interaction is limited. Consequently, point estimates could be unstable, and therefore larger studies should be acquired.

An aspect that should be realized is that the polymorphisms in the ERα and VDR are anonymous; i.e. there is no direct known functional consequence for the ERα and VDR protein. Therefore, when association is found it is assumed that allele(s) of these single nucleotide polymorphisms are in linkage disequilibrium (LD) with one or more of the truly functional polymorphisms elsewhere in the gene. These functional polymorphisms could alter VDR[46,47] or ERα protein structure or might affect the activity of the VDR and ERα 5' promotor and 3' UTR, leading to the expression of altered quantities of VDR[47] and ERα proteins[48] under physiologic conditions. Differential transcriptional activity of the VDR and ERα receptor proteins could then preferentially modulate subsets of target genes in vitamin D and estrogen responsive pathways.

Although the mechanism(s) for the gene-gene interaction we observe is so far unknown, it is conceivable from a physiological point of view. 1,25-$(OH)_2D_3$ is an important factor in estrogen biosynthesis[25] and might thus influence local equilibrium between estrogens and androgens. Furthermore 1,25-$(OH)_2D_3$ regulates ER expression in osteoblast-like cells[28]. In

this way 1,25-$(OH)_2D_3$ might regulate the effect of E_2 on bone metabolism. *In vitro* and *in vivo* studies have shown that several biological responses to treatment with vitamin D, such as intestinal calcium absorption and osteocalcin production, are VDR genotype dependent[49-52]. If 1,25-$(OH)_2D_3$ influences the effect of E_2 on bone metabolism, this effect might also be VDR genotype dependent.

On the other hand, E_2 influences vitamin D metabolism and VDR expression. Sex hormone replacement therapy increases total and free serum 1,25-$(OH)_2D_3$ levels[26,27]. In human fetal osteoblasts E_2 upregulates VDR expression[53]. Also in rat duodenal mucosa E_2 increases VDR expression and bioresponse[29]. In this way E_2 might influence vitamin D regulated processes, like intestinal calcium absorption and osteocalcin production in bone. Several studies have demonstrated that the response to hormonal replacement therapy is ERα genotype dependent[19,54,55]. Therefore, the effect of estrogen replacement on vitamin D regulated processes might also be ERα genotype dependent.

In conclusion, the present study shows an interlocus interaction in relation to BMD and fractures between two important candidate genes in osteoporosis. Recently, we also demonstrated an interaction between VDR and another candidate gene, the COLIA1 gene, with respect to fracture risk[11]. This underscores the polygenic character of osteoporosis and the importance of examining multiple gene interactions. However, for this approach large (multicenter) studies are required to achieve sufficient power.

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Chapter 9

General discussion and further research

In this chapter the most important findings of this thesis are discussed, and put into perspective. The discussion will finish with suggestions for further research.

Effects of 17β-estradiol on intestinal calcium absorption

The first part of the thesis focuses on the effects of E_2 on 1,25-$(OH)_2D_3$ mediated processes with respect to the regulation of calcium homeostasis and bone metabolism. 1,25-$(OH)_2D_3$ acts directly on the intestine to stimulate calcium absorption[1,2]. Decreased intestinal calcium absorption has been attributed to vitamin D deficiency and/or vitamin D resistance[3-6] and is one of the factors implicated in the pathogenesis of osteoporosis[7-9] and increased risk for hip fracture[10]. The data presented in chapters 2 and 3 demonstrate that also E_2 regulates intestinal calcium absorption. In a rat model it was shown that net calcium absorption was significantly reduced in E_2 deficiency. This observation supports previous findings that menopause is associated with decreased intestinal calcium absorption[11,12]. E_2 repletion in OVX rats restored net calcium absorption in the duodenum to the sham level. These direct calcium movement measurements support the calcium absorption data derived from food-intake studies in rats[13]. Furthermore, it is consistent with the observation that in postmenopausal women estrogen supplementation normalizes intestinal calcium transport[11,14,15].

The effect of E_2 on intestinal calcium absorption can be exerted directly or indirectly. The observation that calcium absorption in the E_2 supplemented groups was partially restored to sham level, while serum 1,25-$(OH)_2D_3$ levels were strongly reduced (80-90% lower than in the sham and OVX groups, respectively) suggests that E_2 has a direct effect on intestinal calcium absorption. A direct effect of E_2 on intestinal calcium absorption is supported by the presence of ER in the intestines[16,17] and the finding that E_2 stimulates calcium uptake in cultured rat duodenal enterocytes[18]. The precise downstream mechanism by which E_2 may affect intestinal calcium absorption is however still unknown. Recently, estrogen responsive elements have been found in the promoter sequence of epithelial calcium channels (ECaC2)[19]. Therefore, E_2 might stimulate calcium influx in intestinal epithelial cells by upregulation of calcium channel synthesis. However, duodenal ECaC2 expression in mice appeared E_2 independent[19].

In the E_2 deficient group intestinal calcium absorption was significantly reduced, while 1,25-$(OH)_2D_3$ levels were (not significantly) increased. This suggests that in the absence of E_2 the

intestine may be desensitized for the action of 1,25-$(OH)_2D_3$. Several studies have suggested that E_2 could also regulate intestinal calcium absorption indirectly by modulating 1,25-$(OH)_2D_3$ action. In oophorectomized women E_2 preserves a normal intestinal responsiveness to 1,25-$(OH)_2D_3$[11]. Previously, Ebeling et al. have reported that the decreased intestinal calcium absorption of postmenopausal women could be attributed to a decline in intestinal VDR number[4]. Liel et al showed in a rat model that E_2 upregulates VDR expression and concomitantly calbindin D_{9k} mRNA expression, while E_2 did not affect serum 1,25-$(OH)_2D_3$ levels[20]. In contrast, we found no estrogen dependent differences in VDR expression between sham, OVX and E_2 repleted rats. Our observations on the VDR are consistent with the data from Kinyamu et al., who found no change in intestinal VDR level, while intestinal calcium absorption was decreased in postmenopausal women compared with premenopausal women[6]. Taken together an interaction between E_2 and 1,25-$(OH)_2D_3$ seems not to be exerted via an effect on VDR level. Possibly, E_2 induces VDR phosphorylation, one step in the cascade leading to receptor activation. Subsequently, increased receptor phosphorylation might result in increased hormone binding activity and increased receptor half-life. In this way E_2 might enhance 1,25-$(OH)_2D_3$-dependent transcriptional activation, which may result in increased epithelial calcium channel, calbindin and/or PCMA synthesis. This may finally result in increased intestinal calcium absorption.

Although the studies demonstrate that E_2 facilitates calcium absorption, the reduction in calcium absorption after OVX could not be completely overcome by E_2 supplementation. One explanation is that E_2 also enhances calcium (re-)absorption in another part of the intestine, like the cecum. This segment has been shown to play an important role in calcium absorption in rats[21,22] and expresses the ER[17]. Also in colonic mucosa E_2 could mediate its effects by an increase in responsiveness to endogenous 1,25-$(OH)_2D_3$ through upregulation of VDR expression[23].

Another explanation could be that in addition to estrogens, other hormones or regulatory factors are reduced after OVX, which are not restored after estrogen replacement. 1,25-$(OH)_2D_3$ circulation levels were about 80% decreased in E_2 repleted rats compared to sham rats. The reduction in 1,25-$(OH)_2D_3$ mediated intestinal calcium absorption in E_2 repleted rats may not have been overcome completely by E_2 mediated calcium absorption. IGF-I is another potential factor involved in intestinal calcium absorption. Hope et al. suggested that IGF-I might stimulate intestinal calcium absorption by speeding up the differentiation of immature villus cells such that

they have the function of cells more mature with regard to calcium absorption[24]. In our study serum IGF-I levels in E_2 repleted rats were about 20% lower than in sham-operated rats and might also have accounted for the fact that intestinal calcium absorption was not completely restored to sham level by E_2 repletion.

17β-Estradiol and renal 1α-hydroxylase activity and renal calcium reabsorption

A marked observation in the study described in Chapter 2 is made regarding the 1,25-$(OH)_2D_3$ levels. The slight elevation in serum total 1,25-$(OH)_2D_3$ levels following OVX is in agreement with earlier observations in rats[25,26]. In estrogen-deficient women, both decreased[27,28] and normal serum 1,25-$(OH)_2D_3$ levels[11,29-31] have been reported. The strong reduction in both total and free serum 1,25-$(OH)_2D_3$ levels with E_2 supplementation is in agreement with a study by Kalu et al.[26] in rats, but appears to be in contrast to the human situation. In women, transdermal estrogen administration has no effect on either serum DBP or serum 1,25-$(OH)_2D_3$ levels[25,32,33] or even an increase with hormone replacement therapy was observed[34-36]. These differences in effects of E_2 on serum 1,25-$(OH)_2D_3$ levels between rat and human studies could be attributed to the relatively high and constant serum E_2 levels in rats, which is reflected by increased uterus weight and femoral BMD in rats four weeks after operation.

It is unlikely that the large changes in 1,25-$(OH)_2D_3$ levels can be explained by the relatively small decrease in serum 25-$(OH)D_3$ levels in the E_2 repleted groups. As a consequence, renal conversion of 25-$(OH)D_3$ into 1,25-$(OH)_2D_3$ by 1α-hydroxylase should have been reduced and/or the catabolism of 1,25-$(OH)_2D_3$ and 25-$(OH)D_3$ should have been increased. Because the ER has been demonstrated to be present in the kidney[37,38], E_2 could directly inhibit 1α-hydroxylase activity and/or stimulate 24-hydroxylase activity. Previously, with cultured opposum kidney cells a direct inhibitory effect of E_2 on 1α-hydroxylase activity has been shown[39].

E_2 could also indirectly regulate 1,25-$(OH)_2D_3$ synthesis. We showed that serum PTH levels are not dependent on estrogen status. Therefore, it seems unlikely that E_2 alters 1,25-$(OH)_2D_3$ synthesis via reduction of PTH, a potent stimulator of 1α-hydroxylase activity. However, E_2 may act at the kidney to inhibit PTH-stimulated 1,25-$(OH)_2D_3$ synthesis. In the literature data concerning the effect of E_2 on serum PTH levels and 1α-hydroxylase activity are conflicting. E_2 inhibits the PTH-stimulated intracellular cAMP accumulation in opposum kidney cells[40] and inhibits PTH receptor binding affinity in calvaria and kidney[41]. However, Ash et al. have shown

in a rat model that E_2 promotes PTH-induced synthesis of 1,25-$(OH)_2D_3$[42]. Estrogen substitution in postmenopausal women may result in unchanged[43-45], or increased[15,46] renal 1α-hydroxylase activity either due to increased serum PTH levels[15], an increased renal responsiveness to PTH[46] or due to a direct stimulating effect on 1α-hydroxylase activity[46].

E_2 may also indirectly inhibit 1,25-$(OH)_2D_3$ synthesis by increasing serum calcium levels. High serum calcium levels have been reported to directly inhibit 1α-hydroxylase activity[47]. Immediately after E_2 repletion serum calcium levels may increase as a result of increased intestinal calcium absorption. Increased serum calcium may then be used for bone mineralization, may inhibit 1,25-$(OH)_2D_3$ synthesis or may be lost by urinary calcium excretion. Finally, an equilibrium may exist in which intestinal calcium absorption is increased, serum calcium levels are normal, bone mineralization and urinary calcium excretion are increased, and 1,25-$(OH)_2D_3$ synthesis is decreased. From the present study it remains unclear whether in the rat E_2 inhibits 1α-hydroxylase activity and/or induces 24-hydroxylase activity in a direct and/or indirect manner.

In E_2 repleted rats urinary calcium excretion was increased compared with ovariectomized rats. This may be due to increased intestinal calcium absorption under the influence of E_2 because the rats were pair-fed during nocturnal collection of urine and/or a reduced calcium reabsorption. Stimulation of calcium reabsorption is, like regulation of 1α-hydroxylase activity, a renal PTH response. Urinary calcium excretion was not affected by ovariectomy, while E_2 repletion increased urinary calcium excretion. This supports the concept of reduction of PTH-sensitivity by E_2 in rat kidney. To the contrary, in women estrogen deficiency results in increased urinary calcium excretion[48,49]. Therefore, our observations in rats are not in all aspects compatible with these in postmenopausal women. However, the observed changes in intestinal calcium absorption are in line with those observed in postmenopausal women[12,15,43]. This together with our observations on 1,25-$(OH)_2D_3$ levels shows that the rat is a valuable model to assess E_2 effects on calcium metabolism independent of 1,25-$(OH)_2D_3$, as has also been argued by other investigators[50,51].

17β-Estradiol and bone

1,25-$(OH)_2D_3$ has a dual effect on bone metabolism. On the one hand 1,25-$(OH)_2D_3$ stimulates bone formation and mineralization and on the other hand 1,25-$(OH)_2D_3$ stimulates bone

resorption. This balance is kept by direct and indirect effects on the osteoblast and the osteoclast, respectively. E_2 is also involved in regulation of bone metabolism. So far, the major action of E_2 is believed to be inhibition of bone resorption[52,53]. The studies described in chapters 4 and 5 demonstrate that E_2 may also have additional effects by modulation of bone formation and mineralization.

IGF-I is one of the factors produced *in vitro* by osteoblastic cells and this growth factor has mitogenic and stimulating effects on bone cells, resulting in enhanced collagen production and bone matrix biosynthesis[54,55], thereby also influencing bone structure[56]. We demonstrate in an *in vivo* rat model that IGF-I mRNA is predominantly synthesized in osteocytes, and to a lesser extent in osteoblasts. On basis of the anticipated role of osteocytes in bone it is tempting to speculate that IGF-I is a mediator between mechanical loading and regulation of bone metabolism. Furthermore we show that E_2 has a positive effect on IGF-I mRNA expression.

IGFBP-5 is also produced by bone cells and promotes osteoblast mitogenesis by enhancement of the biological action of IGF-I[57] as well as in an IGF-I independent manner[58,59]. We showed in an *in vivo* rat model that IGFBP-5 mRNA is synthesized in osteoblasts and that E_2 has a positive effect on IGFBP-5 mRNA expression. These data suggest that E_2 does not only inhibit bone resorption, but that E_2 may also have anabolic effects on bone via induction of the IGF-I/IGFBP-5 system. This hypothesis is supported by *in vitro* studies that have shown that the proliferative response of osteoblasts to E_2 is mediated by IGF-I[60,61]. Our data are in line with a study of Suliman et al., who demonstrated that E_2 increases IGF-I protein levels in femura of aged rats[62]. However, Seck et al. found no association between bone matrix IGF-I protein level and menopause in women[63]. This dissociation may be related to differences in bone turnover between rats and humans. It is conceivable that in rats bone turnover after ovariectomy is more rapidly and significantly increased than in women shortly after menopause. So, in a relatively short period new bone matrix may be synthesized with a lower IGF-I content, while in postmenopausal women bone matrix IGF-I is accumulated in many years and may therefore not be significantly changed shortly after menopause.

Several studies have addressed the correlation between serum IGF-I levels and serum E_2 levels in women. So far no consistent data have been reported. Positive[64-66], no[67], and negative correlations[68,69] between serum E_2 and serum IGF-I levels have been reported. There are also contrasting data with respect to the relation between serum IGF-I levels and BMD. Some studies showed a negative correlation between serum IGF-I levels with BMD and vertebral

fracture risk in postmenopausal women[70-72], but others could not confirm this[73,74]. We showed in our rat model that there is dissociation between serum IGF-I levels and IGF-I mRNA expression in bone cells. Serum E_2 level was negatively correlated with serum IGF-I level, while there was a positive correlation with bone IGF-I mRNA expression. Previous studies have also shown reduced serum IGF-I levels in rats subcutaneously given E_2[75]. These data are suggestive for a role of locally produced IGF-I and not of circulating IGF-I in the regulation of bone formation. Therefore it remains questionable to use serum IGF-I levels as a marker to assess the relation between IGF-I and bone turnover.

The IGF-I/IGFBP-5 system is also influenced by 1,25-$(OH)_2D_3$. 1,25-$(OH)_2D_3$ increases IGF-I levels in human bone cell supernatants[76], and causes a small but not significant increase in the release of IGF-I in the supernatant of rat osteoblast-like cells[77]. In rat osteoblasts, 1,25-$(OH)_2D_3$ has been shown to increase IGFBP-5 mRNA expression[78,79].

In the present study there was no correlation between serum 1,25-$(OH)_2D_3$ levels and IGF-I mRNA expression in bone. This might be due to the inverse relationship between serum 1,25-$(OH)_2D_3$ levels and E_2 levels resulting in opposing effects on IGF-I expression in bone. In view of the very low serum 1,25-$(OH)_2D_3$ levels in estrogen repleted rats, it is conceivable that the interplay between E_2 and the IGF-I system on bone is largely independent of 1,25-$(OH)_2D_3$ action.

In Chapter 5 we demonstrate that sera from women with a high BMD induce less *in vitro* mineralization than sera from women with low BMD. This was assessed in an *in vitro* bioassay with human fetal osteoblastic (SV-HFO) cells. Women with high BMD had significantly higher serum E_2 levels than women with low BMD. Recently, we found that *in vitro* mineralization by SV-HFO cells is modulated by E_2[80]. Although not directly expected, increase in mineralization may contribute to the risk of osteoporotic fractures. The optimal mineralization density for bone strength is actually not known[81]. An increased mineralization increases the strength of bone, but overmineralization may increase stiffness and brittleness of bone, which is more liable to fractures[82]. Therefore, it is tempting to speculate that in women with very low serum E_2 levels, newly formed bone may be overmineralized resulting in more brittle bone. Our data suggest a new role for E_2 in bone metabolism, i.e. control of the extent or set point of mineralization, and thereby the elasticity of bone, which potentially contributes to the quality of bone and susceptibility to fracture.

Our observation that postmenopausal women with low BMD had lower serum E_2 levels than women with high BMD is in line with other studies. In a previous study serum E_2 levels correlated positively with lumbar spine and femoral neck BMD in pre- and postmenopausal women[52]. Recently, Heshmati et al. showed that in postmenopausal women variations in bone resorption are related to serum E_2 levels, even when serum E_2 levels are low[53]. These data suggest that after menopause, variations in serum E_2 levels may determine bone turnover and consequently determine (loss of) bone mass, which is actually measured by DEXA. However, the quality of the newly formed bone may also be related to serum E_2 levels, in view of the proposed effects of E_2 on IGF-I synthesis and bone mineralization.

The mechanism by which E_2 would regulate bone mineralization still has to be resolved. Bone mineralization may be modulated by a direct effect of E_2 on osteoblasts, and via interplay between E_2 and other factors involved in bone metabolism. In this study we found no differences in serum PTH and serum 1,25-$(OH)_2D_3$ levels between women with either a low or high BMD. This may suggest a direct effect of E_2 on osteoblasts. However, there may be an interactive effect between the estrogen and vitamin D endocrine system on bone metabolism. Several studies have shown that E_2 upregulates VDR expression in osteoblastic cells[80,83], specifically during the matrix maturation phase[80]. Recently, Gallagher et al. showed that in postmenopausal women, combination therapy with hormone replacement therapy/estrogen replacement therapy and calcitriol therapy increased BMD significantly more in the total hip and trochanter than did hormone replacement therapy/estrogen replacement therapy and calcitriol therapy alone[84]. In rats OVX and vitamin D deficiency independently led to decreases in BMD but only the combination reduced the mechanical femoral neck strength[85].

The studies described in chapters 2 - 5 demonstrate the complex interplay between E_2 and 1,25-$(OH)_2D_3$ in the regulation of calcium homeostasis and bone metabolism. Figure 9.1 tries to summarize the possible effects of E_2 on kidneys, intestines and bone with respect to calcium and bone metabolism.

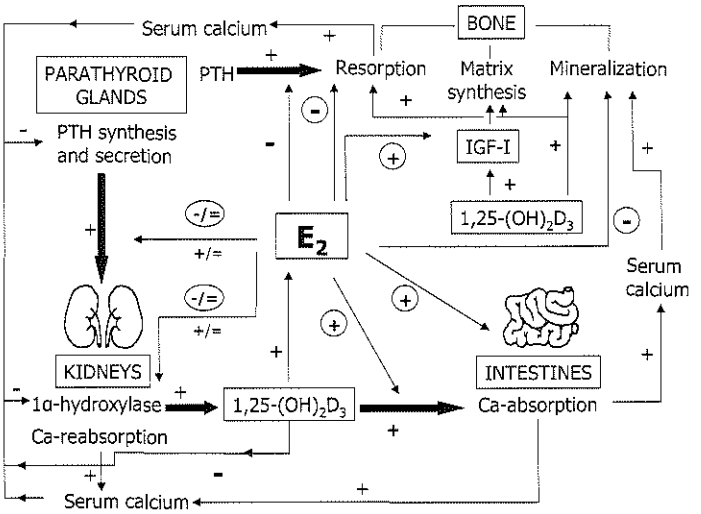

Figure 9.1 Diagram of the main actions of 17β-estradiol (E_2), 1,25-dihydroxyvitamin D_3 (1,25-$(OH)_2D_3$) and parathyroid hormone (PTH) on calcium and bone metabolism and possible interactions between the hormones. E_2 has no effect on PTH synthesis or secretion by the parathyroid glands[86]. In the rat kidney E_2 may inhibit calcium reabsorption and 1α-hydroxylase activity directly, or indirectly by inhibition of renal PTH responsiveness. In humans either no effect[34-36,43-45] or stimulatory effect[15,25,32,33,46] on renal calcium reabsorption and 1α-hydroxylase activity have been reported.

E_2 may stimulate intestinal calcium absorption directly or indirectly by enhancement of 1,25-$(OH)_2D_3$ mediated intestinal calcium absorption. This may be the result of increased intestinal VDR number[20,23] and/or affinity for its ligand. E_2 may also enhance 1,25-$(OH)_2D_3$ mediated intestinal calcium absorption by inducing 1,25-$(OH)_2D_3$ production in humans[15,46], but not in rats. In rats 1,25-$(OH)_2D_3$ may promote aromatase activity[87], thereby stimulating E_2 synthesis. Serum calcium negatively influences 1α-hydroxylase activity, renal calcium reabsorption and PTH synthesis and secretion.

E_2 also has a modulating effect on bone turnover, bone matrix synthesis and bone mineralization. E_2 inhibits (PTH induced) bone resorption[88,89]. Bone matrix synthesis can be increased by a stimulating effect of E_2 on IGF-I and IGFBP-5 synthesis, either directly or by interaction with 1,25-$(OH)_2D_3$. Bone mineralization can also be influenced by the modulating effect of E_2 on intestinal calcium absorption. Main findings from the studies presented in this thesis are encircled. The main effects of PTH and 1,25-$(OH)_2D_3$ on kidney and intestines are given in fat black arrows.

The significance of ERα and VDR gene polymorphisms for the action of vitamin D.

E_2 and 1,25-$(OH)_2D_3$ play an important role in maintenance of bone quantity and bone quality. As described in previous sections, both bone quantity and bone quality determine osteoporotic fracture risk. Therefore ERα and VDR genes are considered osteoporosis candidate genes. In the second part of the thesis (Chapters 6, 7, and 8) we studied the association between VDR and ERα gene polymorphisms with bone turnover, BMD and osteoporotic fracture risk and examined whether there is an interaction between these gene polymorphisms in determination of fracture risk.

Vitamin D receptor gene polymorphisms

The biological actions of 1,25-$(OH)_2D_3$ are mediated largely, if not entirely, by the VDR. 1,25-$(OH)_2D_3$ has a potential therapeutic effect in osteoporotic patients, but the overall effect is not consistent[90]. It is likely that genetic variability of the VDR may explain differences in responsiveness of target tissues to 1,25-$(OH)_2D_3$. Therefore, we have investigated the genetic association of VDR gene polymorphisms with bone turnover and with the response to 1,25-$(OH)_2D_3$ *in vivo* and *in vitro* in postmenopausal women. In Chapter 6 we show that women within the lowest quintile of BMD have a higher rate of bone turnover than women within the highest quintile of BMD. This increased bone turnover rate was associated with VDR haplotype 2 gene polymorphism. However, there were no VDR genotype dependent changes in bone turnover in response to short-term administration of 1,25-$(OH)_2D_3$. In Chapter 7 data from an *in vitro* study with peripheral mononuclear cells (PBMC) are shown. This study demonstrates that the half maximal concentration for 1,25-$(OH)_2D_3$ inhibition of PHA-stimulated growth is significantly different between PBMC characterized by different alleles of the VDR translational start-site while the maximal inhibition is similar for all genotypes. This supports the hypothesis that the 424 amino acids-long VDR is more efficient in exerting 1,25-$(OH)_2D_3$ effects than the 427 amino acids-long VDR. These observations are in line with the observation of others who showed a VDR genotype-dependent change in BMD in response to vitamin D treatment[91-93].

Finally, in Chapter 8 we show that VDR haplotype 1 gene polymorphism is associated with increased vertebral fracture risk. This association was largely independent of BMD. As discussed in previous sections fracture risk is not only dependent on bone quantity but also on bone quality. 1,25-$(OH)_2D_3$ is an important stimulator of bone mineralization and

stimulates type I collagen synthesis. Differences in bone quality related to differences in type I collagen expression may also be ascribed to interaction between VDR gene and COLIAI gene polymorphisms[94].

The studies described in Chapter 6 show that VDR haplotype 2 is associated with increased bone turnover. A previous study demonstrated that VDR haplotype 3 is associated with decreased BMD[95], while in Chapter 8 we show that VDR haplotype 1 is associated with the clinically most relevant endpoint vertebral fractures. Although different VDR haplotypes may be associated with different determinants of osteoporotic fracture risk, one should take into the account the difference in power of the studies. In this respect, the number of subjects in the vitamin D intervention study (Chapter 6) was small and therefore the power of the study is limited.

These findings strengthen the need to find functional polymorphisms and to determine linkage disequilibrium with the analyzed polymorphisms. The alleles of the *Bsm*I and *Apa*I polymorphisms in intron 8 and of *Taq*I in exon 9 are in linkage disequilibrium and haplotypes can be constructed over this 2.2 kb region. The linkage disequilibrium of these RFLPs extends into the 3' untranslated region. None of these VDR polymorphisms affect the structure of the VDR protein itself, but may still have functional consequences. There is some evidence that VDR haplotype 1 is associated with aberrant expression levels of VDR mRNA, possibly through changes in mRNA stability[96-99].

The *Fok*I polymorphism at the translation initiation site is unique among common VDR variants so far, in that it results in an alteration of the VDR structure[100]. This polymorphic site is genetically unlinked to the above *Bsm*I, *Apa*I, *Taq*I[101,102] cluster. This is in line with the observation that the *Fok*I RFLP was associated with the response to 1,25-$(OH)_2D_3$ *in vitro*, whereas the VDR 3' genotype lacked an association. The significance of the *Fok*I polymorphism in bone metabolism *in vivo* is so far unclear. In the literature there are conflicting data on the association between *Fok*I RFLP and BMD[100-105]. Our study sample was too small for statistical analysis regarding association between *Fok*I RFLP and BMD.

The *Bsm*I, *Apa*I, *Taq*I and poly (A) repeat polymorphisms and *Fok*I polymorphism may be in linkage disequilibrium with one or more additional polymorphic variations in the VDR gene that affect(s) VDR activity[106,107]. Recent investigations into the portion of the VDR gene encoding the 5' untranslated region have revealed a complex of at least seven exons with evidence for alternative splicing. It is possible that undiscovered common polymorphic sites exist in this newly described complex of multiple exons at the 5' end of the gene. Such polymorphisms

could alter VDR protein structure[108] or might affect the activity of one of the three proposed VDR promoters, leading to the expression of altered quantities of VDR proteins under physiologic conditions[107]. Differential transcriptional activity of the VDR isoforms could then preferentially modulate subsets of target genes in vitamin D-responsive pathways. Thus, in combination with the gene-environment studies, there is growing evidence that allelic variation in the VDR gene or in a nearby gene involved in vitamin D metabolism, underlies the genotype related differences observed in the association studies.

Interestingly, we found that the VDR *FokI* genotype was associated with ED_{50} of PBMC growth inhibition by 1,25-$(OH)_2D_3$ and not with maximal inhibition of PHA-stimulated growth by 1,25-$(OH)_2D_3$. This observation may have clinical significance. It is conceivable that dependent on the vitamin D status a VDR genotype is present or absent. So, especially under conditions of vitamin D insufficiency, e.g. in elderly women, the biological consequences may become apparent, while at sufficient 1,25-$(OH)_2D_3$ levels the genotype consequences may be absent.

ERα receptor polymorphisms

In chapter 8 we have shown that ERα gene polymorphisms (*PvuII* and *XbaI*) are associated with lumbar spine BMD and vertebral fracture risk. It should be realized that the association study was performed with anonymous polymorphisms. This suggests that the *PvuII*/*XbaI* polymorphisms are in linkage disequilibrium with a functional polymorphism. Indeed, a TA repeat in the promotor region has been found to be in strong linkage disequilibrium with the *PvuII* polymorphism [109-111]. However, it is currently unknown whether this polymorphism has functional consequences.

It is generally accepted that fracture risk is not only determined by bone quantity but also by bone quality[81,112,113]. An interesting observation was that the association between ERα gene polymorphisms and vertebral fracture risk was largely independent of lumbar spine BMD. A role for E_2 in bone quality is also supported by the observations in Chapters 4 and 5 demonstrating E_2 effects on the anabolic agents IGF-I and IGFBP-5 and on bone mineralization. Furthermore, the association of ERα gene polymorphism with BMD was more pronounced at the lumbar spine (mainly trabecular bone), where a relatively high bone turnover exists, compared to the femoral neck (mainly cortical bone). This is supported by the observation that in postmenopausal women ERα gene polymorphisms affect the response to estrogen in the lumbar spine BMD, but

not in the femoral neck[114,115]. Therefore, these association data together with those in Chapters 4 and 5 indicate that E_2 not only controls bone quantity but also bone quality.

Interaction between ERα and VDR gene polymorphisms

Besides gene-environment interaction[116,117], gene-gene interaction may also occur or actually proteins encoded by variants of different genes may interact[94,118,119]. Protein-protein interaction is a very important process in multiple pathways, e.g. in the basic transcription and translation machinery, but also in most metabolic pathways. These gene-gene interactions can be additive, subtractive or synergistic. In Chapter 8 a synergistic interaction between ERα haplotype 1 genotype and VDR haplotype 1 genotype with respect to BMD and vertebral fracture risk is described.

Several mechanisms could explain the interaction between the VDR and ERα gene with respect to lumbar spine BMD and vertebral fracture risk (see Chapter 1.6). A possible mechanism is that the VDR and ERα gene interact by mutual regulation of synthesis of their ligands. In postmenopausal women the main source of E_2 is the conversion of adrenal androgens. It has been demonstrated in human osteoblasts that the expression of P_{450} aromatase, which is responsible for the conversion of androgens to estrogens, is dependent on the amount of VDR mRNA expressed[120]. Therefore E_2 levels might be VDR genotype dependent. In view of the observations that intestinal calcium transport, bone turnover[53] and BMD[52] are correlated with serum E_2 levels, these E_2 dependent processes may be modulated by VDR genotype. On the other hand, E_2 has a potential stimulating effect on 1,25-$(OH)_2D_3$ synthesis and a similar mechanism could be hypothesized for ERα genotype dependent 1,25-$(OH)_2D_3$ mediated processes. However, whether 1α-hydroxylase activity is dependent on ERα expression is currently not known.

The VDR and ERα gene may also interact by mutual regulation of synthesis of VDR and ER. VDR and ER expression are regulated by both 1,25-$(OH)_2D_3$ and E_2. If serum 1,25-$(OH)_2D_3$ levels are ERα genotype dependent, then VDR expression may be ERα genotype dependent. On the other hand, if serum E_2 levels are VDR genotype dependent, then ERα expression may also be VDR genotype dependent.

Another underlying mechanism for the interaction between the VDR and ER may be direct interaction or competition for transcription factors involved in calcium and bone metabolism. The synthesis of proteins involved in these processes may be modulated by interplay of ERα

and VDR. Interestingly, the recently described epithelial calcium channel may be modulated by both 1,25-$(OH)_2D_3$ and E_2[121-123]. Therefore the expression of this protein could be VDR and ERα genotype dependent.

These genetic association studies underline that there is a close interplay between the estrogen and vitamin D endocrine system. Still it is crucial to find functional polymorphisms and to determine LD with the analyzed polymorphisms. The functional polymorphisms may be localized in another gene. However, in view of the physiological interactions between the ER and VDR endocrine system, it is conceivable that the functional polymorphisms are located in the ERα and VDR genes.

Finally, a full recognition of the genetic complexity of ERα and VDR actions in humans may eventually allow accurate prediction of ERα and VDR activity in individual patients based on genotype, along with an enhanced ability to assess disease risk, as well as response to pharmacological agents related to ERα and VDR actions.

SUGGESTIONS FOR FURTHER RESEARCH

In this thesis we demonstrate that intestinal calcium absorption is regulated by E_2. This may be either a direct effect or an indirect effect, possibly by interaction with 1,25-$(OH)_2D_3$. If there is an interaction between E_2 and 1,25-$(OH)_2D_3$ on intestinal calcium transport, then this effect seems independent of VDR number. Possible interactive effects could be investigated in more detail in the 1α-hydroxylase knock-out mouse. In this model the (interactive) effects of 1,25-$(OH)_2D_3$ and E_2 on intestinal calcium transport and proteins involved in intestinal calcium absorption (e.g. epithelial calcium channels, calbindins, PMCA) can be studied.

Another interesting observation is the VDR genotype dependent 1,25-$(OH)_2D_3$ induced growth inhibition of PBMC. This might implicate that there are only VDR genotype dependent differences in 1,25-$(OH)_2D_3$ mediated processes in vitamin D deficient women and not in vitamin D repleted women. By analogy, it would be interesting to study whether E_2 mediated processes are more or less efficiently regulated in women with equal (low) serum E_2 levels depending on ERα genotype. In view of the observed relations between serum E_2 levels with bone turnover rate and BMD, and the association between ERα genotypes and vertebral fracture risk, it is conceivable that postmenopausal women with low serum E_2 levels and homozygous for the ERα risk allele have a higher vertebral fracture risk than women with none

or one of these risk factors. In view of the possible relation between serum E_2 level, ERα genotype and fracture risk, it is also conceivable that homozygous carriers of the risk allele have less beneficial effect of hormone replacement therapy in prevention of vertebral fractures. These hypotheses could further be evaluated.

Another observation is that E_2 not only determines bone quantity but also bone quality. The effect of E_2 on the various determinants of bone quality and whether there are ERα genotype dependent differences in these effects could be studied.

So far, only anonymous polymorphisms in both genes have been found. Finding functional sequence variants and defining haplotype patterns is essential to better understand the associations and interactions between genes. In view of the interaction between VDR and COLIAI gene polymorphism with respect to fracture risk, it would be interesting to study the interaction between VDR, ERα and COLIAI gene polymorphisms with respect to vertebral and nonvertebral osteoporotic fractures. For this purpose large, multicenter studies are necessary.

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Chapter 10

Summary/Samenvatting

SUMMARY

Calcium homeostasis and bone metabolism are regulated by the calciotropic hormones, 1,25-dihydroxyvitamin D_3 (1,25-$(OH)_2D_3$ and parathyroid hormone (PTH), and the sex hormone 17β-estradiol (E_2). 1,25-$(OH)_2D_3$ and E_2 are both steroid hormones and exert their action via receptors, the vitamin D receptor (VDR) and estrogen receptor (ER), respectively. Several studies have shown that 1,25-$(OH)_2D_3$ and E_2 may have a mutual effect on each other's biosynthesis and receptor expression.

1,25-$(OH)_2D_3$ maintains calcium homeostasis by increasing the efficacy of the intestinal cells to absorb dietary calcium. 1,25-$(OH)_2D_3$ also maintains normal serum calcium and serum phosphate levels by inducing bone resorption through enhancement of osteoclastogenesis and osteoclast activity. These actions are thought to be mediated via stimulated secretion of specific factors by the osteoblasts. Furthermore, 1,25-$(OH)_2D_3$ stimulates bone mineralization.

Estrogens are important for bone maturation during the growth phase. E_2 also plays an important role in the control of bone turnover. The major action of E_2 is believed to be inhibition of bone resorption, mostly by indirect actions of E_2 on osteoclast differentiation, activation and apoptosis. Furthermore, E_2 may regulate calcium homeostasis by modulating intestinal calcium transport.

Vitamin D deficiency and estrogen deficiency play key roles in the pathogenesis of postmenopausal osteoporosis. Fractures are the clinically most relevant endpoint of osteoporosis. The risk for fractures is determined by bone quantity and bone quality. Bone mineral density (BMD) is the best quantitative measure. Bone quality is determined by bone architecture, aspects of bone matrix and the rate of bone turnover. Recently, both bone quantity and quality have been shown to be genetically determined, but genes involved remain ill defined.

Aim of the studies in this thesis is to extend our knowledge on the interaction between E_2 and 1,25-$(OH)_2D_3$ and thereby to provide more insight into the significance of E_2 for 1,25-$(OH)_2D_3$ mediated processes in calcium and bone metabolism. Furthermore the significance of VDR genotypes for the biological response to 1,25-$(OH)_2D_3$ and the interaction between ERα and VDR genotypes in relation to BMD and fracture risk are studied.

In chapters 2 and 3 the interaction between E_2 and 1,25-$(OH)_2D_3$ in relation with intestinal calcium absorption, renal calcium excretion, bone turnover and BMD is described. This was studied in an *in vivo* rat model. Rats were divided into a sham-operated group, an ovariectomized (OVX) group, and one or two OVX groups with different dosages of E_2 suppletions. Bone turnover increased and BMD decreased following OVX. E_2 repletion following

OVX resulted in decreased bone turnover and increased BMD. Remarkably, serum 1,25-$(OH)_2D_3$ levels were very low in E_2 repleted rats and not significantly increased following OVX. Although serum 1,25-$(OH)_2D_3$ levels were very low, OVX-induced decreased intestinal calcium absorption was restored by E_2 repletion. There were no differences in renal calcium excretion between E_2 repleted rats and the sham-operated rats. Intestinal and renal VDR levels were similar in the different groups. These results suggest that E_2 regulates calcium homeostasis by direct stimulation of intestinal calcium absorption. In line with this ER has been demonstrated to be present in rat intestines. Furthermore, E_2 might facilitate 1,25-$(OH)_2D_3$ mediated intestinal calcium absorption. Our study suggests that this is not mediated by upregulation of VDR number. Possibly, E_2 increases the affinity of the VDR for its ligand.

Chapters 4 and 5 deal with the effect of E_2 on bone. This has been studied in an *in vivo* rat model and *in vitro* with human fetal osteoblast (SV-HFO) cells. IGF-I is known to increase collagen and bone matrix synthesis. IGFBP-5 promotes these actions. We showed by *in situ* hybridization that IGF-I is predominantly expressed in osteocytes and to a lesser extent in osteoblasts, while IGFBP-5 is expressed in osteoblasts. IGF-I mRNA expression in bone was depressed while IGFBP-5 mRNA expressed was not changed following OVX. E_2 repletion restored IGF-I mRNA expression to sham-level while IGFBP-5 mRNA expression was increased compared to sham-operated rats. These results indicate that the IGF-I system in bone is part of the E_2 control of bone turnover. Furthermore we found dissociation between serum IGF-I levels and IGF-I mRNA expression in bone cells. Therefore it is questionable to use serum IGF-I as a marker to asses the relation between IGF-I and bone turnover.

IGF-I synthesis in bone is also known to be regulated by 1,25-$(OH)_2D_3$. We did not show a correlation between serum 1,25-$(OH)_2D_3$ levels and IGF-I mRNA expression in bone. This might be due to the inverse relationship between serum 1,25-$(OH)_2D_3$ levels and E_2 levels resulting in opposing effects on IGF-I expression in bone. In view of the very low serum 1,25-$(OH)_2D_3$ levels in estrogen repleted rats, it is conceivable that the interplay between E_2 and the IGF-I system on bone is largely independent of 1,25-$(OH)_2D_3$ action.

In Chapter 5 we demonstrate that sera from women with a high BMD induce less *in vitro* mineralization than sera from women with low BMD. Women with high BMD had significantly higher serum E_2 levels than women with low BMD. Recently, we found that *in vitro* mineralization by SV-HFO cells is modulated by E_2. The degree of bone mineralization is related to elasticity of bone and its resistance to fracture. Therefore, it is tempting to speculate that in women with very low serum E_2 levels, newly formed bone may be overmineralized resulting in

more brittle bone. Our data suggest a new role for E_2 in bone metabolism, i.e. control of the extent or set point of mineralization, and thereby the elasticity of bone, which potentially contributes to the quality of bone and susceptibility to fracture.

In the second part of the thesis (Chapters 6, 7 and 8) genetic factors that may modulate the effect of 1,25-$(OH)_2D_3$ on calcium and bone homeostasis are discussed. Both VDR and ERα are important candidate genes in genetic studies on osteoporosis, since they play a major role in calcium homeostasis and bone metabolism. Several polymorphisms in both the VDR gene and ERα gene have been described and associated with BMD. However, data are not consistent. This may be ascribed to interaction between genetic factors and other factors, like environmental factors and other genes. Furthermore, the functional consequence of the various polymorphisms in the VDR gene and ERα gene are unknown. It is likely that genetic variability in the VDR gene and ERα gene may explain differences in responsiveness of target tissues to 1,25-$(OH)_2D_3$ and E_2, respectively.

In light of this we examined *in vivo* and *in vitro* whether there is a genotype dependent difference in biochemical response to 1,25-$(OH)_2D_3$ suppletion. In Chapter 6 we observed a higher rate of bone turnover in women within the lowest quintile of BMD compared to women within the highest quintile of BMD. The increase in bone turnover was associated with VDR gene polymorphism. However, this genotype-dependent difference in bone turnover could not be explained by a divergent response to administration of vitamin D.

In Chapter 7 the *in vitro* response of peripheral blood cells with different VDR genotypes on 1,25-$(OH)_2D_3$ administration are shown. We studied a VDR gene polymorphim that codes either for a 424 amino acids long VDR or a 427 amino acids long VDR. There was no difference in maximal inhibition by 1,25-$(OH)_2D_3$ between the two polymorphisms. The half maximal concentration for 1,25-$(OH)_2D_3$ induced growth inhibition was lower for the shorter VDR. This implies that the 424 amino acids long VDR exerts the effects of 1,25-$(OH)_2D_3$ more efficiently than the 427 amino acids long VDR.

In Chapter 8 we show that both ERα and VDR polymorphisms are associated with vertebral fracture risk in women. These associations are largely independent of BMD, which could be explained by ERα and VDR genotype dependent effects of E_2 and 1,25-$(OH)_2D_3$, respectively, on bone quality (Chapters 4 and 5). Furthermore there appears to be an interaction between ERα genotypes and VDR genotypes resulting in strongly increased vertebral fracture risk in women homozygous for both risk alleles.

In conclusion, these data demonstrate the importance of the estrogen endocrine system in the regulation of calcium and bone metabolism and its interaction with the vitamin D endocrine system.

Samenvatting

Calcium speelt een essentiële rol in een aantal intra- en extracellulaire processen. Derhalve is het van belang om het serum calcium binnen nauwe grenzen te houden. Om deze calcium homeostase te behouden is er een dynamisch evenwicht tussen extracellulair calcium en calcium dat opgenomen en uitgescheiden wordt via de darm, nier en bot. Deze calciumstromen worden strikt gereguleerd door de calciotrope hormonen, 1α,25-dihydroxyvitamine D_3 (1,25-$(OH)_2D_3$), parathyroid hormoon (PTH) en 17β-estradiol (E_2).

1,25-$(OH)_2D_3$ en E_2 zijn beide steroïdhormonen en die hun werking uitvoeren via hun receptoren, respectievelijk de vitamine D receptor (VDR) en oestrogeenreceptor (ER). 1,25-$(OH)_2D_3$ bewaart de calcium homeostase door de efficiëntie van calcium absorptie in de dunne darm te verhogen. Daarnaast heeft 1,25-$(OH)_2D_3$ een belangrijke werking op bot. Wanneer calcium opname onvoldoende is om het serum calcium binnen de normale grenzen te behouden, mobiliseert 1,25-$(OH)_2D_3$ calcium uit de calciumvoorraden in bot. Dit wordt bewerkstelligd door verhoging van de botafbraak door inductie van de osteoclastogenese (vorming van botafbrekende cellen). De osteoclastactiviteit wordt indirect door 1,25-$(OH)_2D_3$ gereguleerd door de actie van 1,25-$(OH)_2D_3$ op osteoblasten (botvormende cellen), die verschillende osteoclast-gevoelige cytokines produceren. Daarnaast stimuleert 1,25-$(OH)_2D_3$ de botmineralisatie.

Oestrogenen zijn belangrijk voor de rijping van het skelet tijdens de groeifase. Na de menopauze, wanneer de serum E_2 concentratie afneemt, treedt een toename van de botombouw op. Dit kan tegengegaan worden door oestrogeen toediening. Hieruit blijkt dat oestrogenen de botstofwisseling beïnvloeden. Oestrogenen worden met name geacht de botafbraak te remmen, grotendeels door een indirect remmend effect op de vorming en activatie, en stimulerend effect op apoptose (geprogrammeerde celdood) van osteoclasten. Daarnaast lijkt E_2 ook de calciumhomeostase te beïnvloeden door regulatie van de opname van calcium via de darm.

Zowel vitamine D deficiëntie als oestrogeen deficiëntie zijn in verband gebracht met het ontstaan van osteoporose (botontkalking). Dit ontstaat met name bij vrouwen na de menopauze. Botbreuken vormen het klinisch meest relevante eindpunt van osteoporose, daar osteoporotische botbreuken gepaard gaat met een belangrijke ziekte, sterfte en kosten voor de gezondheidszorg. Het risico van botbreuken wordt o.a. bepaald door de kwantiteit en kwaliteit van bot. De botmineraaldichteid (BMD) is de belangrijkste maat voor de kwantiteit van bot. De kwaliteit van bot wordt met name bepaald door de bot architectuur, botmatrix aspecten en de

botombouw. Uit recent onderzoek is gebleken dat zowel botkwantiteit als botkwaliteit voor een belangrijk deel genetisch (erfelijk) bepaald zijn.

Eerdere onderzoeken hebben aangetoond dat er een nauwe relatie bestaat tussen het vitamine D en oestrogeen endocriene systeem. E_2 stimuleert de vorming van 1,25-$(OH)_2D_3$ in de nier, terwijl 1,25-$(OH)_2D_3$ de vorming van E_2 stimuleert. Daarnaast zijn er aanwijzingen dat beide hormonen een wederzijds stimulerend effect op elkaars receptor expressie hebben.

Het doel van de studies in dit proefschrift is om meer kennis te verkrijgen omtrent de interactie tussen E_2 en 1,25-$(OH)_2D_3$ en daardoor meer inzicht te verschaffen in het belang van E_2 voor 1,25-$(OH)_2D_3$ gemedieerde processen in de calcium- en botstofwisseling. Tevens werden het belang van VDR genotypen voor de biologische response op 1,25-$(OH)_2D_3$ en de interactie tussen ERα en VDR genotypen in relatie tot BMD en risico op botbreuken bestudeerd.

In hoofdstukken 2 en 3 wordt de interactie tussen E_2 en 1,25-$(OH)_2D_3$ in relatie tot de calcium opname via de darm, calciumuitscheiding via de nier, botombouw en BMD beschreven. Dit is bestudeerd in een rattenmodel. De ratten werden verdeeld in een controlegroep, een geovariectomieerde groep (OVX; eierstokken verwijderd), en een of twee OVX groepen die E_2-suppletie kregen. OVX leidt tot een verhoogde botombouw en verlaging van de BMD, terwijl E_2 suppletie leidt tot verlaging van de botombouw en verhoging van de BMD ten opzichte van OVX. Opmerkelijk zijn de lage serum 1,25-$(OH)_2D_3$ spiegels in E_2-gesuppleerde ratten en licht verhoogde serum 1,25-$(OH)_2D_3$ spiegels in OVX ratten. Ondanks de sterk verlaagde serum 1,25-$(OH)_2D_3$ spiegels, kon de OVX-geïnduceerde verlaagde calcium opname in de darm hersteld worden door E_2 suppletie. Hierbij werd onder invloed van E_2 suppletie geen verschil in calciumuitscheiding via de nier ten opzichte van de controlegroep gevonden. Tevens werden geen verschillen in VDR aantal in darmen en nieren tussen de verschillende groepen gevonden. Deze bevindingen maken aannemelijk dat E_2 de calcium homeostase reguleert door directe stimulatie van de calcium opname in de darm. In lijn hiermee zijn ook ER in het duodenum van de rat gevonden. Daarnaast zou E_2 ook de 1,25-$(OH)_2D_3$ gemedieerde calcium opname in de darm kunnen moduleren, maar dit lijkt niet te verlopen door stimulatie van het VDR aantal. Mogelijk verandert E_2 de affiniteit van de VDR voor 1,25-$(OH)_2D_3$. Hierdoor zou onder invloed van E_2 de 1,25-$(OH)_2D_3$ gemediëerde calcium opname in de darm efficiënter kunnen verlopen.

De hoofdstukken 4 en 5 zijn gewijd aan het effect van E_2 op bot. Dit is allereerst onderzocht in een *in vivo* rattenmodel. IGF-I geeft een verhoogde collageensynthese en botmatrixsynthese. IGFBP-5 versterkt deze effecten van IGF-I op bot. Uit *in situ* hybridisaties blijkt dat IGF-I met name in osteocyten en in mindere mate in osteoblasten tot expressie komt, terwijl IGFBP-5 met name in osteoblasten tot expressie komt. E_2 suppletie brengt de OVX geïnduceerde verlaagde

IGF-I mRNA expressie op controle niveau, en geeft een verhoging van de IGFBP-5 mRNA expressie ten opzichte van de controle groep. Deze bevindingen duiden er op dat E_2 de botturnover mede reguleert door interactie met het IGF-I systeem.

Het is bekend dat ook 1,25-$(OH)_2D_3$ IGF-I expressie in bot stimuleert. In deze studie werd geen correlatie gevonden tussen de serum 1,25-$(OH)_2D_3$ concentraties en IGF-I mRNA expressie in bot. Dit kan toegeschreven worden aan tegengestelde effecten van 1,25-$(OH)_2D_3$ en E_2 op IGF-I mRNA expressie in bot. De verlaagde 1,25-$(OH)_2D_3$ spiegels zouden gepaard kunnen gaan met verlaagde IGF-I mRNA expressie, doch dit effect kan volledig tenietgedaan zijn door de aan E_2 gerelateerde verhoogde IGF-I mRNA expressie in bot. Gezien de zeer lage serum 1,25-$(OH)_2D_3$ spiegels in oestrogeen gerepleerde ratten, is het aannemelijk dat de interactie tussen E_2 en het IGF-I systeem grotendeels onafhankelijk van de werking van 1,25-$(OH)_2D_3$ verloopt.

In hoofdstuk 5 tonen we middels een *in vitro* bioassay aan dat sera van vrouwen met een hoge BMD minder mineralisatie induceren dan vrouwen met een lage BMD. Vrouwen met een hoge BMD hadden significant hogere serum E_2 spiegels dan vrouwen met een lage BMD. Eerder onderzoek heeft aangetoond dat E_2 *in vitro* mineralisatie door SV-HFO cellen moduleert. Daarnaast is bekend dat de mate van botmineralisatie gerelateerd is aan de elasticiteit van bot en de kans op fractuur. Deze data suggeren een nieuwe rol voor E_2 in de bot homeostase, namelijk regulatie van het setpoint van de mineralisatie. Op deze wijze zou E_2 de elasticiteit van bot kunnen moduleren, wat bijdraagt aan de kwaliteit van bot kunnen beïnvloeden, en daarmee de kans op fracturen kunnen beïnvloeden.

In het tweede deel van het proefschrift (hoofdstukken 6, 7 en 8) wordt aandacht besteed aan genetische factoren die het effect van 1,25-$(OH)_2D_3$ op de calcium- en botstofwisseling kunnen bepalen. Zowel de VDR als ERα zijn belangrijke kandidaat genen in studies naar osteoporose daar zij een belangrijke rol spelen in het calcium- en botmetabolisme en in de bepaling van de kwantiteit en kwaliteit van bot. Verschillende polymorphismen (variaties die in meer dan 1% van de bevolking voorkomen) in zowel de VDR als ERα zijn beschreven. In verschillende studies zijn zowel VDR als ERα polymorphismen geassocieerd met BMD. De data zijn echter niet consistent, wat onder meer verklaard kan worden door interactie van genetische factoren met andere factoren zoals omgevingsfactoren en andere genen. Daarnaast is de functionele betekenis van de polymorphismen van het VDR en ERα gen nog onbekend. Het is echter zeer waarschijnlijk dat de genetische variabiliteit van het VDR en ERα gen het verschil in response van doelorganen op respectievelijk 1,25-$(OH)_2D_3$ en E_2 beïnvloedt.

Hiertoe onderzochten wij zowel *in vivo* als *in vitro* of er VDR genotype afhankelijke verschillen in biochemische response op vitamine D toediening bestaan. In hoofdstuk 6 zagen wij dat

vrouwen met een lage BMD een hogere botombouw hebben dan vrouwen met een hoge BMD. Deze toegenomen botombouw blijkt geassocieerd te zijn met VDR gen polymorphismen. Deze bevinding kon echter niet verklaard worden door een genotype afhankelijk verschil in response op kortdurende vitamine D toediening.

In hoofdstuk 7 worden de resultaten getoond van een *in vitro* studie, waarin de response van perifere bloedcellen met een verschillend VDR genotype op 1,25-$(OH)_2D_3$ toediening werd bestudeerd. We hebben een VDR gen polymorphisme onderzocht dat leidt tot een 424 aminozuur-lange VDR of een 427 aminozuur-lange VDR. Voor beide polymorphismen was de maximale remming door 1,25-$(OH)_2D_3$ gelijk. De concentratie 1,25-$(OH)_2D_3$ waarop half-maximale remming van de groei optreedt was echter voor de 424 aminozuur lange VDR lager dan voor de 427 lange VDR. Dit betekent dat de korte VDR efficiënter het effect van 1,25-$(OH)_2D_3$ uitoefent dan de lange VDR.

In hoofdstuk 8 wordt aangetoond dat zowel ERα als VDR polymorphismen geassocieerd zijn met fractuurrisico bij vrouwen. Dit risico is grotendeels onafhankelijk van de BMD. Dit kan verklaard worden uit de eerder beschreven effecten van E_2 en 1,25-$(OH)_2D_3$ op de botkwaliteit. Daarnaast blijkt er een interactie te bestaan tussen ERα genotype en VDR genotypen resulterend in sterk verhoogd risico op het ontstaan van wervelfracturen.

Concluderend tonen deze data aan dat E_2 een belangrijke effect heeft op de regulatie van calcium homeostase en botmetabolisme en er een samenspel is met het vitamine D endocriene systeem in de regulatie hiervan.

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Curriculum vitae

Edgar Colin werd op 26 januari 1968 te Amsterdam geboren. Na het behalen van het Gymnasium B diploma aan het Pius X Lyceum te Amsterdam, werd in 1986 aangevangen met de studie geneeskunde aan de Universiteit van Amsterdam. In 1993 werd het artsexamen afgelegd. Aansluitend was hij als AGIKO werkzaam op de afdeling inwendige geneeskunde van het Erasmus MC te Rotterdam. De basis van het proefschrift werd gelegd op het Calcium- en Botstofwisselings Onderzoeks-laboratorium onder leiding van Dr. J.P.T.M. van Leeuwen, Prof.dr. H.A.P. Pols en Prof.dr. J.C. Birkenhäger†. In november 1997 werd aangevangen met de opleiding tot internist in het Reinier de Graaf Gasthuis te Delft (opleider E. Maartense). Vanaf 1 januari 2000 is hij werkzaam als internist i.o. op de afdeling inwendige geneeskunde van het Erasmus MC te Rotterdam (opleider Prof.dr. H.A.P. Pols).