# MODULATION OF THE INTESTINAL VITAMIN D RECEPTOR AND CALCIUM ATPase ACTIVITY BY ESSENTIAL FATTY ACID SUPPLEMENTATION

BY

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***Philippians 4:13***

*I can do all things through Christ, which strengtheneth me.*

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# ABSTRACT

Essential fatty acid (EFA) deficient animals develop severe osteoporosis. Studies in humans showed that ratio supplementation of EPA (eicosapentaenoic acid) and GLA (gamma-linolenic acid) plus calcium ($Ca^{2+}$) in elderly osteoporotic patients causes increases in bone density. Similarly ratio supplementation in male and female rats caused increases in bone calcium and bone density. The physiological mechanisms by which the EFAs exert these effects on $Ca^{2+}$ retention have not been clarified.

The hormonal form of vitamin D, $1,25(OH)_2D_3$ is by far the most significant factor controlling intestinal calcium transport in all three steps of transcellular transport. The vitamin D receptor (VDR) is essential for the functioning of the hormone, thus the regulation of the intracellular VDR concentration and binding affinity are important mechanisms by which the response of the target tissue can be modulated. It is proposed that steroid hormone receptors may have a binding site for endogenous modulators such as fatty acids, which could change the conformation of the receptor. The activity of the $Ca^{2+}$ ATPase in the basolateral membrane correlates with the degree of $Ca^{2+}$ absorption. Membrane fluidity has a direct influence on the conformation of the active sites of membrane-associated enzymes. Changes in the fluidity of membranes after supplementation with EFAs have been reported. Therefore changes in ATPase activity as well as VDR availability due to EFAs may influence calcium retention.

The loss of oestrogen has profound effects on $Ca^{2+}$ metabolism. Previous studies with EFA supplementation of female Sprague Dawley rats after OVX, showed an increase in bone $Ca^{2+}$ and bone density. The objective of this study was to investigate the effect of long-term nutritional supplementation with

EFAs on $Ca^{2+}$ absorption, VDR binding and bone status after the loss of oestrogen induced by OVX, to verify the possible mechanism by which EFAs could exert their effect.

The main findings of this study were that long-term supplementation of EFAs has a prophylactic effect on bone loss as induced by OVX. Short-term supplementation previously reversed OVX induced bone loss to a certain extent, but the longer term feeding had a more pronounced effect as measured in bone $Ca^{2+}$ and density. Low ATPase activity was measured due to OVX and could result in low $Ca^{2+}$ absorption which in turn may be responsible for an increase in PTH levels observed with OVX. An increase in PTH may be accompanied by an increase in $1,25(OH)_2D_3$ levels which upregulates it's own receptor levels. VDR number was upregulated due to OVX possibly trying to compensate for the lowering in $Ca^{2+}$ absorption, or induced by PTH. EFAs increased the ATPase activity back to sham levels, while the VDR number decreased back to sham levels. EFAs reduced affinity of the VDR for vitamin D, possibly due to non-competitive inhibition.

EFAs are considered a nutritional supplement and not a drug regime for the treatment of osteoporosis. These above-mentioned results could be of importance in designing a food supplement containing $Ca^{2+}$ and EFAs for the prevention of osteopenia or maintenance of peak bone mass.

*Keywords:* Essential fatty acid, $1,25(OH)_2D_3$, calcium, vitamin D receptor, parathyroid hormone, $Ca^{2+}$ ATPase activity, OVX, bone $Ca^{2+}$, eicosapentaenoic acid, membrane fluidity, osteoporosis, gamma-linolenic acid.

# OPSOMMING

Diere met 'n tekort aan essensiële vetsure (EV), ontwikkel erge osteoporose. Kliniese studies het getoon dat die supplementering van eikosapentanoësuur (EPA) en gamma-linoleensuur (GLA) in 'n spesifieke verhouding, 'n verhoging in beendigtheid in bejaarde osteoporotiese pasiënte tot gevolg het. 'n Soortgelyke verhoging in beenkalsium en beendigtheid waardes het ook plaasgevind met die supplementering van mannetjie- en wyfierotte. Die spesifieke fisiologiese meganisme waarmee EV kalsiumretensie veroorsaak, is egter nog onduidelik.

Die hormonale vorm van vitamien D nl. $1,25(OH)_2D_3$ is by verre die mees betekenisvolle faktor in die beheer van al drie die transsellulêre stappe van intestinale $Ca^{2+}$ transport. Die vitamien D reseptor (VDR) is noodsaaklik vir die funksionering van die hormoon, daarom is die regulering van die intrasellulêre konsentrasie asook die bindingaffiniteit baie belangrike meganismes waarmee die reaksie van die teikenorgaan gereguleer word. Daar is voorgestel dat steroïedhormoonreseptore, bindingsplekke vir endogene moduleerders soos bv. vetsure, besit wat die konformasie van die reseptor kan verander. Die aktiwiteit van die $Ca^{2+}$ ATPase in die basolaterale membraan, korreleer goed met die mate van kalsiumabsorpsie. Die vloeibaarheid van membrane het 'n direkte invloed op die vorm van die aktiewe eenhede van membraan-geassosieërde ensieme. Die veranderinge in ATPase aktiwiteit sowel as die vitamien D reseptor beskikbaarheid, wat teweeg gebring word deur EV, mag kalsiumretensie beïnvloed.

'n Verlies aan estrogeen het 'n groot effek op kalsiummetabolisme. In vorige studies waar vetsure aan wyfie Sprague Dawley rotte gesupplementeer is, nadat hulle geovariektomiseer is, het 'n toename in beenkalsium en beendigtheid

getoon. Die doel van die studie was om die effek van essensiële vetsuur supplementering na OVX, oor 'n langer termyn op kalsiumabsorpsie, vitamien D reseptor binding en beenkalsium te ondersoek, om moontlike meganismes waarmee essensiële vetsure hulle effek veroorsaak, te vind.

Die hoofbevindinge van die studie was dat die langtermyn supplementering van essensiële vetsure 'n profilaktiese effek op die OVX geïnduseerde beenverlies gehad het. Korttermyn supplementering kon met vroeër studies die verlies van been, in 'n mate omkeer, maar die langer termyn het 'n groter effek gehad, gesien uit die gemete waardes van beenkalsium en beendigtheid. Lae ATPase aktiwiteit is gemeet as gevolg van die OVX, wat lae kalsiumabsorpsie tot gevolg mag hê, en wat verantwoordelik kan wees vir die waargeneemde verhoging in paratiroïedhormoonvlakke (PTH). Verhoogde PTH vlakke mag gepaard gaan met 'n verhoging in die $1,25(OH)_2D_3$ vlakke, wat verantwoordelik is vir die opregulering van sy eie reseptor. Die aantal reseptors is opgereguleer deur die OVX moontlik om te kompenseer vir die verlaagde kalsiumabsorpsie, of dalk deur PTH geïnduseer. Essensiële vetsure het die ATPase aktiwiteit verhoog na dieselfde vlak as die van die sham-geopereerde groep, so ook het die affiniteit vir vitamien D afgeneem in die VDR, nie-kompeterende inhibisie mag 'n moontlike rede vir die verlaging in affiniteit wees.

EV word beskou as 'n voedingssupplement eerder as medikasie vir die behandeling van osteoporose. Die bogenoemde resultate mag van belang wees in die ontwikkeling van 'n voedingssupplement wat essensiële vetsure en kalsium bevat, om osteopenie te voorkom of selfs vir die handhawing van piek beenmassa.

*Sleutelwoorde*: Essensiële vetsure, $1,25(OH)_2D_3$, kalsium, vitamien D reseptor, paratiroïed hormoon, $Ca^{2+}$ ATPase aktiwiteit, OVX, beenkalsium, eikosapentanoësuur, membraanvloeibaarheid, osteoporose, gamma-linoleensuur.

# ABBREVIATIONS

| | |
|---|---|
| $1,25(OH)_2D_3$ | 1,25-dihydroxyvitamin $D_3$ |
| $24,25(OH)_2D_3$ | 24,25-dihydroxyvitamin $D_3$ |
| $25(OH)D_3$ | 25-hydroxyvitamin $D_3$ |
| AA | Arachidonic acid |
| ALA | Alpha-linolenic acid |
| ATP | Adenosine 5'-triphosphate |
| BBM | Brush border membrane |
| BGP | Bone GLA-containing protein |
| BLM | Basolateral membrane |
| $Ca^{2+}$ | Calcium |
| CaBP | Calcium-binding protein |
| Calbindin-D9k | 9kD-calbindin protein |
| cAMP | Cyclic adenosine 5'-monophosphate |
| CBC | Calcium-binding complex |
| cDNA | Complementary deoxyribonucleic acid |
| D6D | Delta-6-desaturase |
| DBD | DNA-binding domain |
| DBP | Vitamin D-binding protein |
| DGLA | Dihomogamma-linolenic acid |
| DHA | Docosahexaenoic acid |
| DNA | Deoxyribonucleic acid |
| EFA | Essential fatty acid |
| EGTA | Ethylene glycol-tetraacetic acid |
| ELISA | Enzyme-linked immunoassay |
| EPA | Eicosapentaenoic acid |
| FA | Fatty acid |
| FFA | Free fatty acid |

| | |
|---|---|
| GLA | Gamma-linolenic acid |
| HAP | Hydroxylapatite |
| IGF-I | Insulin-like growth factor I |
| IL-2 | Interleukin 2 |
| LA | Linoleic acid |
| LBD | Ligand-binding domain |
| LDL | Low-density lipoprotein |
| mAB | Monoclonal antibody |
| MMP | Metalloproteinase |
| mRNA | Messenger ribonucleic acid |
| NADPH | Nicotinamide adenine dinucleotide phosphate (reduced) |
| NEFA | Non-esterified fatty acid |
| PG | Prostaglandin |
| PMCA | Plasma membrane calcium ATPase |
| PTG | Parathyroid gland |
| PTH | Parathyroid hormone |
| PUFA | Polyunsaturated fatty acid |
| UV | Ultraviolet |
| VDR | Vitamin $D_3$ receptor |
| VDRE | Vitamin D responsive element |

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# CHAPTER 1

# STUDY OBJECTIVES

## 1.1 Motivation for the study

The hormonal form of vitamin D, $1,25(OH)_2D_3$ is by far the most significant factor controlling calcium transport in all three steps of transcellular transport e.g. entry, transfer, and extrusion. [1] One possible mechanism of action is the non-genomic alteration in fatty acid composition of both the brush border membrane (BBM) and the basolateral membrane (BLM). [2]

Studies show that membrane fluidity has a direct influence on the conformation of the active sites of some membrane-associated enzymes. The significant activation of the $Na^+$-$K^+$ ATPase activity of the membranes isolated from fish oil (eicosapentaenoic acid, EPA) supplemented rats found by Coetzer et al. [2] may be extrapolated to the $Ca^{2+}$ ATPase, as both enzymes are located in the BLM. Changes in the fluidity of membranes after supplementation with essential fatty acids (EFAs) have been reported, and measured as the unsaturation index. [2]

The vitamin D receptor (VDR) is essential for the functioning of the hormone. The fact that the receptor was originally discovered in the major vitamin D target organ namely the intestinal mucosa, and was subsequently revealed in other sites of mineral translocation or regulation i.e. parathyroid gland, bone and kidney adds credence to its proposed role in vitamin D action. The $1,25(OH)_2D_3$ receptor system seems to fit perfectly with the prevailing steroid hormone dogma of a two step process of hormone binding in the cytoplasm and subsequent localisation in the nucleus. [3] The synthesis of a vitamin D binding protein in the nucleus that follows localisation of the receptor-vitamin D complex, is also an important factor in calcium transport. The regulation of the intracellular VDR concentration is thus an important mechanism by which the response of the target tissue can be modulated.

It is of interest to understand the mechanism and the regulation of $Ca^{2+}$absorption, as it is important in whole body homeostasis. The concentration of calcium in the blood is maintained between 2.2 and 2.5 mM. [4] Bone tissue plays an important role in $Ca^{2+}$ homeostasis, for 99% of the body's calcium is stored in bone and teeth.

When $Ca^{2+}$ is needed by an organism, parathyroid hormone (PTH) secretion is stimulated which in turn activates the production of $1,25(OH)_2D_3$ which causes calcium to be absorbed by the intestine. If, for any reason the calcium cannot be absorbed and the level of $Ca^{2+}$ in the blood continues to be low, secretion of

high levels of PTH together with elevated levels of $1,25(OH)_2D_3$ will cause the mobilisation of calcium from the bone. [5]

It is proposed that steroid hormone receptors may have a binding site for endogenous modulators such as fatty acids in the receptor fragment containing the hormone-binding domain and certain C-terminal sequences of the DNA-binding domain. [6] Long chain fatty acids bind non-competitively to the receptor and it changes the conformation of the receptor inhibiting the binding of its steroid. [7] An increase in the level of fatty acid (FA) in the liver cytosol has been reported to be accompanied by a decrease in the binding capacity of liver glucocorticoid receptors for glucocorticoids, because of a decrease in the binding constants and the total number of binding sites. [6] All previous studies on the effect of EFAs on steroid receptor binding have been done *in vitro* with isolated cells. No study has ever been done where EFAs were supplemented to animals and membranes and receptors then purified and tested for vitamin $D_3$ binding and affinity.

The loss of oestrogen due to an ovariectomy has profound effects on $Ca^{2+}$ metabolism. Ovariectomy causes significant bone loss which has been previously shown to be reversed by short-term (6 weeks) supplementation with the active metabolites of the n-6 and n-3 families of EFAs e.g. gamma-linolenic acid (GLA) and eicosapentaenoic acid (EPA). [8] Oestrogen has also been shown to increase the number of $1,25(OH)_2D_3$ receptors in the rat uterus. [9] Therefore

in this study, the loss in oestrogen was induced by OVX and the effect thereof tested on the number of receptors in the intestine as well as receptor binding. Long term effects of dietary supplementation with EFAs on $Ca^{2+}$ absorption, receptor binding and bone status were also investigated.

## 1.2 Objectives

### 1.2.1 Overall aim:

The broad aim of the study was:

- to investigate the effect of long-term fatty acid supplementation before and after ovariectomy on the vitamin $D_3$ receptor availability, binding and bone status in the female rat.

### 1.2.2. Specific aims:

The following specific aims were formulated:

- measurement of intestinal $Ca^{2+}$-ATPase activity in order to assess calcium absorption during fatty acid supplementation
- to measure intestinal vitamin $D_3$ receptor availability and binding as influenced by OVX and FA.
- to assess calcium status of the rats by measuring PTH levels as well as bone calcium content and density.

## 1.3 Importance of the study

Previous studies done on the supplementation of female rats after OVX show an increase in bone $Ca^{2+}$ plus a decrease in urinary deoxypyridinoline excretion. Supplementation of EPA and GLA as a diester in conjunction with oestrogen replacement therapy were shown to enhance the positive effect of oestrogen on bone, but EFAs also had a significant effect on their own. [8]

Supplementation of EFAs in specific ratios especially EPA and DHA (docosahexaenoic acid) together with calcium, has been proven previously by members of our laboratory to be of great importance in increasing bone density in elderly osteoporotic patients.

The physiological mechanisms by which the EFAs exert these effects have not been clarified. EFAs are also considered a nutritional supplement and not a drug regime for the treatment of osteoporosis. We investigated the effect of long-term nutritional supplementation with EFAs on bone, as well as possible mechanisms of action. The results obtained may be of importance in future designing of food supplements containing calcium and EFAs for the prevention of osteopenia or maintenance of peak bone mass.

# CHAPTER 2

# INTRODUCTION

## 1. Vitamin D

### 1.1 History

Since the earliest days of the 19th century, the importance of sunlight in the sturdiness of the skeletal structure has been suggested. [10] Glisson in 1650 or Whistler in 1645 was the first to recognise rickets as the first, true bony disease, but it wasn't until the 1900's when this disease appeared in epidemic proportions in Northern Europe, North America and Northern Asia, [10] that it was given a scientific basis. At that time the concept of a vitamin was a new area of investigation.

Some of the basic work leading to the discovery of the vitamin must be attributed to Magendie and Hopkins, who reasoned that by knowing the chemical composition of foodstuffs would make it possible to support life. It was Funck [11] who introduced the idea of "vital amines" that is necessary to support life. In addition to this, Sir Edward Mellanby [12] demonstrated rickets to be at least in part a nutritional disorder that could be healed by cod liver oil.

The discovery of vitamin A,B and C between 1911-1917 undoubtedly inspired Mellanby, and he concluded that fat-soluble vitamin A prevented rickets because McCollum [13] had found fat-soluble vitamin A in cod liver oil. However, McCollum recognised that the properties of the anti-rachitic substance discovered by Mellanby, must be different from those of the growth-promoting fat-soluble vitamin A.

By destroying vitamin A in cod liver oil, while retaining the ability to prevent or cure rickets, a new fat-soluble vitamin was discovered, which he then called vitamin D. [14]

However, nutrition alone failed to explain the occurrence of rickets only in industrialised towns. Children who had poor nutrition and lived in the countryside or in underdeveloped countries of the world did not develop the dreaded disease. These findings led to the hypothesis that the cause of rickets in children was the lack of exposure to sunlight. Hess, [10] proved the curative effects of UV radiation, with a demonstration that exposure to sunlight, or to a mercury vapour arc lamp, could cure rickets in children.

In 1922-1923, the field was confused because both cod liver oil and sunlight prevented rickets. Steenbock provided the first established basis of ultraviolet activation of provitamin D. He clearly showed that ultraviolet irradiation not only of animal skins but also of their food showed an antirachitic activity. [15,16]

Windaus and his colleagues were the first to isolate a crystalline substance that they termed vitamin $D_1$ (lumisterol), [17] an artifactual addition of vitamin $D_2$ (ergocalciferol), which was discovered in 1932. [18] For many years vitamin $D_2$ was the major synthetic form of vitamin D used for the prevention and cure of rickets in man. Steenbock and his colleagues noted that birds didn't react well to solutions of irradiated ergosterol, when compared to either cod liver oil or solutions of irradiated cholesterol. [19]

This observation led to the postulation of another vitamin D that could be produced by the irradiation of impure cholesterol solutions. [20] The Windaus group chemically synthesised 7-dehydrocholesterol, [21] and upon irradiation, produced the other major form of vitamin D, namely, vitamin $D_3$. [22]

Thus in 1937 the isolation and identification of the vitamin D nutritional compounds had been completed, ending this important era of vitamin D investigation. The idea however that vitamin D was active without metabolic alteration, [23] was later to be reversed with the introduction of modern biochemical techniques, which have now clearly demonstrated that vitamin D must be metabolically altered and in fact converted to a hormone, to function in calcium and phosphorus metabolism. [24]

## 1.2 Formation of cholecalciferol in the skin

Vitamin D was the first vitamin to be identified as a precursor of a steroid hormone. [25] The first evidence of this, was the demonstration that vitamin

$D_3$ (cholecalciferol) is normally produced in the skin from provitamin $D_3$ (7-dehydrocholesterol) by ultraviolet light originating from the sun. [26] Vitamin D is not an essential dietary factor either, but a prohormone, [27] that needs to be modified by hydroxylation reactions before being able to function as an active metabolite e.g. $1,25(OH)_2D_3$. [28]

Vitamin D together with its metabolites is seco-steroids, with molecular structures closely related to that of classic steroid hormones (e.g. estradiol, cortisol, aldosterone) which has a root cyclopentanoperhydrophenanthrene structure. Seco-steroids are those steroids in which one of the rings of the cyclopentanoperhydrophenanthrene structure has undergone breakage of a carbon-carbon bond. [28] In vitamin D, the 9-10 carbon bond of ring B is broken (figure 1).

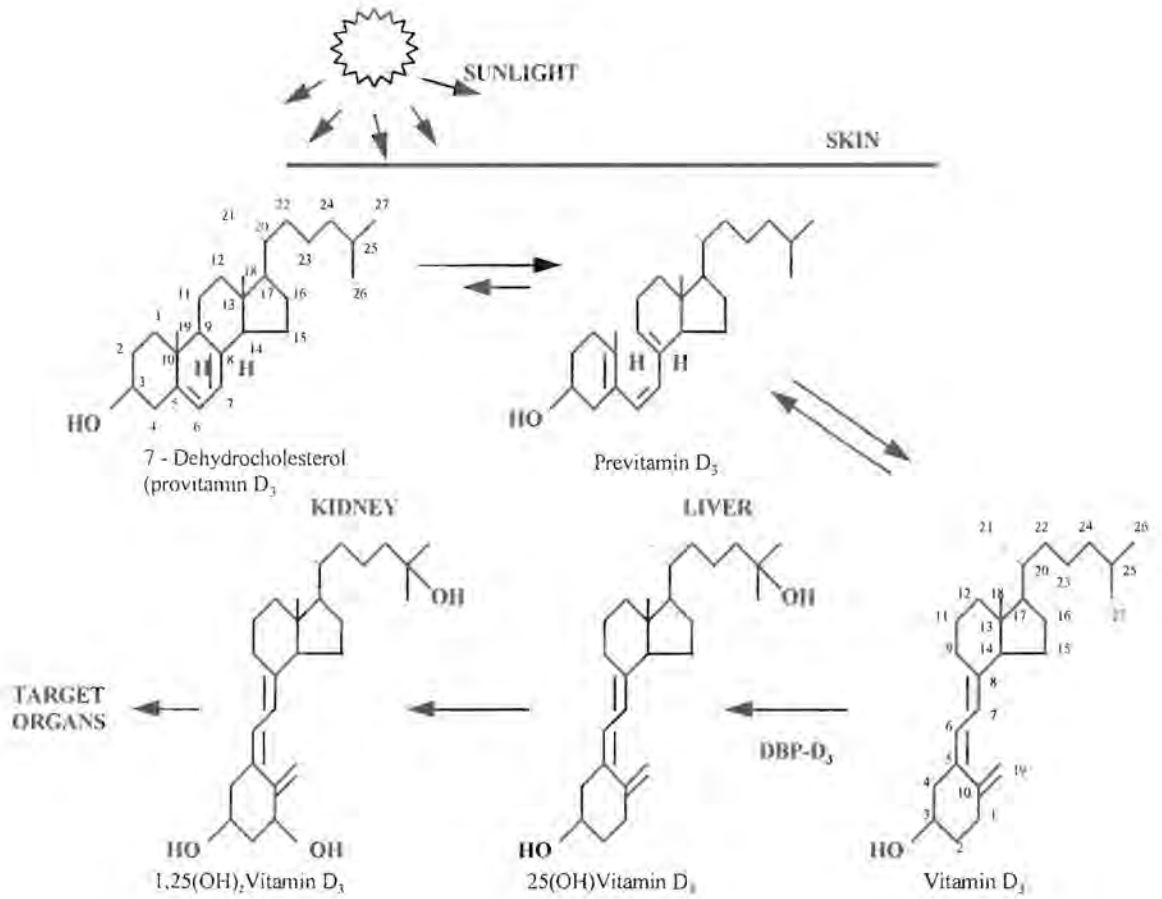


**Figure 1:** The photochemical production of Vitamin $D_3$ and the metabolising to its major biologically active metabolites. DBP: Vitamin D-binding protein
(Adapted from 27)

The skin consists of two primary layers: the inner dermis, composed largely of connective tissue, and the outer epidermis, which is much thinner than the dermis. The epidermis contains five strata: strata corneum, lucidum, granulosum, spinosum and basale, from outer to inner layer respectively. [27] Although half of the provitamin $D_3$ (7-dehydrocholesterol) content in human skin is found in the dermis and the other half in the epidermis, greater than 90% of the previtamin $D_3$ synthesis still occurs in the epidermis. [29]

The highest concentrations of 7-dehydrocholesterol in the epidermis are found in the stratum basale and spinosum.

When human skin is exposed to sunlight, the high-energy ultraviolet photons with energies between 290-315 nm, penetrate into the epidermis, reaching the provitamin $D_3$ deep in the stratum basale and spinosum, photolysing it into previtamin $D_3$. [30,31] The two principle determinants, are the quantity (intensity) and quality (appropriate wavelength) of the ultraviolet irradiation. Provitamin $D_3$ absorbs UV light most efficiently over the wavelengths of 270-290 nm.

Thermal isomerisation of the previtamin results in the formation of vitamin $D_3$. [32] Because of the seco nature of vitamin D, the A ring is inverted, with rotation occurring around the bond between C-7 and C-8 (figure 1). [3] Previtamin $D_3$ is a thermally labile compound, while vitamin $D_3$ is a more stable isomer. [29] Because of its thermal lability, it is susceptible to photodegradation by sunlight. The major photoproducts that result from this photodegradation reaction, are biologically inert isomers, lumisterol and tachysterol. [33] Time thus, play an inhibitory role in the production of vitamin $D_3$. The process of photolysis takes about three days to complete, which leaves enough time for degradation. It is for this reason suggested that vitamin D binding protein, a 52 kD to 58 kD protein, transports vitamin $D_3$ out of the skin into the systemic circulation, and this maintains relatively low concentrations of vitamin $D_3$ within the skin. [32] This allows the thermal isomerisation of previtamin $D_3$ to vitamin $D_3$ to continue at a more rapid

rate by decreasing the concentrations of vitamin $D_3$ within the skin, partially eliminating the time factor.

## 1.3 Metabolism of vitamin D

### 1.3.1 25 hydroxylation of vitamin $D_3$ in the liver

Vitamin $D_3$ accumulates in the liver, [34] where it undergoes its first obligatory reaction, namely 25-hydroxylation. 25-hydroxylation is the first metabolic reaction required for all subsequent metabolisms of vitamin $D_3$. [35] The enzyme that catalyses this reaction is present both in liver microsomes as well as mitochondria. [36] The microsomal system has been solubilised and its components resolved into two enzymes, including a flavoprotein, presumably an NADPH-dependent cytochrome P450 reductase, and a cytochrome P450.[37]

The mitochondrial system has been solubilised and shown to be a three-component mixed function mono-oxygenase involving an iron sulphur protein, a flavoprotein and a cytochrome P450. The mitochondrial system, however, is not specific for vitamin D, since it carries out other cholesterol hydroxylation reactions. [38] With a lower Michaelis constant (Km) the microsomal enzyme can be better regulated than the mitochondrial enzyme with the higher Km. The mitochondrial enzymes being poorly regulated, will form significant quantities of $25(OH)D_3$ at higher concentrations of vitamin $D_3$. It is for this reason that the circulating level of $25(OH)D_3$ is a

good index of the vitamin $D_3$ reserves of the organism. [32] The $25(OH)D_3$ rapidly leaves the liver bound to the plasma transport protein.

### 1.3.2 1α Hydroxylase in the kidney

The kidney was identified as the site of $1,25(OH)_2D_3$ synthesis –and a site of $24,25(OH)_2D_3$ synthesis over two decades ago. Ghazarian [38] showed that a solubilised kidney mitochondrial preparation contained both cytochrome P450 and 1α hydroxylase activity. It seemed that 1-hydroxylase (and probably the 24-hydroxylase as well) is similar to classical mitochondrial– as in the liver- mixed function oxidases which hydroxylate endogenous steroids (figure 2). [39]

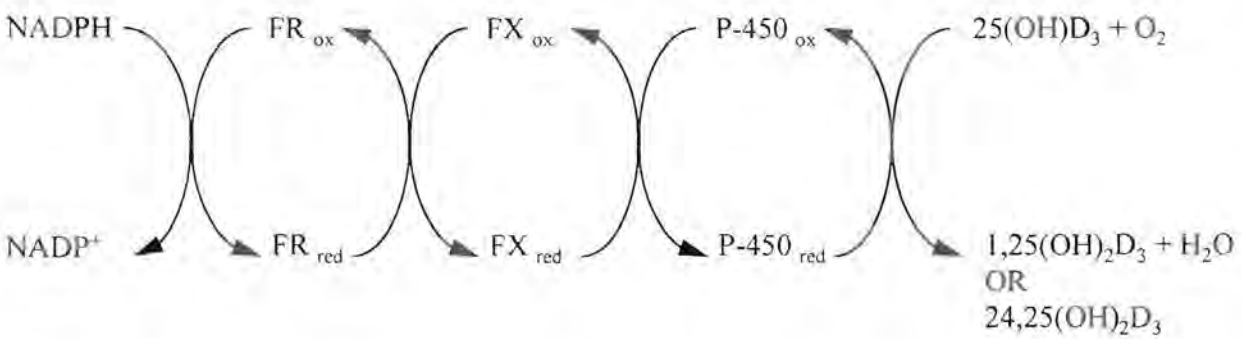


**Figure 2:** General scheme of mitochondrial mixed function oxidases [39]

$1,25(OH)_2D_3$ is formed in the mitochondria of the proximal tubules of the nephron. [32] The mixed function oxidase is dependent on NADPH and the electron carrying proteins, ferrodoxin reductase and ferrodoxin, whereas cytochrome P450 (Mw 55 000-60 000) is an integral protein of the inner mitochondrial membrane, ferrodoxin (Mw 12500-14 000) is a matrix

protein. [40] Although it has occasionally been speculated that 1- and 24 hydroxylase activity might exist in the same cytochrome P450, with its hydroxylation activity switched between the two positions by allosteric changes in the protein, the physical separation of the two activities argues against the possibility. [39]

## 1.4 Regulation of vitamin D metabolism

The 25-hydroxylase activity of the liver does not appear to be as strictly regulated as the 1α hydroxylase of the kidney. Thus the activity of the latter enzyme is strictly regulated by the $1,25(OH)_2D_3$ status of the cell. [3] In the vitamin D-deficient state, the production of $1,25(OH)_2D_3$ are maximal and $24,25(OH)_2D_3$ production is minimal/undetectable. The situation is reversed in the presence of $1,25(OH)_2D_3$. Very soon after the addition of calcitriol to cultures of chick kidney cells in serum-free medium, 1α hydroxylase activity begins to decline and shortly thereafter 24-hydroxylase activity increases. [41] The effect of $1,25(OH)_2D_3$ on both enzymes is inhibited by cycloheximide and actinomycin D, suggesting genomic regulation.

Other hormonal factors, such as parathyroid hormone, calcitonin, oestrogen and the pituitary hormones as well as plasma levels of calcium and phosphorus, also regulate the production of $1,25(OH)_2D_3$. [42] Calcitriol plays a crucial role in the maintenance of blood calcium and phosphorus levels and in normal skeletal mineralisation. [43] Low calcium diets and hypocalcemia in intact animals result in a marked elevation of the

25(OH)D-1α hydroxylase. Constant oral calcium loading in humans results in decreased plasma calcitriol concentration, and chronic calcium restriction results in increased plasma calcitriol concentration. [44]

This has been interpreted as suggesting an effect of secondarily decreased/increased parathyroid hormone (PTH) levels rather than direct effects of small but undetectable changes in plasma calcium concentration. However Trechsel et al. [45] showed that chronic calcium restriction results in increased plasma calcitriol concentration even in thyroparathyroidectomised rats, thus hypocalcemia acts directly to stimulate 1α hydroxylase activity. As the level of $1,25(OH)_2D_3$ decreases with an increase in calcium intake, the level of $25(OH)D_3$ increases. This latter effect may be secondary to a decrease in the concentration of $1,25(OH)_2D_3$ or due to the increased flux of calcium per se. [46] Calcitriol may inhibit 25- hydroxylase in the liver or it may accelerate the metabolism of $25(OH)D_3$.

Low blood calcium can also stimulate the parathyroid glands to secrete the parathyroid hormone, which in turn increases production of the vitamin D hormone in the proximal convoluted tubule cells of the kidney. [28] PTH is required for the mobilisation of calcium from bone, and is required for renal conservation of calcium (figure 3).

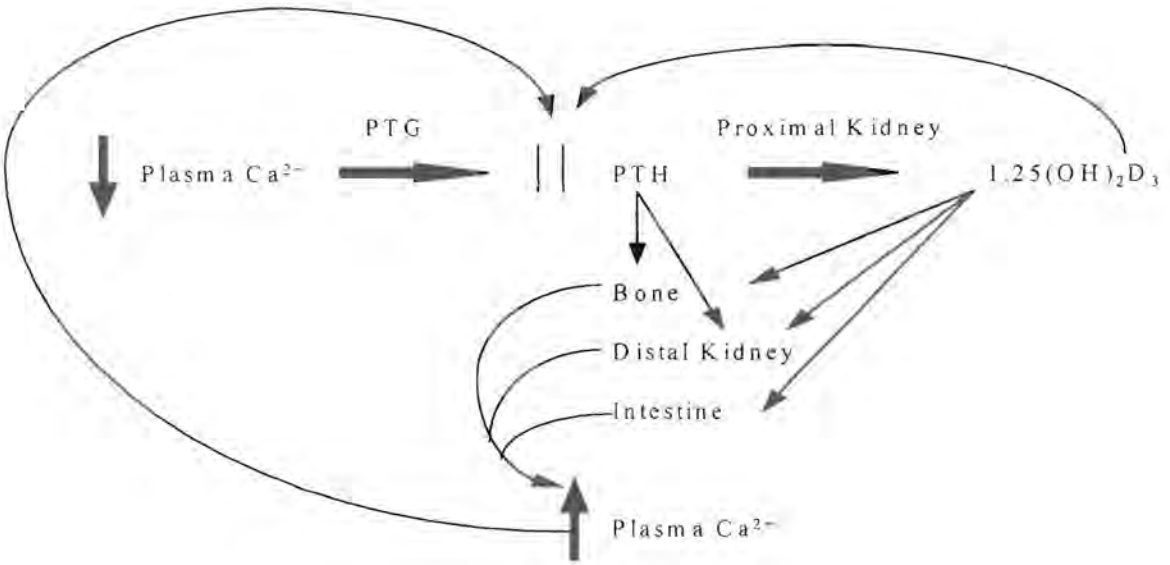


**Figure 3:** Regulation of the plasma $Ca^{2+}$ contents through the parathyroid gland (PTG) and the vitamin D system. (Adapted from 28)

Reconstitution experiments suggest that acute regulation of 1α hydroxylase activity by PTH is achieved through reversible phosphorylation of the ferrodoxin component. Pre-treatment of renal slices from low Ca fed, vitamin D deficient, parathyroidectomised rats with PTH result in dephosphorylation of ferrodoxin and a consequent stimulation of 1α hydroxylase activity when ferrodoxin is reconstituted with ferrodoxin reductase and mitochondrial cytochrome P450. [47] These data suggest the existence of an endogenous PTH-responsive phosphatase that activates the 1α hydroxylase by dephosphorylating ferrodoxin. It appears that parathyroid hormone stimulates production of $1,25(OH)_2D_3$ through a cAMP mechanism. PTH rapidly stimulates cAMP generation and cAMP-dependent protein kinase activity in the kidney, $1,25(OH)_2D_3$ production has been

correlated to intracellular cAMP levels. It is suggested that the putative phosphatase that is responsible for dephosphorylation of ferrodoxin is a phosphoprotein that is regulated by a cAMP-dependent kinase.

Low phosphorus diets and hence hypophosphatemia markedly stimulate the 1α hydroxylase measured both *in vivo* and *in vitro*. [35] The need for phosphorus is translated by the vitamin D endocrine system to improve intestinal absorption of phosphorus and to mobilise phosphorus from bone. [28] In patients with moderate renal insufficiency, restriction of dietary phosphorus induce an increase in serum calcitriol despite inducing a decrease in the levels of iPTH. Thus, in a number of metabolic circumstances in humans, the effect of phosphorus on the renal production of vitamin D can override that of PTH. However, it should be noted that stimulation with phosphate deprivation measured *in vitro*, is much less than that seen with low calcium diets. [35] Thus, it appears that hypophosphatemia, in addition to stimulating the 1α hydroxylase, may affect vitamin D metabolism by some other mechanism.

Other factors that have been implicated as regulators of the renal vitamin D hydroxylase include: calcitonin, oestrogen and growth hormone. Initially it was believed that the stimulation of $1,25(OH)_2D_3$ synthesis observed following calcitonin injections into rats, depended on the presence of the parathyroid glands. [4]

During egg laying in the chicken and pregnancy in the human, the need for calcium is increased. In both conditions, oestrogen is known to be elevated and plasma $1,25(OH)_2D_3$ is enhanced. It has been shown, that a decrease in parathyroid sensitivity to hypocalcemia occurs in osteoporotic women treated with oestrogen. [48] At the same time an apparent increase in renal 1α hydroxylase and renal tubular sensitivity to the smaller change in PTH occurs. Oestrogen appears to protect the skeleton, therefore in part by sparing the skeleton from calcium-liberating stimuli, while maintaining the calcium-conserving PTH responses from other target organs. [4]

Hypophysectomy results in a suppression of the plasma level of $1,25(OH)_2D_3$ that can be partially restored by growth hormone. [49] The increase in renal $25(OH)D_3$ 1α hydroxylase in response to growth hormone seems to be mediated by phosphate depletion. The stimulatory effect elicited by a maximal dose of IGF-I and phosphate depletion was additive, suggesting that the IGF-I induced stimulation and phosphate depleted stimulation of the enzyme activity, occur by a different mechanism. [50] These findings do not support the hypothesis that IGF-I is specifically involved in the modulation of 1α hydroxylase activity in phosphate depletion. IGF-I may merely stimulate basal renal 1α hydroxylase activity by facilitating cell growth or differentiation induced by various hormonal and metabolic factors. [51]

## 1.5 Functions of vitamin $D_3$

### 1.5.1 Intestinal $Ca^{2+}$ Transport

The intestinal absorption of $Ca^{2+}$ has been proposed to occur by a saturable (transcellular) process where $Ca^{2+}$ is transferred through the enterocyte as well as an unsaturable process (paracellular) where $Ca^{2+}$ moves between the cells of the intestinal epithelium. [52] The attention will be focused on the transcellular transport that consists of three steps: a. Entry of calcium into the enterocyte across the brush border membrane (BBM) b. Translocation of calcium bound to calbindin through the cytosol to the basolateral membrane (BLM) c. Active extrusion of $Ca^{2+}$ via an ATP-dependent $Ca^{2+}$ pump, exocytosis and a $Na^{+}/Ca^{2+}$ exchanger. [1]

$1,25(OH)_2D_3$ exerts two effects on the small intestine which allows active calcium absorption to occur genomic and non-genomic. [53] The synthesis of $1,25(OH)_2D_3$-induced binding proteins is very important in the transport of $Ca^{2+}$, actinomycin D/cycloheximide (protein synthesis blockers) decrease the intestinal absorption. Two types of intestinal calcium-binding proteins dependent on vitamin D, have been described. [54] A soluble calcium-binding protein (CaBP) with molecular weight of 9000 was identified in chicken mucosal homogenates, the other mucosal calcium-binding protein dependent on vitamin D was found in a membrane particulate isolated from rat intestinal homogenates. This calcium binding complex (CBC) correlates

well with calcium transport, it has been suggested to be a membrane component of the translocation mechanism for calcium. [54]

On the other hand, intestinal perfusion experiments performed, have shown that following the infusion of $1\alpha,25(OH)_2D_3$, there is a rapid increase in calcium movement from the intestinal lumen into the extracellular fluid independent of new protein synthesis (non-genomic). [55] $1,25(OH)_2D_3$ can change the lipid composition of the BBM. Phospatidyl choline synthesis is increased both by de novo synthesis of phosphatidyl choline and an increase in the methylation of phosphatidyl ethanolamine, resulting in an increase in the fluidity of the BBM and an increase in the numbers of surface calcium channels that allow flux of $Ca^{2+}$ into the enterocyte. [56]

Due to the impermeability of lipid membranes it is reasonable to suspect that a calcium channel may reside in the BBM. An interesting finding was made with an experiment testing vitamin D-deficient as well as vitamin D-replete animals. In vitamin D-deficient rat enterocytes, calcium accumulated and obstructed the microvillar membrane whereas in enterocytes with sufficient vitamin D, calcium was mobilised from this environment by means of vitamin D-dependent mechanisms (CaBP). A possible reason for this occurrence is that the calcium-binding protein, calmodulin that is found in the microvillar area, binds to the accumulated $Ca^{2+}$ and to a myosin-1-complex and draws the microvillar membrane closer to f-actin filaments. This actin-myosin-1-complex can close the so-called $Ca^{2+}$ channels and therefore change the permeability of the microvillar membrane. [1] In the

vitamin D-replete animals the continual removal of $Ca^{2+}$ from this region by CaBP causes the release of $Ca^{2+}$ from the calmodulin-myosin-I-actin complex, restoring the $Ca^{2+}$-channels or transporters to the open (active) state (figure 4).

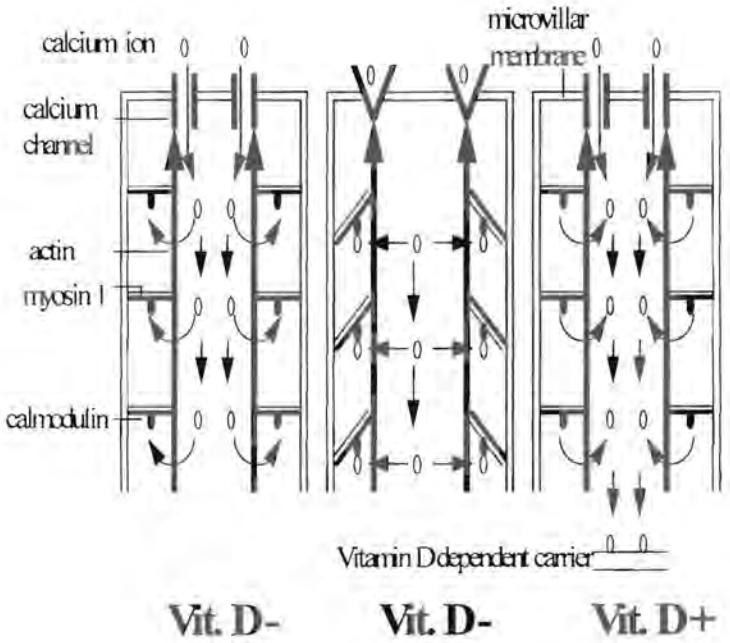


**Figure 4:** Regulating model for the control of $Ca^{2+}$ entry at the microvillar membrane. (Adapted from 1)

The rate of self-diffusion of calcium in the enterocyte occurs too slowly therefore use is made of calbindin-D9k that binds two calcium molecules dependent on vitamin $D_3$. [53]

Calcium out-flow occurs at a fair thermodynamic gradient. Two systems associated with the BLM are available to extrude $Ca^{2+}$ against the considerable gradient: an ATP-dependent plasma membrane $Ca^{2+}$ pump

(PMCA) and a $Na^+/Ca^{2+}$ exchanger. [1] The PMCA has a molecular weight of 130-140 kDa, transports one $Ca^{2+}$ per ATP and has a Km of approximately 0.2 μM in the presence of calmodulin. The $Ca^{2+}$ ATPase is activated by a ATP-dependent phosphorylation of an aspartyl residue, and the activity increased by the ATP-dependent phosphorylation of serine and threonine residues catalysed by protein kinase A and C. [1]

The effect of cholecalciferol on the time course of $Ca^{2+}$ uptake by isolated chick basolateral membrane vesicles clearly show that $1,25(OH)_2D_3$ increases both the rate of uptake of $Ca^{2+}$ and the total amount of $Ca^{2+}$ accumulated by the BLM vesicles. This increased $Ca^{2+}$ uptake effect could be due either to the synthesis of new pump units, the recruitment of pre-existing $Ca^{2+}$ pumps to the BLM, or the direct activation of the pump or perhaps a combination. [52] Cai et al. [58] have shown that the increase in amount of PMCA in response to $1,25(OH)_2D_3$ is mediated by an increase in the transcription of the PMCA isoform - 1 gene and a subsequent increase in synthesis.

Calmodulin was shown previously to increase both the $Ca^{2+}$ affinity and maximal transport rate of the erythrocyte $Ca^{2+}$ pump. [52] Calbindin-D9k is homologous to calmodulin and activates the $Ca^{2+}$ pump. The effect of calbindin on the PMCA is non-specific, because various other calcium-binding proteins together with EGTA (which usually buffers $Ca^{2+}$) stimulate the $Ca^{2+}$ pump as well. The high-affinity binding sites for $Ca^{2+}$ are shielded by positive charges, when complexed to EGTA or the $Ca^{2+}$-binding proteins,

$Ca^{2+}$ is presumably more accessible to the pump $Ca^{2+}$-binding site. Contrary to these findings a calbindin-D9k binding domain in the erythrocyte plasma membrane calcium pump was found recently, thus suggesting that the interaction between calbindin and the $Ca^{2+}$ pump might be more complex. [59]

### 1.5.2 Bone calcium homeostasis

Vitamin D exerts two major effects on bone. $1,25(OH)_2D_3$ increases mineralisation or formation of new bone that can be visualised by the decrease in the amount of osteoid. [5] The other effect is the loss of bone matrix as well as mineral which is termed resorption. In a vitamin D deficient state the loss in mineralisation is far greater than the loss in bone resorption activity. An opposite effect can be seen with excessive vitamin D. The great loss in bone matrix due to resorption can lead to several bone disorders.

#### 1.5.2.1 Stimulation of mineralisation

##### Role of the osteoblast

Osteoblast cells are derived from pluripotent mesenchymal stem cells of the bone marrow. [60] These cells form a mineralised matrix and are situated on top of osteoid (layer of bone matrix not yet calcified) seams, they eventually become surrounded by the matrix they synthesise. The cells are then termed osteocytes. Osteoblasts are responsible for the production of several

products including alkaline phosphatase, collagen type 1, the principal structural organic component of bone involved in the anabolism of bone, and production of osteocalcin [61] that is incorporated into the extracellular matrix of bone. Osteocalcin, a bone GLA protein, contains 3 gamma carboxyglutamic acid residues that bind calcium.

A matter that is unresolved is whether $1,25(OH)_2D_3$ exerts an effect on mineralisation in a direct or indirect manner, thus whether decreased intestinal transport of calcium and phosphorus is the basis for impaired bone mineralisation or whether $1,25(OH)_2D_3$ has a direct effect on the osteoblast cells. [5] Reports have shown nuclear localisation of administered $1,25(OH)_2D_3$ in osteoblasts, chondrocytes and osteocytes. [62] Vitamin $D_3$ induces the production of osteocalcin [63] and stimulates osteoblast bone formation while it inhibits collagen synthesis. [64] this inhibiting effect is related to the nuclear localisation, $1,25(OH)_2D_3$ inhibits nuclear transcription of the collagen gene via osteoblastic $1,25(OH)_2D_3$ receptors that bind nuclear chromatin. [64]

While vitamin $D_3$ inhibits collagen synthesis, even at low concentrations, no reduction in the rate of bone matrix formation or maturation is noted, thus there could have been diminished collagen synthesis at the time when the osteoid was remineralising. The state of bones in vitamin D-deficiency can be radiographically seen as a widening of the metaphyseal growth plate and an irregular appearance at the end of the metaphysis due to uneven mineralisation. As soon as $1,25(OH)_2D_3$ is administered, an increase in the

percentage of osteoid surface having a calcification front, a rise in plasma phosphate and an increase of osteoclast count can be observed. [5]

### 1.5.2.2 Stimulation of bone resorption

#### Role of the osteoclast

Osteoclasts are derived from hemapoietic granulocyte-macrophage colony forming units of the bone marrow. [60] In contrast to the osteoblast cell, the osteoclast is a giant multi-nucleated cell and is usually found in contact with a calcified bone surface and within a lacuna, a result of its resorption. The contact zone of the osteoclast with the bone is described as a ruffled border (folding of the plasma membrane). The clear zone possibly corresponds to the formation of the ringed structure that consists of F-actin rings that facilitates osteoclast attachment to the bone's calcified surface. [65] The ultra-structure features of this cell are an abundance of Golgi complexes around the nucleus, mitochondria and transport vesicles loaded with lysosomal enzymes. These enzymes together with cysteine proteinases such as cathepsin K and metalloproteinases (MMP-9) are pumped into the resorption lacunae where they respectively degrade organic bone matrix as well as collagen type 1. [65]

The bone resorbing effect of $1,25(OH)_2D_3$ is mediated at the bones themselves. [5] The sensitivity of isolated bones in organ culture to

administered $1,25(OH)_2D_3$ is evidence of a direct effect, as little as $10^{-11}$ M $1,25(OH)_2D_3$ can start resorption of rat fetal bones. [66] *In vivo* experiments suggest that PTH is required for the bone resorbing effect of vitamin D and vice versa, in contrast either agent can produce resorption *in vitro*. [67] High-affinity binding of $1,25(OH)_2D_3$ has been found in whole bone homogenates and isolated bone cells. [68] $1,25(OH)_2D_3$ together with PTH and calcitonin stimulate osteoclastic resorption. The binding protein for $1,25(OH)_2D_3$ in bone could represent the bone cell receptor for the bone-resorbing effects of the vitamin D metabolites. The metabolites of vitamin D do not directly increase cAMP in bone as is the case with PTH. [5]

$1,25(OH)_2D_3$ is essential for osteoclast differentiation from precursor cells [65] as well as increasing lysosomal enzyme release, osteoclast number and size, nuclear area, ruffled border and clear zone. [69]

### 1.5.3 Effects of vitamin D and its metabolites on the kidney

The effect of vitamin $D_3$ and its various metabolites on ion transport are much less marked on the kidney than on the intestine. [32] What is known is that administration of vitamin $D_3$ causes an increase in the tubular reabsorption of phosphate and calcium. The effect of $1,25(OH)_2D_3$ on phosphate transport has been suggested to be mediated by changes in lipid composition of the membranes. The enzyme 25-hydroxyvitamin $D_3$-1α-hydroxylase has previously been located in the proximal tubule, receptors for $1,25(OH)_2D_3$ have been found in the distal tubule. Experiments done on

calcium binding protein in the kidney has located it in the distal tubule as well, thus $1,25(OH)_2D_3$ exerts a significant effect on the distal tubule by inducing the synthesis of a calcium binding protein. [32]

## 2. The vitamin D receptor (VDR)

### 2.1 Discovery of the VDR

The vitamin D receptor (VDR) was first revealed in 1969 as a chromosomal protein in intestinal mucosal nuclei that specifically bound the most active metabolite of the parent vitamin. [70] It was later discovered that the active metabolite is $1,25(OH)_2D_3$, the hormonal ligand that binds to the VDR *in vivo*. [71] After this, Tsai and Norman [72] showed that $1,25(OH)_2D_3$ association with chromatin in reconstituted systems, was facilitated by a soluble factor. This factor was conclusively shown to be receptor-like, by elucidating three critical characteristics of this macromolecule: a) It binds vitamin D analogues in a rank order corresponding to their biologic potencies, [73] b) sediments at 3.0-3.5 S in high salt-sucrose gradients and c) displays saturable high-affinity binding *in vitro*. [74]

One of the important properties of the receptor was discovered in 1979, it was identified as a DNA-binding protein, being purified by utilising DNA cellulose chromatography. [75]

## 2.2 Prevalence

That the receptor was originally discovered in the major vitamin D target organ, namely the intestinal mucosa, and was subsequently revealed in other sites of mineral translocation or regulation i.e. parathyroid gland, [76] bone, [77] and kidney [78,79,80] adds credence to its proposed role in vitamin D action. These tissues persist as locations with the highest concentrations of VDR, but many target tissues and cell types for $1,25(OH)_2D_3$ have been identified by biochemical detection of receptor and by way of autoradiographic localisation of $^3H$ $1,25(OH)_2D_3$ *in vivo*. [81] Many of these new targets are mineral transport sites, endocrine organs (pancreas, pituitary) and reproductive tissues (breast, placenta). The skin, where the photobiosynthesis of vitamin D occurs, is also a location for the VDR. [82]

The vast majority of the target tissues appear not to be primarily related to calcium metabolism, but rather to the activation and regulation of exo- and endocrine secretory and somatotrophic processes such as cell differentiation and proliferation. [83]

It is conceivable that vitamin D affects all cells during a certain period of their differentiation. Thus in fully differentiated intestinal epithelial cells, $1,25(OH)_2D_3$ is a potent inducer of calcium transport and this action correlates with the appearance and escalation of the receptor during embryonic and neonatal development in the chick [84] and rat. [85] Thus what was originally considered an antirachitic vitamin, is now recognised as the

precursor to a powerful sterol hormone capable not only of affecting calcium and skeletal homeostasis, but also of fundamental actions on cell proliferation and differentiation. [86]

**2.3 Nature**

Biochemical studies of the $1,25(OH)_2D_3$ receptor indicate that the mode of action of the vitamin D sterol is similar to that of steroid and thyroid hormones, with the active metabolite complexing with a selective, high-affinity binding protein, that concentrates the hormone in the nucleus. [86] With the subcellular distribution of the VDR, nuclear localisation is supported indirectly by the striking nuclear occurrence of tritiated $1,25(OH)_2D_3$ by way of autoradiography [81] and the original biochemical detection of the receptor protein in nuclear chromatin after administration of labelled vitamin D *in vivo*. [70] Evidence was also gathered to suggest that the unoccupied receptor was primarily a cytosolic molecule, with the $1,25(OH)_2D_3$ receptor complex migrating to the nucleus upon hormone binding. [74] Thus, the $1,25(OH)_2D_3$ receptor system seemed to fit perfectly with the prevailing steroid hormone receptor dogma of a two step process of hormone binding in cytoplasm and subsequent localisation in the nucleus. [87]

Although nuclear localisation of occupied $1,25(OH)_2D_3$ receptor remains undisputed, in vitamin D and several other steroid hormone systems, the subcellular distribution of the unoccupied receptor is less well defined. Walters and associates [88] observed that as much as 90% of the unoccupied

$1,25(OH)_2D_3$ receptors are associated with purified nuclei or chromatin under low ionic strength fractionation conditions. Thus, the nature of the VDR may approach that of the thyroid hormone receptor, which is an intrinsic non-histone chromosomal protein. [89] Unoccupied receptors are thought to have affinities for homologous nuclei in the following order: steroid hormones < $1,25(OH)_2D_3$ < thyroid hormones. [83] The present working hypothesis is that the VDR, like other steroid receptors, is a loosely associated chromosomal protein, with affinity for nuclear components increasing upon hormone binding. [83]

## 2.4 Structure

### 2.4.1 Avian receptors

The most extensively studied VDR is that from chick intestinal mucosa. [90] The chick intestinal receptor is biochemically indistinguishable from that found in other avian tissues like the parathyroid gland, bone, pancreas and ovaries. It is an acidic protein, with a pI of 6.2 and possesses distinct domains for $1,25(OH)_2D_3$-binding ($Kd = 10^{-10} - 10^{-11}$ M) and for association with DNA. [91] Both the $1,25(OH)_2D_3$-binding region and the DNA-binding domain contain essential sulphhydryl groups. [86] The receptor has been shown to have a mw of 63000 Dalton, [92] or 64000 Dalton through gel electrophoresis under denaturing conditions. The receptor is unstable and both hormone and DNA-binding capacity decay in a temperature and time-dependent fashion. It has been found that endogenous protease cleaves the 60000-Mr receptor into a fragment of Mr 45000 that binds hormone but not

DNA. [86] It is likely that this fragment is the cytosolic form of the receptor that has been reported not to bind DNA. [93]

Much additional information is available about the DNA or polynucleotide binding characteristics of the chick intestinal $1,25(OH)_2D_3$ receptor, [86] the data indicates that DNA-binding is not solely electrostatic, but involves hydrophobic interactions with the major grooves of the DNA double helix. [94]

### 2.4.2 Mammalian receptors

Mammalian receptors are remarkably similar to their avian counterparts, possessing corresponding dissociation constants for $1,25(OH)_2D_3$-binding of $10^{-11} - 10^{-16}$ M, and virtually identical specificity in that other prominent circulating vitamin D metabolites like $25(OH)D_3$ and $24,25(OH)_2D_3$ bind less than 4% as effectively as the hormone. [86] The mammalian $1,25(OH)_2D_3$ receptor has DNA-binding properties indistinguishable from the avian receptor, which reveals that both the hormone- and DNA-binding domains are conserved in these two species.

The major form of the $1,25(OH)_2D_3$ receptor in pig intestine has a molecular weight of approximately 55000 Da. [95]

Although there has been many characterisations of various mammalian receptors including rat intestinal mucosa receptors, [96] the biochemical

properties such as $1,25(OH)_2D_3$-binding affinity and specificity, as well as DNA-binding characteristics, are indistinguishable for the receptor in all tissues from fish to humans. This indicates that the molecule is highly conserved and that similar mechanisms constitute the action of $1,25(OH)_2D_3$ in each of its target cells. [86]

As a result of the low abundance of the receptor in target tissues, including the intestine, significant advances in studying its structure, function and regulation were prevented. [97] A major advance occurred with partial purification of the receptor, allowing production of monoclonal antibodies. These monoclonal antibodies provided an important tool for cloning the cDNA's, for recombinant receptor expression, purification, regulation and phosphorylation. Perhaps the most important use of the receptor antibodies was in the cloning of receptor cDNA from different species. Thus, the amino-acid sequences were deduced for a portion of the chicken receptor, and the full-length human and rat receptor. [97]

The rat VDR, currently being studied in the laboratory of Strugnell and DeLuca, [98] is a 55 kD protein made up of 423 amino acids. As can be expected, there is a certain amount of analogy with other members of the superfamily.

## 2.5 Mode of action of the vitamin D receptor

Based upon a number of biochemical experiments, a map of the avian $1,25(OH)_2D_3$ receptor has been constructed and can be seen in figure 5. The $1,25(OH)_2D_3$ hormone binds in a hydrophobic pocket encompassing the C-terminal 30 kDa of the protein, although only a few amino acids by carboxypeptidase cause dissociation of $1,25(OH)_2D_3$. [99] There is an exposed region to the centre of the molecule that is extremely sensitive to proteolysis. This domain is known as the "hinge" region because it forms a barrier between the ligand-binding domain (LBD) and the DNA-binding domain (DBD). Relatively little is known about the structural aspects of this region. It is however apparent that it is highly immunogenic because it is the antigenic site for several monoclonal antibodies. [100] The DBD has zinc-fingers (fingers anchored via Zn atoms co-ordinated by sulfhydryl groups of cysteine residues) which associate with the DNA. [99] It has been shown that sulfhydryl reagents dissociate $1,25(OH)_2D_3$ from the DNA and chelators such as EDTA destroy the DNA binding function of the receptor.

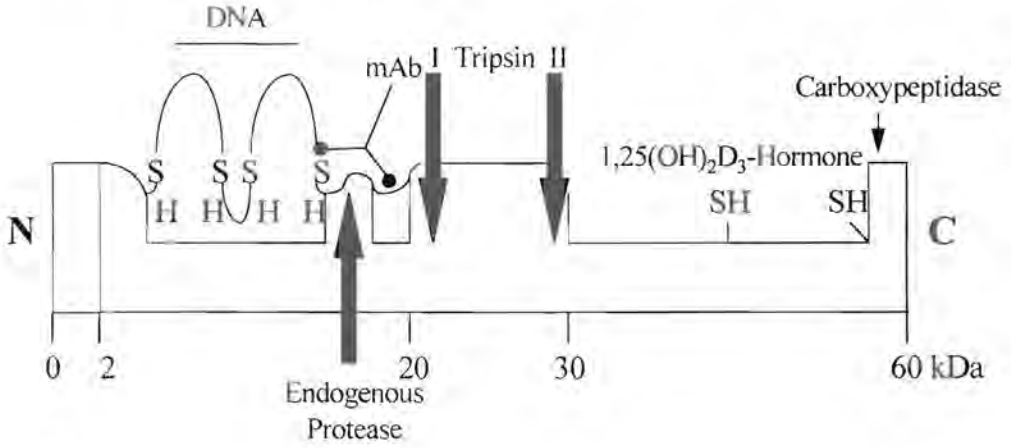


**Figure 5:** Structure of the avian receptor. mAb: monoclonal antibody (Adapted from 99)

A fraction of the unoccupied receptor is hypothesised to be present in the cytoplasm, but the equilibrium favours the nuclear compartment, where the receptor exists as a loosely associated chromosomal protein. [86] When the receptor is unoccupied, the trans-activation domain is silent and the Zn fingers are repressed so that optimal binding to vitamin D responsive elements (VDRE) is not achieved. Upon binding to $1,25(OH)_2D_3$ the receptor is phosphorylated at multiple sites. Although phosphorylation of serines could conceivably turn off the transcriptional activation processor and drive the receptor from the DNA, positive events do occur. The phosphorylation, following the binding of the hormone create highly acidic patches on the receptor that attract or complex with positive domains in rate limiting transcription factors. The result of such a process would be stimulation of the expression of genes such as CaBP and BGP (bone GLA containing protein).

There are number of proteins that are induced by the binding of vitamin D to its receptor (figure 6).

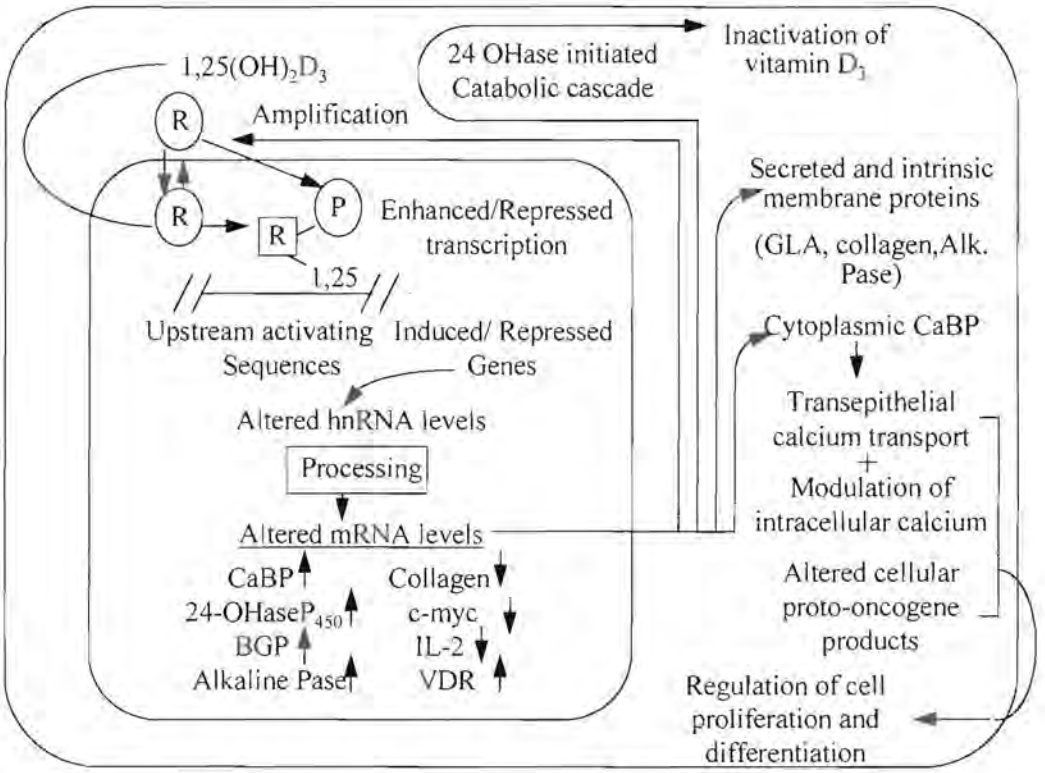


**Figure 6:** Model for receptor-mediated action of vitamin $D_3$ at the molecular level.(Adapted from 86)

In the kidney, $1,25(OH)_2D_3$ is inactivated through the induced 24-hydroxylase initiated cascade. Modulation of proteins such as BGP, collagen and alkaline phosphatase, required for bone mineralisation and remodelling, is the apparent response of bone cells. CaBP that is formed, plays a pivotal role in intestinal $Ca^{2+}$ transport as well as the modulation of intracellular $Ca^{2+}$ in other target organs. Certain hemapoietic and transformed cells respond to $1,25(OH)_2D_3$ by differentiation and suppression of malignant phenotypes. Thus, evidently in combination with its receptor, $1,25(OH)_2D_3$ is a potent regulator of cell growth and maturation. [86]

## 2.6 Factors influencing the $1,25(OH)_2D_3$ receptor

### 2.6.1 $1,25(OH)_2D_3$

Several studies have indicated that $1,25(OH)_2D_3$ can regulate the VDR. Costa et al. demonstrated in 1986 [101] that $1,25(OH)_2D_3$ upregulated the VDR *in vivo*. Two organs were used to determine the level of receptors: kidney (a critical organ in the control of hormonal synthesis) and the intestine (a classical target organ of vitamin D action). The results obtained indicate substantial differences in the response of these organs. An increase in specific binding of up to 336% was seen after 5 days of $1,25(OH)_2D_3$ administration in the kidney. Intestinal receptors increased only 130% after 5 days' treatment, which suggests differential importance of upregulation in various organs. There is uncertainty about whether administration of $1,25(OH)_2D_3$ affects the receptor synthesis or mRNA levels encoding for the VDR, or whether the receptor becomes more stable. *In vivo* studies done by Huang et al. [102] indicated that constant exposure to elevated blood levels of $1,25(OH)_2D_3$ does not result in an alteration in intestinal receptor mRNA. In cycloheximide-blocked cells, the degradation rate of previously formed receptor was markedly decreased by the administration of $1,25(OH)_2D_3$. [103] The homologous upregulation is not primarily due to new receptor synthesis but may be the result from events that result in increased receptor stability, e.g. ligand-binding.

### 2.6.2 Age

Age has been shown to affect several physiological events related to calcium and vitamin D metabolism. [104] Horst et al. [104] have shown that a reduced calcium absorption and bone loss are accompanied by a reduction in unoccupied intestinal and bone VDR. An age-dependent reduction in receptor numbers was also associated with a decrease in calcium transport. [105] Although Liang et al. [106] found a decline in $1,25(OH)_2D_3$ serum levels accompanied by a low expression of receptor in aged animals, it is not certain whether it is the constant exposure to the low $1,25(OH)_2D_3$ that induces the age-related deficit in the expression of VDR. [106] An attenuated response to $1,25(OH)_2D_3$ (VDR upregulation) was found in aged animals with a significant increase in VDR; [105] thus the reduction in receptor number can be partially reversed by $1,25(OH)_2D_3$ supplementation in aged subjects.

### 2.6.3 Oestrogen

Walters [9] found $1,25(OH)_2D_3$ receptors in chromatin extracts of ovariectomised rat uterus in 1981. This uterine receptor component is similar to $1,25(OH)_2D_3$ receptors of other tissues in sedimentation coefficient and steroid specificity. The administration of oestrogen to the ovariectomised uterus had a stimulatory effect on the receptor binding of $^3H$ $1,25(OH)_2D_3$ which could not be stimulated in the chick intestine, which suggests once again that the control of the VDR is a property of the target tissue rather than an inherent property of the receptor species. [9]

### 2.6.4 Dietary calcium

$1,25(OH)_2D_3$ increases intestinal calcium transport through events that include binding of $1,25(OH)_2D_3$ to the intracellular vitamin D receptor. Under physiological conditions as during dietary calcium restriction, calcium transport increases, it is likely to assume that the increased calcium transport accompanies increased VDR content. Favus et al. [107] showed a threefold increase in the VDR content in the intestine with a dietary calcium restriction; therefore dietary calcium restriction upregulates the vitamin D receptor.

## 3. Essential Fatty Acids (EFAs)

### 3.1 Background

Fatty acids are aliphatic chains of carbon atoms with terminal methyl ($-CH_3$) and carboxyl (-COOH) groups. [108] Unsaturated fatty acids have one or more double bonds, which have a cis-configuration, with two hydrogen atoms on the same side of the C-chain plane. Cis-configurations usually occur in polyunsaturated fatty acids (PUFAs) with two or more double bonds. Trans-isomers have the hydrogen atoms on opposite sides of the C-chain plane.

PUFAs are classified according to the location of the first double bond. Thus, n-3 PUFA have the first double bond three carbon atoms from the

omega or methyl end. Two PUFAs, linoleic acid or LA (18:2n-6), an omega 6 PUFA and alpha- linolenic acid or ALA (18:3n-3), an omega 3 PUFA, are called essential fatty acids (EFAs) because they cannot be synthesised by the body and have to be supplied through the diet. The first number indicates the number of carbon atoms in the molecule, then after the colon the number of double bonds, and the third number after the n, the position of the first double bond starting from the omega or methyl end of the chain. [109]

### 3.2 Metabolism

Within the body LA and ALA are metabolised by the series of alternating desaturations (removing 2 hydrogen atoms and inserting an extra double bond) and elongations (adding two carbon atoms) as shown in figure 7. [110]

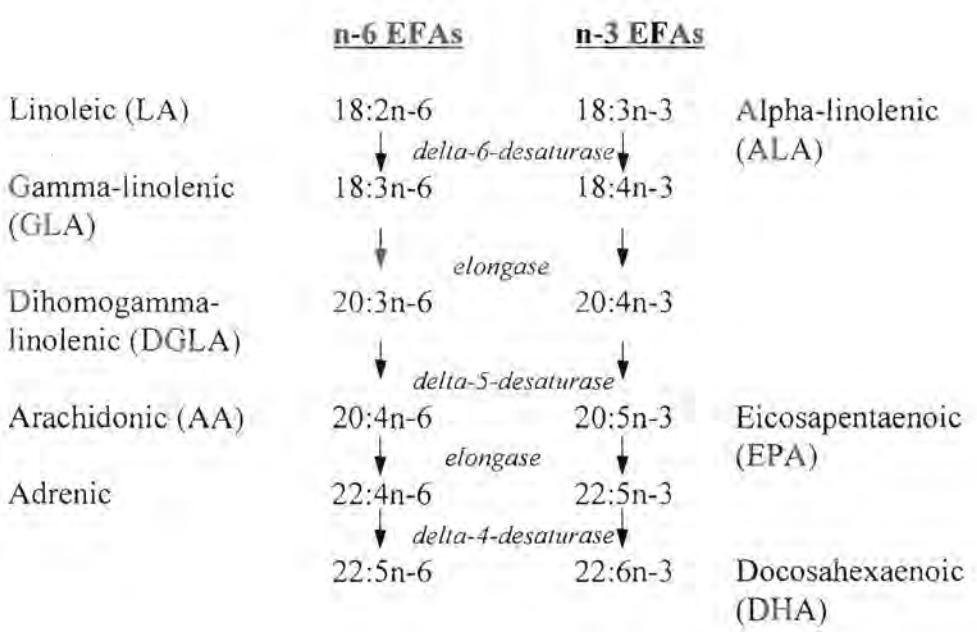


**Figure 7:** Pathways of metabolism of essential fatty acids of the n-6 and n-3 series. [110]

It is widely believed that the enzyme sequences metabolising LA and ALA are identical. [111] The desaturation steps tend to be slow and the elongation steps to be rapid. This is particularly true of the first desaturation, which is very slow, and the first elongation, which is very rapid. Thus, GLA of the n-6 series and stearidonic acid (18:4n-3) of the n-3 series are found in the body in only very small quantities: this is because they are formed very slowly by desaturation and then immediately metabolised by elongation. [112]

The conversion of LA and ALA to the metabolites is rate limited at the first step, delta-6-desaturation. The affinity of fatty acids for the delta-6-desaturase (D6D) is higher, the greater the number of double bonds. The n-3 and n-6 EFAs are competitive inhibitors of each other's metabolism. [113,114] On the whole, the n-3 EFAs are more effective at inhibiting desaturation of the n-6 EFAs than vice versa. Thus, the presence of linolenic acids or higher members of the n-3 family inhibit the desaturation of linoleic acid. [108]

The delta-6-desaturase (D6D) is the key first step in the metabolism of linoleic acid, the main dietary EFA, and is likely to play an important role in attempts to modulate prostaglandin (PG) synthesis by dietary manipulation. [110] Several factors influence the activity of this enzyme. A variety of hormones modulate the action for instance glucagon, adrenaline and thyroxin. There is evidence that D6D activity is lost or at least substantially reduced as an animal grows and ages. The deterioration in cardiovascular function and decreased T-suppresser cell function can all be associated with

the loss of GLA as a result of a decreased activity of the delta-6-desaturase. Administration of ethanol has consistently shown to increase the ratio of linoleic acid to arachidonic acid, suggesting an inhibition of D6D at some point. Other factors such as: zinc, cell transformation, and trans fatty acids may also play a role.

A number of vitamins such as vitamin C, $B_6$, and vitamin E is known to interact with EFA metabolism although in no case are the nature of the interaction clearly defined, as well as a few minerals such as zinc, which is a co-factor in the metabolism of EFAs. [109] The relationship between EFAs and vitamin E is well known but unexplained. As the amount of PUFA increase in the diet, the requirement for vitamin E, a fat-soluble anti-oxidant vitamin, also increases. Essential fatty acids are more easily oxidised in the body than are more saturated fatty acids, and they are very easily peroxidised in air, vitamin E inhibits peroxide formation.

### 3.3 Principal dietary sources of EFAs

LA is present in substantial amounts in dairy products, organ meats such as liver, and notably vegetable seed oils such as sunflower, safflower, corn and soy. Margarine that contains liquid oil as the first ingredient is also a good source of LA. The main sources of GLA are the seeds of the evening primrose, borage and blackcurrant. Moderate amounts of DGLA are found in human milk. [115] Meat, egg yolks and some seaweeds contain arachidonic acid. [116] Alpha-linolenic acid is the predominant fatty acid of leaves and is

found in some vegetable oils (for example linseed, canola and soya). EPA and DHA are mainly found in fish and fish oils. Fatty fish such as mackerel, herring and salmon are especially rich in n-3 acids.

### 3.4 Physiological roles of the EFAs

The EFAs play a few major roles in the body with a number of derivative functions arising because of these major roles.

3.4.1 The EFAs are essential structural components of phospholipids in every cell membrane. The phospholipids normally contain an EFA in the 2-position, and sometimes both positions are occupied by EFAs. [108] The precise physio-chemical characteristics and therefore functions of phospholipids such as phosphatidylcholine, etc. are strongly influenced by precisely which fatty acids are in the sn-1 and sn-2 position. [117] Because of their unsaturation, they confer on membrane properties of fluidity, flexibility and permeability. [112] Because they modulate the properties of the environment in which membrane proteins is embedded, they change the functioning of such proteins as receptors; enzymes such as ATPase and ion channels. For example, in *in vitro* studies increasing unsaturation of the membrane environment reduces the binding of steroid and other hormones to their receptors. [118,119]

3.4.2 The EFAs are the precursors for the so-called eicosanoids, prostaglandins (PG's) leukotrienes and other oxygenated derivatives, derived in particular from DGLA (series 1 PG's), AA (series 2 PG's), EPA (series 3 PG's). The main enzymes involved in the metabolism of the EFAs to eicosanoids are the cyclo-oxygenase and related systems, which give rise to prostaglandins and thromboxanes, and the 5-,12- and 15-lipoxygenases, which give rise to a variety of oxygenated metabolites including the leukotrienes. $PGE_1$ (from DGLA): has a wide range of desirable effects, including inhibiting platelet aggregation, inflammation, producing vasodilatation and inhibiting phospholipase. Thromboxane $A_2$ (from AA): is potent proaggregatory and a vasoconstrictor. Leukotrienes (from AA) contract smooth muscle and are strongly proinflammatory, to name but a few functions of the derivatives.

3.4.3 The EFAs are also part of most of the second messenger signalling systems within the cell. Fatty acids (FAs) have been shown to act both as modulators and messengers, particularly of signals triggered at the level of cell membranes. Enzymes and proteins of the cyclic AMP and the protein kinase C signalling pathways and those involving ion fluxes and mobilisation is both inhibited or activated by fatty acids. They can also participate in a feedback control mechanism since phospholipases are themselves modulated by FAs. FAs, in particular AA liberated from membrane

phospholipids, are also second messengers in signal transduction, a good example is the activation of protein kinase C. [120,121,122]

3.4.4 EFAs are involved in cholesterol transport and metabolism. The esters formed with the EFAs are consistently more soluble and more easily dispersed than the esters formed with other fatty acids. [123]

### 3.5 The relative importance of n-6 and n-3 EFAs

The possible health benefits of fish oil have caused the n-3 EFAs to come under the spotlight. [112] The n-6 EFAs such as GLA has been totally overshadowed by the enormous attention paid to EPA and DHA. Experimental evidence has proven the importance of n-6 EFAs in that biological and biochemical abnormality only occurs in n-6 deficient diets [124] and the abnormalities can be rapidly reversed by addition of n-6 EFAs alone. [125,126]

Nevertheless, there is evidence that n-3 fatty acids may have unique functions in various animals. [127] It is known that dietary alpha-linolenic acid will improve growth in EFA-deficient rats, although it will not cure EFA deficiency, particularly infertility and dermatitis. [128] There is some evidence available that suggests that realistic doses of fish oil (EPA + DHA) will undoubtedly lower triglycerides but raise the blood concentration of low-density lipoprotein (LDL) cholesterol. [129] Dietary EPA can suppress the

production of thromboxane $A_2$ by reducing platelet phospholipid AA stores and by competitively inhibiting cyclo-oxygenase. [130] The lipids of warm-blooded animals usually contain relatively little 22:6n-3 (DHA), except in the brain where 22:6n-3 is located with great specificity in particular parts. [127] The retina is another organ into which DHA is selectively incorporated in many species of animals. [131]

It has also been shown that DHA is a strong inhibitor of prostaglandins. Thus n-3 EFAs may reduce or inhibit factors involved in cardiovascular disease, inflammatory and immune disorders. [132]

The question of what the ratio between n-6 and n-3 EFA supplementation should be, arises. In most tissues in the body, the ratio of n-6 to n-3 EFAs lies within the range 3:1-9:1. [110] It is important to remember that a progressive increase in EPA levels in diets, leads towards a decrease in n-6 derived EFAs in tissues, thus n-3 EFAs accumulating to a higher percentage in tissues than n-6. This may be the result of the converting enzyme's preference for n-3 EFAs especially in the desaturation steps. [133,134]

The relative ratio of these fatty acids in the plasma or tissue may affect the proportions of n-3 and n-6 eicosanoids. Several researchers [135,136] have indicated reduction in AA levels with a resultant decrease in $PGE_2$ levels in animals fed a n-3 rich diet. Claassen et al. [133] have found that supplementation of GLA and EPA in an appropriate ratio may be of benefit in enhancing the calcium metabolism, and GLA and EPA are more effective

in modulating calcium metabolism than their precursors. Thus, supplementation of n-6 and n-3 fatty acids in specific ratios seem therefore an appropriate treatment regime to modulate membrane EFA composition, n-6 and n-3 eicosanoid production and possibly calcium metabolism.

## 3.6 The importance of EFAs in calcium homeostasis

### 3.6.1 Intestinal $Ca^{2+}$ Absorption

The functional role of vitamin D in $Ca^{2+}$ transport has been discussed previously. It has been postulated that vitamin D affects all three steps involved in transcellular calcium absorption. [137] The BBM ensures directionality being located at the luminal surface, Rasmussen et al. [138] proved that vitamin D could induce a profound alteration in the lipid composition of the microvillar membrane. There was no change in the proportion of phospholipids, only an increase in the long chain essential polyunsaturated fatty acid concentration. O'Doherty [139] reported that the intestinal phosphatidylcholine deacylation-reacylation cycle could be altered by the administration of 1,25-dihydroxyvitamin $D_3$, retailoring the fatty acid composition of the BBM. By changing the composition, properties such as fluidity and permeability, the structure and function of the membrane associated proteins also changes. [139] Increases in phospholipase $A_2$ activity together with an increased incorporation of arachidonic acid into the phosphatidylcholine followed the administration of $^3H$ $1,25(OH)_2D_3$.

The relationship between vitamin D, long chain essential fatty acids and calcium transport have been well established. [117] Putkey et al's [140] study to investigate the effect of an EFA deficiency on vitamin D-stimulated intestinal $Ca^{2+}$ transport was conducted both *in vivo* and *in vitro*. An EFA deficiency in both vitamin D-deficient and replete chicks resulted in a subsequent decrease in LA levels with a compensatory increase in non-essential unsaturated fatty acids. The EFA deficiency was unable however to affect the ability of the vitamin D-deficient chicks to respond to vitamin D, with a two-fold increase in serum $Ca^{2+}$ and a four to five-fold increase in $Ca^{2+}$ transport. Dietary vitamin D had no detectable effect on the lipid fluidity or polarity in either the BBM or BLM, suggesting that increased $Ca^{2+}$ transport were mediated via other mechanisms using vitamin D. [140]

The results that Kreuter et al. [141] obtained re-emphasised the necessity of EFAs for the action of vitamin D. Administration of vitamin D to vitamin D-deficient, EFA-deficient chicks and vitamin D-deficient control chicks, led to the same increase in calcium transport *in situ*. There was a temperature sensitivity in the *in vitro* system. With brush border vesicles, an increased temperature of 34°C from $1,25(OH)_2D_3$-treated EFA-deficient chicks accumulated calcium at a faster rate than the other vesicles.

The rate of $Ca^{2+}$ uptake into isolated vesicles from $1,25(OH)_2D_3$ treated EFA-deficient chicks correlated with the amount of linoleic acid in the BBM. These results, confirmed that fatty acids are important elements in the control of brush border $Ca^{2+}$ transport. A change in the phospholipid

composition of the BBM may affect permeability and calcium-ATPase associated with the BLM. [142] The feasibility of modulating intrinsic intestinal membrane functions *in vivo* by means of dietary content of fatty acid has been previously shown to have an influence on the fatty acyl composition of the brush border phospholipids as well as the activity of enterocyte microsomal desaturases. [143]

The profound stimulating effect on the $Na^+$-$K^+$ ATPase activity of the membranes isolated from the fish oil (EPA) supplemented groups in an experiment done by Coetzer et al. [2] may be extrapolated to the calcium ATPase, as both enzymes are located in the BLM. Stimulation of $Ca^{2+}$-ATPase by unsaturated fatty acids has also previously been shown. [144] Results show that membrane lipid fluidity has a direct influence on the conformation of the active site of some membrane-associated enzymes, with the result that such enzymes display a higher activation energy when the membrane lipids are comparatively more fluid. This suggests that some proteins may phase separate with the more fluid lipids at low temperatures.

### 3.6.2 EFAs and Bone

Prostaglandins (PG's) of the E series, primarily $E_1$ and $E_2$, are produced by bone and have the greatest activity in bone. [145] Following the discovery of their ability to stimulate bone resorption *in vitro*, investigations found PG's at sites of localised bone resorption associated with inflammatory lesions *in vivo*. In contrast to this finding, *in vivo* studies of PGE infusions into either

blood or bone resulted in bone formation. [117] In mature animals given pharmacological doses of PG's, there is a generalised activation of remodelling with increased formation in the remodelling cycle. [145] PG's have an initial, inhibitory effect on osteoclast function. However, the major long-term effect in bone organ culture is to stimulate bone resorption due to an increase in replication and differentiation of new osteoclasts. [146] PG's have a biphasic effect on bone formation; the replication and differentiation of osteoblasts are stimulated at low concentrations leading to increased bone formation. At high concentrations collagen synthesis is inhibited probably at the level of transcription of the collagen gene. [146]

Taking into consideration all the above-mentioned effects of PG's on bone, it is likely that EFAs, which are PG modulators, will also have potent bone-stimulating actions. EFA deficiency leads to typical patterns such as a decrease in the levels of palmitoleic and dihomogamma-linolenic acid (DGLA) with profound inhibiting effects on the level of development and degree of mineralisation of the bone. [147]

Katz et al. [148] demonstrated the effects of various EFAs at different concentrations. DGLA could not stimulate bone resorption at low concentrations, but inhibited resorption at a concentration of $10^{-4}$ M. Arachidonic acid (AA) also inhibited resorption at $10^{-4}$ M, but at lower concentrations $10^{-5}$ - $10^{-7}$ M stimulated active resorption. This effect of AA can be related to a rise in $PGE_2$. The concentration of PG was the highest in the first 24 hours, unless these were removed or suppressed by

indomethacin, no response to exogenous $PGE_2$ could be demonstrated. [148] Besides the resorptive effect of AA it was also noted by Raisz et al. [149] that AA stimulated calcium entry into the osteoblast at low concentrations, but inhibited entry at higher concentrations. EPA is also responsible for bone resorption in organ culture, [149] EPA is a much less effective precursor for $PGE_3$ than AA is for $PGE_2$. It has been found recently that EPA could stimulate bone formation in chicks associated with a decrease in $PGE_2$ production. [150]

Zinc is a major co-factor in the metabolism of EFAs and it is apparently required for the first rate-limiting step, the delta-6-desaturation of LA and ALA. [138,151] It is therefore likely that zinc could assist EFAs with the development and mineralisation of bone. Odutuga. [152] showed that low zinc status accentuated signs of EFA deficiency such as reduction in growth rate and reduced weight of bones. Vitamin E, which protects EFAs from peroxidation, may enhance the effects of EFAs in chicks. [153] It was shown in 1994 that dietary modification could alter the levels of fatty acids in rat alveolar bone. [147]

It is therefore important to investigate the influence of different ratios of their metabolites GLA (n-6) and EPA (n-3). [133]

The effect of ratio supplementation on bone metabolism was investigated by Claassen et al. [133] with important results. Studies where EPA has been supplemented in combination with DHA and GLA have also shown positive

effects on bone metabolism. Both EPA and DHA supplementation prevented an increase in bone fragility that can be expected in diabetic, osteopenic rats [154] respectively. Studies done on male rats where different ratios of GLA:EPA + DHA were supplemented [155] gave interesting results where it would appear that the supplementation with the ratio 3:1 gave the best effect on bone calcium content. Pyridium cross-link excretion was significantly decreased, with reduced hydroxyproline levels, both sensitive markers for bone resorption. These results suggest that supplementation of EFAs, with the ratio 3:1 is the most effective in inhibiting bone resorption in the male rat. [156]

However, in the ovariectomised female rat, a trend of the 1:3 supplemented ratio of GLA:EPA + DHA to increase bone parameters was shown [157] There was a strong correlation between DGLA, DHA and EPA and bone $Ca^{2+}$ and a reduction in deoxypyridinoline excretion. [158]

The possible explanation for such a difference between the male and female animal models, may be the level of $PGE_2$ generated. OVX causes a profound increase in $PGE_2$ levels, contributing to bone resorption. Supplementation with n-3 EFAs competitively inhibits AA production from precursor metabolites and therefore inhibits the production of $PGE_2$. Inhibition of $PGE_2$ production might be instrumental in preventing bone loss as well as calcinosis. [132,159]

# CHAPTER 3

# GENERAL MATERIALS AND METHODS

## 3.1 Materials

Reagents were obtained from Sigma (Pty.) Ltd. (St. Louis, USA), Saarchem (Pty.) Ltd. S.A. Scientific, Sterilab Services cc, Zymed Laboratories Inc. (California, USA). All chemicals used were of the purest grade. Holpro (Pty.) Ltd. (SA) provided the mineral salt and the vitamin mixture was a generous gift from Truka (Pty.) Ltd. (SA). Pan Vera Corporation (Wisconsin, USA) provided us with recombinant vitamin $D_3$ receptor and the 1α, 25-dihydroxy [26,27-methyl-$^3$H] cholecalciferol was obtained from AEC Amersham (Pty.) Ltd. The laboratory of Prof. H.F. DeLuca, University of Wisconsin, Madison WI, generously donated the monoclonal antibodies IVG 8CII and VD2F12. (USA). Scotia Pharmaceuticals (Pty.) Ltd. (UK) manufactured and supplied all the essential fatty acid dietary supplements.

## 3.2 Methods

### 3.2.1 Ovariectomy Study

Female Sprague Dawley rats (age = 21± 2 days; n = 40) were obtained from the University of Potchefstroom. On arrival they were randomly divided into 4 groups (n = 10) and fed a fat-free semi-synthetic diet and

demineralised water ad lib for one week. The rats used for each dietary group had no familial relation. Rats were kept separately in hanger cages in a temperature – and day/night – controlled room at the Pretoria Biomedical Research Centre.

The experiment lasted for 15 weeks. After one week, the rats, aged 28 days, from all the different groups were fed 13g of a semi-synthetic diet containing 8% fat and 1% calcium (Table 1). The different oils were mixed into the diets daily. Gamma-linolenic acid (18:3n-6) and eicosapentaenoic acid and docosahexaenoic acid (EPA and DHA) were supplemented in ratios of 3:1 and 1:3 (w/w). Linoleic acid (LA in sunflower oil) and α-linolenic acid (ALA in linseed oil) were supplemented as a control in the ratio 3:1 (v/v) to a sham control as well as OVX control group, while ratios were only supplemented to OVX groups (Table 2). Aliquots (15 ml) of the EFA mixtures were stored under nitrogen to prevent their oxidation.

At age = 77 days, experimental rats underwent a bilateral ovariectomy (OVX) (3 groups; n = 10) or sham operation (1 group; n = 10) that was done from the dorsal approach. Anaesthesia was induced by inhalation of 1,5 – 2% halothane.

Food supplementation was increased to 15g dry food according to Wronski et al. [160] one week after the ovariectomy until the end of the experiment, the purpose being to prevent OVX induced weight gain. Animals were sacrificed at 18 weeks (age = 126 days) by cardiac puncture after being

anaesthetised with a mixture of sodium pentobarbitone 6% m/v (Sagatal; Rhone-Poulenc, SA) and demineralised water.

**Table 1: Basic semi-synthetic diet**

| Ingredients | Mass concentration (g/kg diet) |
|---|---|
| Sugar | 550 |
| Vitamin mixture | 10 |
| Salt mixture | 50 |
| Cellulose | 10 |
| Casein | 150 |
| Maize flour | 100 |
| Water | 50 |
| Supplemented oil | 80 |

**Table 2: Supplementation of the different essential fatty acid ratios to the four different groups**

| Groups | EFA ratio | Procedure |
|---|---|---|
| 1 | LA/ALA 3:1 (control) | Sham |
| 2 | LA/ALA 3:1 (control) | OVX |
| 3 | GLA/ EPA+DHA 3:1 | OVX |
| 4 | GLA/ EPA+DHA 1:3 | OVX |

### 3.2.2 Sampling Procedures

#### 3.2.2.1 Serum analysis

The successfulness of the ovariectomy was verified by visualising uterus atrophy or weighing of the ovarian tissue at the time of sacrifice as well as serum oestrogen levels. Whole blood (2.5 ml) was drawn from the vena cava inferior, allowed to coagulate and then centrifuged at 2300 rpm for 10 minutes to separate erythrocytes and serum. The serum was frozen away at -20°C until analysis.

#### 3.2.2.2 Plasma and erythrocyte fatty acid profile

EDTA-blood (2.5 ml) was drawn from the inferior vena cava and stored on ice. The samples were then centrifuged at 2300 rpm for 10 minutes to separate the plasma and the erythrocytes. The plasma was siphoned off and stored at –70° C for later analysis. The remaining erythrocytes were washed with an equal volume of 0.9% NaCl and centrifuged for a further 10 minutes at 2300 rpm. Following centrifugation, white blood cells and saline were removed and remaining erythrocytes were washed again in 0.9% NaCl and then stored at 4° C for later analysis of the fatty acid profile.

### 3.2.2.3 Femur Analysis

Right femurs were dissected out following sacrifice, stripped of the soft tissue using gauze and stored for later analysis at 4° C. After the final experimental procedure, all femurs were ashed for 8 hours in a muffle furnace at 550° C. Following the ashing, femurs were weighed, measured and dissolved in 2 ml 6N HCl after excess debris had been removed with a soft paint brush. Of this mixture, 20 µl was added to 8 ml of demineralised and deionised water (400x dilution). Left femurs were dissected out following sacrifice, stripped of the soft tissue using gauze so that the bone's surface remained unmarked and stored in 70% ethanol at 4° C.

### 3.2.2.4 Isolation of samples for $Ca^{2+}$ ATPase studies

The rats were not fasted before determinations. The proximal second 5 cm of the duodenum was removed from the rats under anaesthesia, and rinsed with ice-cold saline (0.9% NaCl), slit open along the mesenteric line, and flushed again with saline. The intestinal mucosa was gently scraped with a glass slide to remove the cells. The scrapings were collected directly into a centrifuge tube with 10 ml ISP buffer (10 mM Imidazole, 0.32 M Sucrose, pH = 7.4) containing 0.2 mM PMSF (0.1 M phenyl methylsulfonyl fluoride in isopropanol-stock) and stored on ice. The scrapings were homogenised 2 x 15 seconds in an Ultrathorax polytron and centrifuged at 2000 rpm for 15

minutes at 4° C. The supernatant, containing partially purified basolateral membranes, was decanted and stored at –70°C. The membrane protein concentration, was measured by using the method of Biorad. The Biorad assay, based on the Bradford dye-binding procedure [161] is a simple colorimetric assay for measuring total protein concentration (see manufacturer's manual – Biorad, 1990)

### 3.2.2.5 Isolation of samples for vitamin $D_3$ receptor studies

The proximal first 5 cm of the duodenum was removed from the rats under anaesthesia, and rinsed with ice-cold TED-Saline (TED: 50 mM Tris-HCl, pH = 7.4, 1,5 mM EDTA, 5 mM dithiotreitol + 150 mM NaCl), slit open along the mesenteric line, and flushed once more with TED-Saline. The intestinal mucosa was gently scraped with a glass slide to remove the cells, after which it was rinsed once with two volumes of TED-Saline by suspension and centrifugation at 1000 rpm for 5 minutes. Then a 33% (v/v) homogenate was prepared in TED-K300 buffer (TED + 300 mM KCl, 1 mM phenylmethylsulfonyl fluoride) with a glass-teflon homogeniser. This homogenate was centrifuged at 9 000 x g at 4° C for 30 minutes. The supernatant, minus the fluffy lipid layer, was quick frozen in liquid nitrogen and stored at –70° C. The membrane protein concentration was measured by using the method of Biorad. The Biorad assay, based on the Bradford dye-binding procedure [161] is a simple colorimetric assay for measuring total protein concentration (see manufacturer's manual – Biorad 1990).

### 3.2.3 Experimental Procedures

#### 3.2.3.1 Determination of Oestrogen levels

Serum oestrogen levels were quantitatively measured using the $I^{125}$ radio-immunoassay of Double Antibody Estradiol from Diagnostics Products Corporation, Los Angeles.

#### 2.2.3.2 Determination of plasma essential fatty acid content

Fatty acid analyses were done by Dr. Marius Smuts, National Research Programme for Nutritional Intervention. In short the methods were as follows: Lipids were extracted from the plasma with chloroform/methanol (2:1,v/v). Heptadecanoic acid was added to each of the fractions and acted as an internal standard. Fatty acid methyl esters were then prepared from these fractions and analysed by gas chromatography. [162,163] Individual fatty acid concentrations were calculated from response factors obtained from a standard fatty acid mixture (14:0 – 22:6). [164]

#### 3.2.3.3 Determination of Erythrocyte membrane EFA content

Erythrocytes were prepared for fatty acid analysis by haemolysis with different phosphate buffers. [165,166] Lipids were extracted from erythrocyte membranes with chloroform/methanol (2:1, v/v). [167] Fatty acid methyl esters

were then prepared by transmethylation of an aliquot of the extract with 2.5 ml methanol-18M sulphuric acid (95.5,v/v) at 70° C for two hours. The esters were then analysed on a Varian model 3700 Gas Liquid Chromatograph using silica megabore DB 225 columns (J&W Scientific, Folsom, CA, USA). [162] Individual fatty acid methyl esters were identified by comparison with the retention times of a standard mixture of free fatty acids (14:0 – 22:6). Erythrocyte membrane fatty acid composition was quantified using heptadecanoic acid (17:0) as internal standard

### 3.2.3.4 Determination of PTH levels

The PTH levels were determined by using the Rat PTH (IRMA) kit from the Nichols Institute Diagnostics, San Juan Capistrano, CA. Two different goat antibodies to the N-terminal region of rat PTH had been purified by affinity chromatography. One of the antibodies was immobilised onto plastic beads to capture the PTH molecules and the other was radiolabelled for detection. The sample containing the PTH was incubated simultaneously with an antibody-coated bead and the $^{125}$I-labelled antibody. Both intact PTH and N-terminal PTH contained in the sample were immunologically bound by both the immobilised antibody and the radiolabelled antibody to form a sandwich complex. At the end of the incubation period, the bead was washed to remove any unbound labelled antibody and other components. The radioactivity bound to the bead was then measured in a gamma counter. The radioactivity of the antibody-complex bound to the bead is directly

proportional to the amount of PTH in the sample. Plotting the CPM versus the respective PTH concentration (pg/ml) for each standard on logarithmic scales generated a standard curve. The concentration of PTH in the samples was determined directly from this curve.

### 3.2.3.5 Femur Analysis

Femur calcium concentration was determined using atomic absorption spectroscopy.

### 3.2.3.6 Bone density measurement

Bone density was measured of the single bones using the Small Animal Program supplied by Hologic ® USA, on the Hologic ® QDR 1000 W DXA machine. Femurs were submerged in 1 cm water for scanning.

### 3.2.3.7 $Ca^{2+}$- $Mg^{2+}$ ATPase Study

$Ca^{2+}$- $Mg^{2+}$ ATPase activity was measured by the release of inorganic phosphate, following incubation of partially purified basolateral membranes in a medium containing 250 mM Imidazole, 15 mM $MgCl_2\,6H_2O$ (IM Buffer); 5 mM Ouabain and 5mM EGTA in the presence and absence of 1 μM free calcium. Tubes prepared according to the protocol, were incubated for 5 minutes at 37° C. After adding 100 μl ATP (3 mM ATP.$Na_2$, final concentration) all tubes were vortexed and incubated for 10 minutes at 37°C

to start the enzyme reaction. The reaction was stopped by adding 750 µl ice cold 8.3% TCA (Trichlor acetic acid) to the tubes, and placing the tubes on ice for 5 minutes. All tubes were then centrifuged at 2000 rpm at 4° C. 500 µl of the resulting supernatant was transferred to 1 ml distilled water. Liberated inorganic phosphate was then complexed by adding 500 µl of an ammonium molybdate solution (5% ammonium molybdate and 60% perchloric acid; 4:1), 3 ml Isobuthanol:benzene (1:1) was added and vortexed for 10 seconds to separate the two phases, tubes were then centrifuged for 30 seconds in a bench centrifuge. 2 ml of the upper phase was transferred to a clean set of tubes, after which 1 ml 96% Ethanol:98% $H_2SO_4$ (33:1) was added. The complexed phosphate was reduced with $Sn^{2+}$ by adding 200 µl $SnCl_2$ solution (40% $SnCl_2$ in 32% HCl; diluted daily 1:200 with water) for the colour reaction. Each tube was vortexed for 10 seconds after $SnCl_2$ had been added. Optical density was read at 715 nm against the blank.

Table 3: **$Ca^{2+}$- $Mg^{2+}$ ATPase activity determination on duodenal basolateral membranes.**

| Solutions | Blank | Standard EGTA $KH_2PO_4$ | EGTA enzyme Blank | $Ca^{2+}10^{-6}$ enzyme |
|---|---|---|---|---|
| IM Buffer | 100 µl | 100 µl | 100 µl | 100 µl |
| 5 mM Ouabain | 100 µl | 100 µl | 100 µl | 100 µl |
| 5 mM EGTA | 100 µl | 100 µl | 100 µl | |
| 1mM $Ca^{2+}$ in EGTA | | | | 100 µl |
| ISP Buffer | 100 µl | | | |
| Enzyme in ISP Buffer | | | 100 µl | 100 µl |
| $KH_2PO_4$ (0.1 µmol) | | 100 µl | | |
| Total volume | 400 µl | 400 µl | 400 µl | 400 µl |

### 3.2.3.8 Vitamin $D_3$ receptor availability study – ELISA

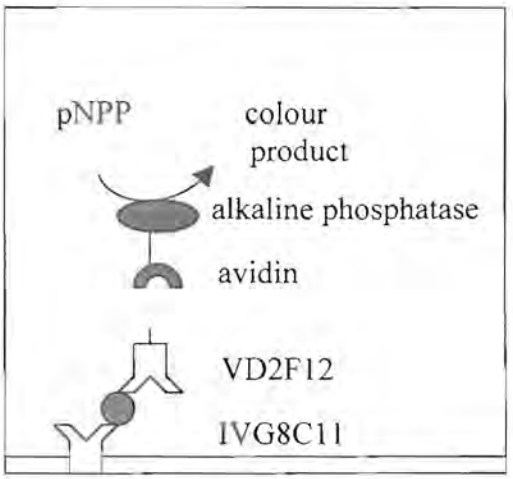


Figure 8: **Schematic assay depicting the antibody binding to the VDR. Derived from Uhland-Smith et al. [168]**

The wells of polystyrene ELISA plates (Costar RIA plate, Sterilab) were coated with 50µl of the monoclonal antibody IVG8C11 – 20 µg/ml in a carbonate/bicarbonate buffer (40 mM $Na_2CO_3$, 60 mM $NaHCO_3$, pH 9.6) for two hours at 37° C. The remaining protein binding sites were blocked with 5% NFDM (non-fat dry milk) in a carbonate/bicarbonate buffer, pH 9.6 for two hours at 37° C. After this step, the plates were washed three times with TBST (TBS: 140 mM NaCl, 3 mM KCl, 50 mM Tris-HCl, pH 8 + 0.05% Tween-20, at room temperature). 50 µl of the biotinylated antibody VD2F12 was added, 0.01 mg/ml in dilution buffer (1% NFDM, 0.05% Tween-20, 0.05% $NaN_3$ in TBS, pH 8.0 + 5mM DTT added fresh before each assay). A dilution range in dilution buffer was made of the Pan Vera recombinant receptor (750 pmol) serial diluted to 10 000 and then 100 fmol in dilution buffer, with a starting concentration of 0 fmol and an end concentration of 50 fmol. (Table 4)

**Table 4** **Dilution range of the standard Pan Vera recombinant receptor.**

| [Std.recep.]=100 fmol (µl) | 0 | 50 | 62.5 | 75 | 87.5 | 100 | 112.5 | 125 | 150 | 175 | 200 | 250 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Dilution Buffer (µl) | 500 | 450 | 437.5 | 425 | 412.5 | 400 | 387.5 | 375 | 350 | 325 | 300 | 250 |
| Receptor concentration (fmol) | **0** | **10** | **12.5** | **15** | **17.5** | **20** | **22.5** | **25** | **30** | **35** | **40** | **50** |

100 µl of each standard dilution as well as 100 µl of each sample, derived from the animals (diluted 1:10 with dilution buffer) was added after the biotinylated antibody and incubated overnight at 4° C. The next morning the plates were washed three times, using TBST at 4° C. 100 µl of the avidin-

alkaline phosphatase conjugate was added, diluted 1:1000 in dilution buffer. The plates were incubated for 2 hours at 37° C. After 2 hours the plates were washed four times with TBST (at room temperature). 100 µl substrate pNPP (p- nitrophenyl phosphate) (100mg/ml diluted 1:100 with 0.75 M 2-amino-2-methyl-propandiol) was added, and the reaction was stopped after 70 minutes with 100 µl of a 0.1 M EDTA solution, pH 8.0. The absorbency was read on a dual wavelength 410, 650 nm on an automatic plate reader (Analytical and Diagnostic Products). The samples containing the VDR were calculated from the standard curve.

### 3.2.3.9 Vitamin $D_3$ receptor binding study – Hydroxylapatite Assay

A 50% slurry of hydroxylapatite was prepared by adding 10g of Bio-Gel HTP (Biorad) to 60 ml of TE buffer (50 mM Tris-HCl, 1.5 mM EDTA, pH 7.5), with gentle swirling. The suspension was allowed to settle for 10 minutes and the supernatant decanted off. Fresh buffer was added and the hydroxylapatite resuspended and allowed to settle two more times. The final slurry was allowed to equilibrate overnight at 4° C. By carrying out the equilibration in a graduated flask, a 50% slurry was made by adding a volume of buffer equivalent to the volume of the resin. Before each use the settled hydroxylapatite was resuspended by gentle swirling. VDR samples (diluted 1:10 with binding buffer; (50 mM Tris-HCl, 1.5 mM EDTA, + 300 mM KCl, pH 7.5) was added to $^3$H 1α, $25(OH)_2D_3$ (2 nM) in the presence - Non-specific binding or absence - Total binding of >400 molar excess non-radioactive 1α, $25(OH)_2D_3$ and vortexed to begin the reaction. The

incubation was carried out overnight at 4° C and terminated by moving the incubation tubes to an ice bath and immediately adding 100 µl HAP slurry. The tubes were vortexed and left on ice for 15 minutes with vortexing every 5 minutes. The samples were then centrifuged for 5 minutes at 4° C. The hydroxylapatite pellets were washed three times with VDR Wash buffer (50 mM Tris-HCl, 0.5% Triton - X100, 1.5 mM EDTA, pH 7.5) by vortexing and centrifuging as above. The final washed hydroxylapatite pellet was transferred quantitatively to scintillation vials. 4 ml scintillation fluid was added per vial and counted. Two "total" samples of 3.12 µl hot VDR + 100 µl HAP slurry + 400 µl ethanol was included to get total possible counts.

The calculation of the concentration of vitamin D receptor that specifically bound to 1α, 25$(OH)_2D_3$ is as follows:

Total binding - non-specific = binding specific binding (dpm).

DPM x fmol/ 352 dpm (receptor conversion) = fmol/ 100 µl

(100 µl the amount of receptor sample in the tube) x100

(dilution factor = 10) to convert to fmol/ ml.

Divide the answer by the protein concentration (mg/ml) to get a final answer of fmol/ mg prot.

### 3.2.4 Statistical Analysis

#### One-way Anova

Anova assumes normal distribution, consequently data were tested for normality using Bartlett's test. When Anova revealed a difference, the

location of that difference was determined using a LSD multiple comparison test. If data were not normally distributed, a Van Wanderen transformation was performed on the data. Data were again tested for normality using Bartlett's test to confirm normal distribution before the LSD multiple comparison test was performed.

# CHAPTER 4

# RESULTS

## 4. Ovariectomy study

### 4.1 Oestrogen content

Serum oestrogen concentration (pmol/l) was determined for each of the four groups and is illustrated in Figure 9. The OVX control (34.3 ± 23.07), OVX 3:1 (26.9 ± 8.05) and OVX 1:3 (22.5 ± 13.60) groups had lower serum concentrations of oestrogen when compared to sham control (99.2 ± 50.83) group.

### 4.2 Uterus mass

Uterus mass (mg) was determined for each rat and is illustrated in Figure 9. Uterus mass was found to be significantly lower in the OVX control (141.9 ± 64.49), OVX 3:1 (190.66 ± 83.95) and OVX 1:3 (155.01 ± 46.62) groups when compared to sham (635.35 ± 118.05). The low oestrogen levels and low uterus mass confirm the model of induced ovariectomy.

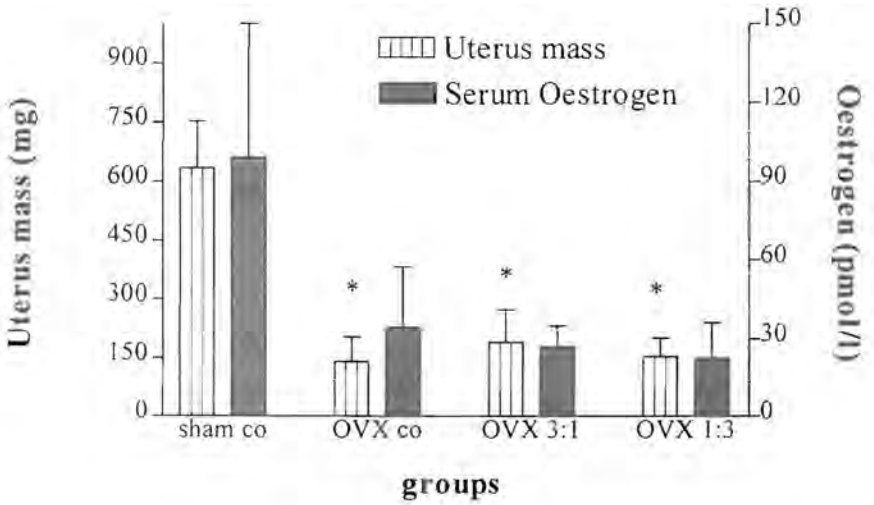


**Figure 9:** Uterus mass (mg) and serum oestrogen concentration (pmol/l) of the four different groups after 15 weeks of EFA supplementation. (* $p < 0.05$ compared to sham control.)

### 4.3 Plasma essential fatty acid content

Essential fatty acid content of the plasma was measured in each of the four groups (Table 5). Table 5 also depicts the relative ratios of n-6 (18:3) to n-3 (20:5 and 22:6) EFAs in the rat's plasma in response to the different diets. When sham was compared to the OVX control, the sham group showed significantly higher levels of 18:2n-6, 18:3n-3 and 22:6n-3 than the OVX control, it was only for the 20:4n-6 EFA profile that the sham group showed significantly lower values compared to OVX control. The OVX 1:3 group showed significantly higher levels of 20:5n-3 (EPA) and 22:6n-3 (DHA) when compared to sham and the OVX control. For the 18:3n-6 series both

OVX 3:1 and OVX 1:3 groups showed significantly higher values compared to sham and OVX control, while for the 18:3n-3 profile, the values for both OVX 3:1 and 1:3 groups were significantly lower when compared to the sham- and OVX control.

### 4.4 Erythrocyte membrane essential fatty acid content

The erythrocyte membrane essential fatty acid concentrations were determined for each of the four groups and are depicted in table 6. For the 18:3n-6 and 20:3n-6 EFA profiles, both OVX 3:1 and OVX 1:3 groups showed significantly higher values compared to sham and OVX control. The OVX 1:3 group showed significantly lower values than the sham and OVX control in both 20:4n-6 and 22:5n-6 profiles, but showed significantly higher values in both 20:5n-3 and 22:6n-3 profiles. These results correlate with the plasma EFA results, where the same pattern was seen. Lower levels were observed in the OVX 3:1 group for 22:5n-3 compared to the sham and OVX control, while the OVX 1:3 group had higher levels. The OVX 3:1 group also had higher levels of 22:5n-6, than the OVX 1:3 group when compared to the sham and OVX control. Lastly, in the 20:5n-3 and 22:6n-3 series, lower levels were observed for the OVX 3:1 group when compared to sham.

**Table 5:** Mean(standard deviation) of plasma EFA concentrations (µg/ml) in the 4 different groups. (* = p<0.05 compared to the sham control; + = p<0.05 compared to the OVX control)

| Fatty acid | Sham | OVX | | |
|---|---|---|---|---|
| | Control | Control | 3:1 | 1:3 |
| 18:2n-6 | 21.75(2.47)+ | 18.45(2.70)* | 20.97(1.78) | 17.49(2.66)* |
| 18:3n-6 | 0.24(0.09) | 0.25(0.08) | 1.51(0.14)*+ | 1.13(0.26)*+ |
| 18:3n-3 | 1.34(0.35)+ | 1.00(0.27)* | 0.13(0.05)*+ | 0.20(0.06)*+ |
| 20:3n-6 | 0.44(0.05) | 0.46(0.07) | 0.56(0.17)* | 0.95(0.15)*+ |
| 20:4n-6 | 27.47(3.42)+ | 31.11(3.29)* | 33.79(2.79)* | 26.11(3.06)+ |
| 20:5n-3 | 0.86(0.11) | 0.77(0.18) | 0.76(0.07)* | 5.02(1.26)*+ |
| 22:5n-6 | 0.05(0.04) | 0.03(0.03) | 0.10(0.09) | 0.04(0.04) |
| 22:5n-3 | 0.43(0.07) | 0.49(0.07) | 0.40(0.05)+ | 1.14(0.11)*+ |
| 22:6n-3 | 3.72(0.60)+ | 3.01(0.41)* | 3.26(0.31) | 4.75(0.53)*+ |
| EPA + DHA: GLA | 19.08 | 15.12 | 2.67 | 8.65 |

**Table 6:** Mean(standard deviation) of erythrocyte membrane EFA concentrations (µg/ml) in the 4 different groups. (* = p<0.05 compared to the sham control; + = p<0.05 compared to the OVX control)

| Fatty acid | Sham | OVX | | |
|---|---|---|---|---|
| | Control | Control | 3:1 | 1:3 |
| 18:2n-6 | 9.35(1.03) | 9.19(0.52) | 9.14(0.46) | 9.03(0.72) |
| 18:3n-6 | 0.04(0.02) | 0.04(0.03) | 0.19(0.04)*+ | 0.19(0.03)*+ |
| 18:3n-3 | 0.25(0.14) | 0.23(0.07) | 0.15(0.06) | 0.17(0.1) |
| 20:3n-6 | 0.44(0.22) | 0.35(0.12) | 0.70(0)7)*+ | 0.83(0.05)*+ |
| 20:4n-6 | 26.45(4.25) | 26.81(3.61) | 26.72(0.56) | 21.37(0.97)*+ |
| 20:5n-3 | 0.48(0.09) | 0.43(0.10) | 0.38(0.04)* | 3.05(0.32)*+ |
| 22:5n-6 | 0.34(0.03)+ | 0.38(0.04)* | 0.67(0.03)*+ | 0.10(0.05)*+ |
| 22:5n-3 | 2.09(0.30) | 2.20(0.18) | 1.43(0.13)*+ | 3.56(0.25)*+ |
| 22:6n-3 | 3.63(0.64)+ | 2.89(0.34)* | 2.98(0.24)* | 4.60(0.50)*+ |
| EPA + DHA: GLA | 102.75 | 83.00 | 17.68 | 40.26 |

## 4.5 PTH levels

The PTH levels (pg/ml) of the four groups are shown in figure 10. There was an increase in PTH levels in both OVX co. (154.1 ± 72.8) and OVX 3:1 (150.9 ± 61.6) groups when compared to the sham co group (111.2 ± 74.6), though not statistically significant. The OVX 1:3 (141.3 ± 74.4) group had lower values than the other two OVX groups, but still higher than the sham control.

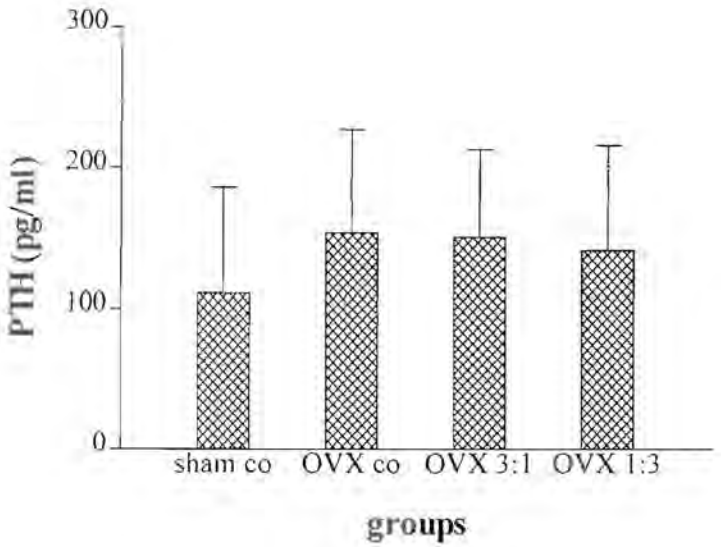


**Figure 10:** PTH levels (pg/ml) for the four groups after 15 weeks of EFA supplementation.

## 4.6 Bone status

Femur calcium (mg/femur) was recorded for each group and is depicted in figure 11. A decrease in calcium was found in the OVX control (96.9 ± 4.6) group as well as in the OVX 3:1 group (96.9 ± 5.1) when compared to sham

control (101.1 ± 4.9). The OVX 1:3 group (101.8 ± 4.1) showed an increase compared to OVX control, back to sham control values. Femur densities of the four groups are also shown in figure 11. Bone density decreased from sham (0.0947 ± 0.0063) to OVX control (0.0919 ± 0.0049) as could be expected. The OVX 3:1 group (0.0903 ± 0.0025) decreased compared to the sham control (0.0947 ± 0.0063). The OVX 1:3 group had a bone density of 0.0941 ± 0.0036) increasing back to sham levels and higher than OVX control. All changes were significant to the level of $p < 0.1$, therefore a definite trend.

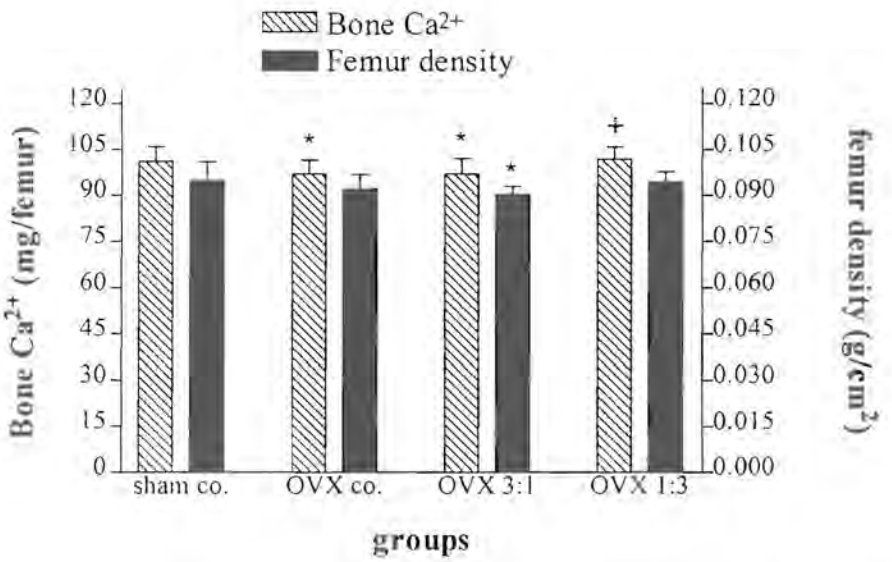


**Figure 11:** Bone $Ca^{2+}$ (mg/femur) and femur density ($g/cm^2$) of the four different groups after 15 weeks of EFA supplementation (* $p < 0.1$ compared to sham control; + $p < 0.1$ compared to OVX control.)

## 4.7 $Ca^{2+}$- ATPase activity

$Ca^{2+}$ ATPase activity (µmol Pi/mg prot/min) values are depicted in figure 12. A significant decrease in activity can be observed in the OVX control group (0.046 ± 0.011) when compared to sham control (0.059 ± 0.008). In the dietary group OVX 1:3 (0.071 ± 0.03) there was a significant increase of 54% in activity compared to the OVX control (0.046 ± 0.011), and a 20% increase compared to sham control (0.059 ± 0.008).

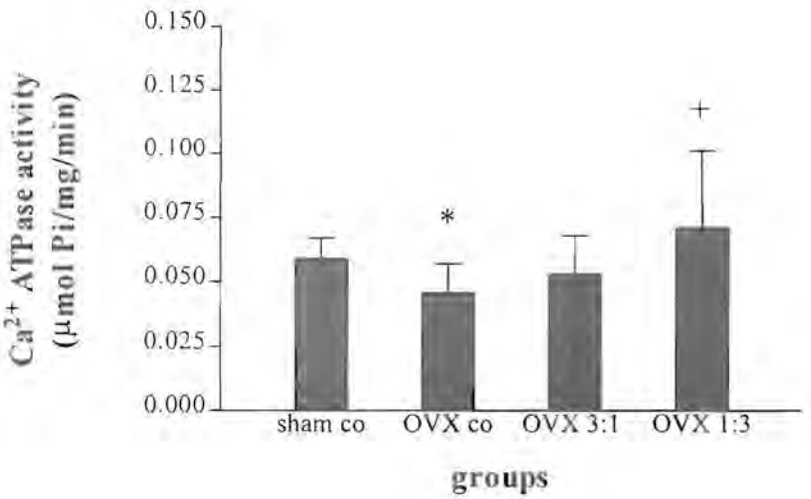


**Figure 12:** $Ca^{2+}$-ATPase activity (µmol Pi/mg prot./min) of the four different groups. * $p < 0.05$ compared to the sham control. + $p < 0.05$ compared to OVX control.

## 4.8 Vitamin $D_3$ receptor availability – ELISA

The vitamin $D_3$ receptor availability was measured (fmol/mg prot), the concentrations are shown in figure 13. There was a significant increase in the number of vitamin $D_3$ receptors available for binding in the OVX

control group (826.68 ± 188.6), compared to the sham control (624.29 ± 520.9) group. The OVX 1:3 group (373.21 ± 113.0) had a significantly lower number of receptors available (55%) compared to OVX control (826.68 ± 188.6) group. There was no significant difference in availability of receptors between the OVX 1:3 group (373.21 ± 113.0) and the sham control (624.29 ± 520.9), nor was there any difference between the OVX 3:1 (818.84 ± 314.21) group and the OVX control (826.68 ± 188.6).

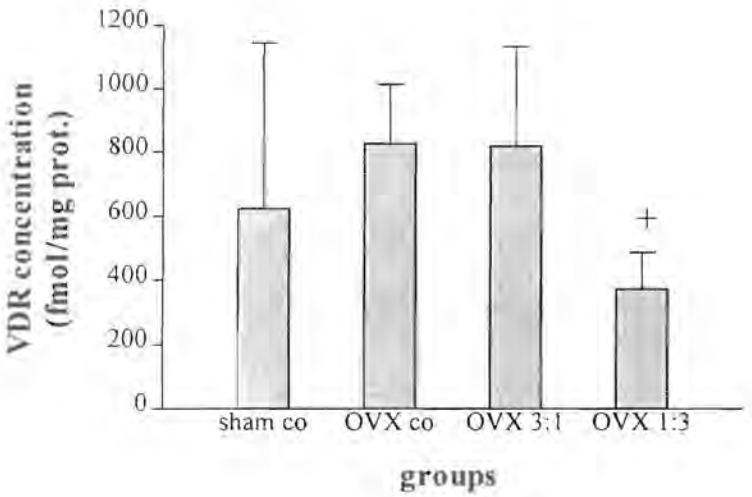


**Figure 13:** Vitamin $D_3$ receptor availability (fmol/mg prot) of the four different groups. + = $p < 0.05$ compared to the OVX control.

## 4.9 Vitamin $D_3$ receptor binding – HAP ASSAY

Figure 14 depicts the concentrations (fmol/mg prot) of vitamin $D_3$ receptors that bound to $^{3H}1,25(OH)_2D_3$. There was no significant difference in the number of receptors actively binding to their steroids between the OVX control group (24.674 ± 4.9) and the sham control group (35.24 ± 15.2). The OVX 1:3 (12.17 ± 7.7) group however, showed a significant decrease in

binding (51%), compared to both the OVX control (24.67 ± 4.9) and the sham control group (35.24 ± 15.2). The OVX 3:1 (14.4 ± 7.9) had significantly lower concentrations of VDR binding compared to the sham control (35.24 ± 15.2) group.

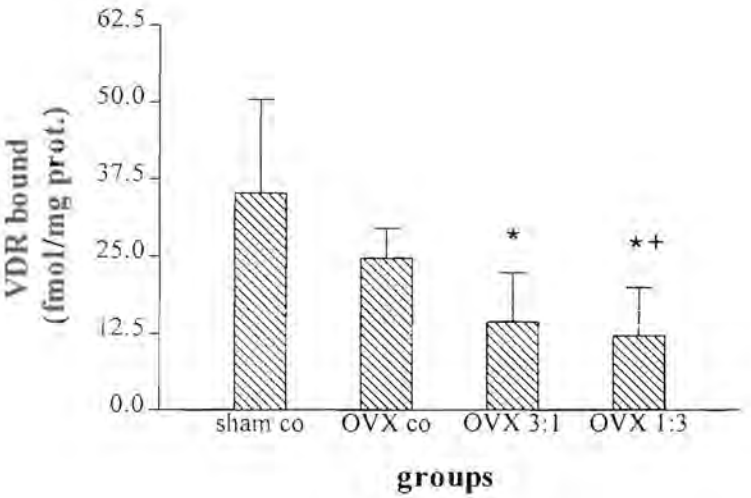


**Figure 14:** VDR binding (fmol/mg prot) for the four different groups. * = $p < 0.05$ compared to the sham control, + = $p < 0.05$ compared to the OVX control.

# CHAPTER 5

# DISCUSSION

## 5. Ovariectomy Study

Based on earlier effects on bone metabolism obtained with the supplementation with EFAs of ovariectomised female rats, [8] we decided to investigate the effects that EFA supplementation might have on the active extrusion of $Ca^{2+}$ at the BLM via an ATP-dependent $Ca^{2+}$ pump and the concentration or binding of the VDR that would lead to a change in $Ca^{2+}$ metabolism.

### 5.1 The OVX female rat model

The uterus mass (mg) as well as the oestrogen blood levels were measured as shown in figure 9. to test if the model of induced ovariectomy was indeed successful. In all three OVX groups including OVX control, OVX 3:1 and OVX 1:3 the effect of the OVX was to reduce the uterus mass by approximately 70%.

The oestrogen levels were significantly lower in all three OVX groups compared to the sham control group. This illustrates that the ovariectomy was successful and thoroughly executed.

## 5.2 Blood analysis

The erythrocyte membrane fatty acid levels were used as a measure to investigate the amount of fatty acids incorporated into membrane phospholipids, where it exerts an effect, and the amount of fatty acids in the plasma to determine the degree of absorption of fatty acids in the small intestine (Table 5 and 6).

For the n-6 EFA family both the OVX 3:1 and OVX 1:3 supplemented groups showed significantly higher values for GLA as well as higher values for DGLA in the plasma compared to sham and OVX control groups. GLA is found in only small quantities in the body as it is slowly formed by D6D and rapidly metabolised to DGLA by elongation. [112] The importance of GLA has been totally overshadowed by EPA and DHA, but experimental evidence have proven the importance of the n-6 family. [125,126] Claassen et al. [133] have found that supplementation of GLA and EPA in an appropriate ratio may be of benefit in enhancing $Ca^{2+}$ balance, and GLA and EPA are more potent modulators of $Ca^{2+}$ metabolism than their precursors. The n-3 and n-6 EFAs are competitive inhibitors of each other's metabolism, [113,114] but on the whole the n-3 EFAs are

more effective at inhibiting the desaturation of the n-6 EFAs than vice versa. OVX seems to increase the metabolism of LA into AA, which is reversed by the supplementation of the 1:3 ratio. Levels of AA return to sham levels but still higher levels of GLA and EPA are maintained in the 1:3 group. The AA levels are significantly reduced in the OVX 1:3 that proves the fact that dietary EPA can reduce the AA levels by inhibiting the delta-5-desaturase of the n-6 family. [110] The ratio of GLA:EPA + DHA is the highest in the OVX 1:3 group compared to OVX 3:1.

The erythrocyte membrane fatty acid levels were very similar to that found in the plasma. The GLA and DGLA levels were significantly higher in the OVX 3:1 and OVX 1:3 supplemented groups compared to the sham and OVX control groups. The AA in the OVX 1:3 group was significantly lower than in all three of the other groups, which again proves that EPA can block GLA metabolism to AA. The EPA metabolism to DHA is significantly higher in the erythrocyte and the plasma of the OVX 1:3 group when compared to sham and OVX control groups. The GLA:EPA + DHA ratio increases 5 to 6-fold in the OVX 1:3 group compared to that seen in the plasma, which is proof for the fact that n-3 EFAs are more readily incorporated into membranes. Such modulation of the n-6 and n-3 EFAs may lead to changes in prostaglandin synthesis with the balance being shifted from the 2-series towards the 1- and 3-series. [110] Figure 15 shows that DGLA that is converted to $PGE_1$, inhibits the release of AA from the membrane, so that levels of AA decrease. 15-OH DGLA inhibits lipoxygenase

while EPA inhibits cyclo-oxygenase that shifts the balance of PG synthesis towards the 1 or 3 series.

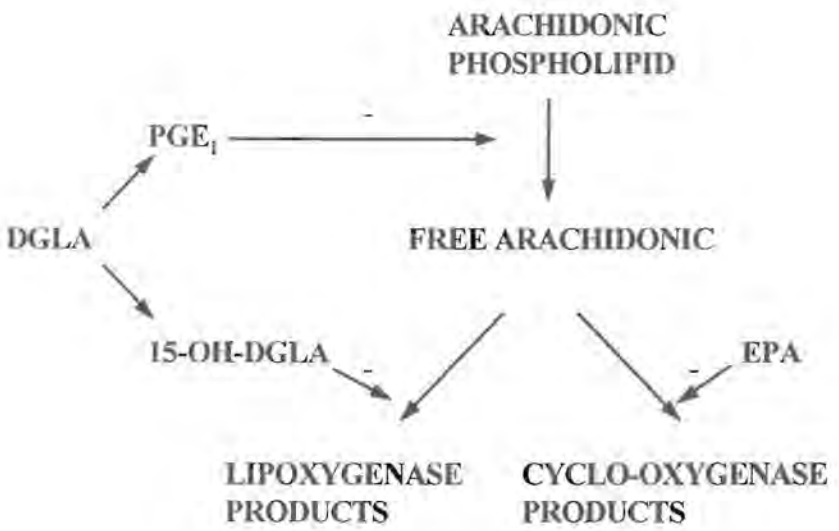


**Figure 15:** The effect of DGLA and EPA on AA metabolism. [110]

## 5.3 PTH levels

From figure 10 it is clear that the blood PTH levels are increased in the OVX control and OVX 3:1 supplemented groups compared to the sham control group. Loss of oestrogen due to OVX seems to increase PTH levels, which may be a reflection of a compromised calcium status. Loss of oestrogen increases skeletal sensitivity to PTH, which would, in this case, result in bone loss. The increase in PTH is slightly reversed by EFA supplementation but no definite conclusion can be reached regarding EFAs and PTH. A reduction in PTH with EFA supplementation has previously been seen in osteoporotic patients. [155]

Low blood calcium can also stimulate the parathyroid gland to secrete PTH, which in turn increases the production of the vitamin D hormone.

## 5.4 Bone Status

The effect of OVX has been previously shown to lower calcium per femur from sham levels. [169,170] This is apparent when we look at figure 11 where osteopenia is induced by OVX. In both OVX control and OVX 3:1 supplemented groups the bone $Ca^{2+}$ as well as the bone density ($g/cm^2$) was lower compared to sham control. Studies done on male rats where different ratios 3:1 and 1:3 of GLA:EPA + DHA were supplemented, the ratio 3:1 had the best effect on bone. [155] Reduced pyridium cross-link excretion and hydroproline levels, both sensitive markers for bone resorption, suggested that supplementation of EFA in the ratio 3:1 was the most effective in inhibiting bone resorption in the male rat.

However, our study done on the ovariectomised female rat shows a different trend, which agrees with previous studies done by Kruger et al. [157] where supplementation with the ratio 1:3 increased the bone calcium content of the OVX female rat. These contrasting findings may be due to the fact that prostaglandins from the E series primarily $E_1$ and $E_2$ have profound, although contrasting effects on bone. PGEs can stimulate bone resorption *in vitro*, *in vivo* studies with PGE infusions into either blood or bone resulted in bone formation.

[117] EFAs are modulators of PG's and can possibly influence bone through PG synthesis. Dietary modification by supplementing EPA was shown in 1994 [147] to alter the levels of fatty acids in rat alveolar bone. EPA supplementation inhibits the metabolism of AA from LA and competitively inhibits the production of AA metabolites such as $PGE_2$ that increases bone resorption. [130]

Interestingly, EPA is also responsible for bone resorption through the action of $PGE_3$, [149] but EPA is a much less effective precursor for $PGE_3$ than AA is for $PGE_2$. The stimulation of bone formation [150] is thus associated with the decrease in $PGE_2$. Direct effects by EFAs on bone are also possible.

Supplementation with EFAs (especially EPA and DHA) increases membrane fluidity. It is evident from the erythrocyte fatty acid levels that EPA and DHA had the highest incorporation into the membrane in the OVX 1:3 group. Membrane changes show modulation of the membrane where they can act as second messengers and affect gene transcription. This altered transcription may result in increased stimulation of osteoblasts [171] that eventually results in bone formation. [171] Fatty acids also interact with protein kinase C, an enzyme whose activity is stimulated by diacylglycerol and unsaturated fatty acids, [172] and requires phosphatidylserine. Protein kinase C contains a cysteine-rich domain with one or two zinc fingers characteristic of many transcription activation factors such as the super family of steroid hormone receptors. [173] Fatty acids can be modulators of two different cell-signalling pathways by interacting

directly with sites on receptor and enzyme proteins, which are crucial mediators of the pathways. [173]

It is also important to keep in mind that the PTH levels were increased in the OVX group with the lower bone $Ca^{2+}$ and density values. PTH is required for the mobilisation of $Ca^{2+}$ from bone. Thus, PTH could be responsible for the decreased bone parameters in the OVX control, the ratio supplementation had a non-specific effect in lowering PTH towards sham, but could have contributed to the bone sparing effects of the EFAs.

## 5.5 $Ca^{2+}$-$Mg^{2+}$ ATPase activity

Fatty acids are important factors in $Ca^{2+}$ transport specially the active extrusion of $Ca^{2+}$ at the BLM, being membrane components that are incorporated into the fatty acyl chains of phosholipids. A change in the phospholipid composition of membranes may affect the permeability as well as the activity of the $Ca^{2+}$ ATPase activity. Arachidonic acid has been reported to increase the ATPase activity in the sarcoplasmic reticulum. [144]

ATPase activity decreased significantly due to OVX probably resulting in lower intestinal absorption of $Ca^{2+}$. There was a profound increase in $Ca^{2+}$-$Mg^{2+}$ ATPase activity in the OVX 1:3 supplemented group possibly leading to an increase in $Ca^{2+}$ absorption in the intestine. It is likely to assume that the more

EPA and DHA, both being highly unsaturated fatty acids are incorporated into a membrane, the more fluid and permeable it will become. The effect on the $Na^{+}$-$K^{+}$ ATPase activity of the membranes isolated from fish oil (EPA) supplemented rats in experiments done by Coetzer et al. [2] supports the above mentioned results, both enzymes are located in the BLM. The fact that the GLA: EPA + DHA ratio increases 5-6 fold in the erythrocyte membrane compared to the plasma, explains that EFAs are more readily incorporated into the membrane.

Previous results obtained [144] show that membrane fluidity has a direct influence on the conformation of the active sites of some membrane-associated enzymes, these enzymes show a higher activation energy. This suggests that certain proteins may phase separate with more fluid lipids. The feasibility of modulating intrinsic intestinal membrane functions by means of dietary content has been shown to influence the FA composition of the BBM as well as the enterocyte microsomal desaturase, the change in these microsomal enzyme activities could change the phospholipid metabolism for which it is responsible. [143]

The fact that the bone density increased in the corresponding supplemented group in which the $Ca^{2+}$ ATPase activity increased, may be an indication that the increased circulating $Ca^{2+}$ is stored in the bone.

## 5.6 Vitamin $D_3$ receptor availability (ELISA)

The values measured in fmol/mg protein are lower than those reported in the literature. That the receptor is unstable and both hormone and DNA binding capacity decay in a time and temperature-dependent fashion could account for the low fmol/mg prot. values observed. [86]

There is a marked increase in the availability of the VDR in the OVX control as well as OVX 3:1 supplemented groups, compared to the sham control group. Loss of oestrogen due to ovariectomy may decrease intestinal absorption of $Ca^{2+}$, which in turn may upregulate PTH (figure 10) as well as vitamin $D_3$. The vitamin may be responsible for upregulation of its receptors. However, $1,25(OH)_2D_3$ levels were not measured in this study.

The fact that higher values of VDR concentration were reported for the OVX control and OVX 3:1 groups is not conclusive of new VDR synthesis. The ELISA technique uses 2 monoclonal antibodies that recognise different sites on the VDR. This method is able to detect any part of the receptor whether being occupied or denatured, all possible receptors are detected. Elevated blood levels of $1,25(OH)_2D_3$ in the study done by Huang et al. [102] could not alter the receptor mRNA. The degradation rate of the previously formed receptor was markedly decreased by the administration of $1,25(OH)_2D_3$ in cycloheximide-blocked cells, which proves that upregulation is not primarily due to new receptor

synthesis but may be the result from events that result in increased receptor stability, e.g. ligand-binding. [103] EFAs may have caused increased receptor stability.

## 5.7 Vitamin $D_3$ receptor binding (HAP ASSAY)

Evidence suggests that non-esterified fatty acids (NEFAs) or free fatty acids (FFAs) are involved in the action of steroid hormones, having effects both on their plasma transport and on their intracellular activity. [174] The action of FFA can take place at the level of:

5.7.1 biosynthesis and metabolism of steroids

5.7.2 the serum steroid binding proteins

5.7.3 the intracellular, transmembrane (nuclear) transfer of the hormone that can occur by diffusion, by binding of the complex formed by the steroid and its specific binding protein to a membrane receptor and internalisation of this complex.

5.7.4 Transfer of the hormone to a cytoplasmic or nuclear receptor that activates the receptor and permits its binding to the hormone responsive element acting on the transcriptional activity of a gene. [120]

A modulator is a substance that acts at a precise location for a very short time in a reversible manner to modify the characteristic of a signal. [120] FAs have

characteristics attributed to modulators. Metabolism, incorporation into phospholipids, binding to a fatty acid binding protein is mechanisms by which the modulatory effect of the modulator disappears.

FFAs can also compete with the binding of steroid hormones to their plasma-binding proteins. [175] In the study done by Bouillon et al. [175] it was found that polyunsaturated but not saturated fatty acids or prostaglandins markedly decreased the affinity of vitamin D metabolites for vitamin D-binding protein (DBP). It is well known that FFAs can bind several plasma proteins with albumin as the main serum protein responsible for FFA transport. It is likely that DBP also belonging to the albumin gene family will bind FFA.[175] Arachidonic acid (20:4) greatly decreased the binding of vitamin D metabolites to DBP but its further cyclic unsaturated metabolites ($PGA_1$ and $PGE_1$) did not influence the ligand DBP interaction. It was also shown by Nunez [120] that the modulation by FFA can be enhanced by the inhibition of FFA metabolism into PGs. Results show that the inhibitory effect that AA have on steroid binding to its receptor is potentiated in the presence of cyclo-oxygenase and lipoxygenase inhibitors that limit the metabolism of C20:4 into PGs which indicates that C20:4 is acting per se and not through its metabolites. [120]

Long chain NEFAs have been shown to modify the structure or conformation of the glucocorticoid receptor. Results obtained by Vallette et al. [176] suggest that the fatty acid interact with the glucocorticoid receptor at a different site from

that of dexamethasone to induce a change in the conformation of the receptor. Modulation by FFAs can be negative or positive for the binding of steroid to serum proteins and intracellular receptors with the following characteristics:

Dose-dependent

Degree of unsaturation;

thus, long chain polyunsaturated fatty acids are the most effective. [120]

NEFAs inhibit the binding of dexamethasone to glucocorticoid receptors, with the best inhibitors being AA (C20:4) and DHA (C22:6). [176] These findings support the trend of increasing inhibition of VDR binding which can be seen in figure 14. In the OVX 1:3 supplemented group the inhibition of VDR binding was the highest and contained the highest concentration of unsaturated fatty acid, for example EPA and DHA.

In *in vitro* studies using isolated receptors plus exogenously added EFAs both the Ka and the number of binding sites decreased in the presence of increasing concentrations of C20:4 and C22:6 with nonlinearly variation in the Kd indicating that the inhibition is non-competitive and at a different binding site from that of the hormone binding site. [176] One reported study also found that fatty acids and phospholipids could inhibit the binding of $1,25(OH)_2D_3$ to its receptor [172] which evidently belongs to the same super family of erb-A receptors as the steroid hormone receptors; they also concluded that the

inhibition of the binding of $1,25(OH)_2D_3$ to its receptor was purely non-competitive.

The intestinal receptors for $1,25(OH)_2D_3$ do not require phospholipids for their binding activity, on the contrary phospholipids that inhibit $1,25(OH)_2D_3$ binding are more likely to play a regulatory role than a structural role in receptor function. [172]

Fatty acids bind to a second site on the glucocorticoid and thus on the $1,25(OH)_2D_3$ receptor as well, and change the conformation of the hormone binding site to reduce the affinity for the hormone either partially or completely depending on the concentration of the fatty acid. [176] In the search for a putative site of interaction of polyunsaturated fatty acids (PUFAs) on a steroid hormone receptor. Sumida et al. [173] used the glucocorticoid receptor as model. That unsaturated fatty acids did not inhibit the non-specific binding ruled out the possibility that the fatty acids may be interacting with another protein rather than directly with the receptor protein, which is shown in the glucocorticoid receptor as well. [173]

The interaction of unsaturated fatty acids with steroid hormone receptors not only varies with the fatty acid but also with the steroid and the target organ studied. This is evident with the administration of oestrogen to the ovariectomised rat uterus containing the $1,25(OH)_2D_3$ receptor. The oestrogen

stimulated the receptor that could not be stimulated in the chick intestine. [9] The control of the VDR is a property of the target tissue rather than an inherent property of the receptor species.

The hydroxylapatite assay is more limited than the more advanced ELISA technique, measurements of the receptor protein are based on ligand-binding activity in tissue and cell extracts. In other words only the part of the receptor population capable of binding radio-ligand excluding occupied, denatured and partially proteolysed receptors can be detected.

The efficient separation of bound from free ligand used in this assay makes it possible however to examine a number of aspects of the binding of the steroid hormone to its cytoplasmic receptor, in this case the specific influence of EFAs on the binding of $1,25(OH)_2D_3$ to its cytoplasmic vitamin $D_3$ receptor. The low values observed can yet again be contributed to the fact that the receptor is unstable and both hormone and DNA binding capacity decay in a time and temperature-dependent fashion, another factor could be endogenous protease cleaving the 60000-Mr receptor into a fragment of Mr 45000 that binds hormone but not DNA. [86]

## 5.8 Conclusion

From our study it is clear from the erythrocyte membrane EFA concentrations that n-3 EFAs are more readily incorporated into membranes, which researchers in our laboratory have previously showed. [8] Supplementation of EFAs in the ratio 1:3 stimulated the $Ca^{2+}$-$Mg^{2+}$ ATPase activity in the BLM. This is a novel finding as it has only been shown previously that high concentrations of fish oil containing EPA and DHA could stimulate the $Na^{+}$-$K^{+}$ ATPase activity.

In confirmation $Ca^{2+}$ transport as described by Coetzer et al. [2] should be measured as a further proof that unsaturated membranes, $Ca^{2+}$ ATPase and transport could be related. Measuring the unsaturation index of the erythrocyte or enterocyte membrane could prove to be of great importance.

Dietary supplementation of EFAs resulted in changes in the number of receptors available, normalising the increase in receptors caused by OVX. A profound decrease in the binding capacity of the VDR was also found in the supplemented group with the highest degree of unsaturated fatty acids namely the OVX 1:3 group.

The VDR availability was consequently the lowest in the corresponding OVX 1:3 group, but the fact that the VDR concentration was higher in the other two

OVX groups does not indicate new synthesis of receptor protein, it can also indicate receptor stability due to ligand-binding.

In this study, long-term supplementation of EFAs from weaning until age = 126 days, was shown to have a prophylactic effect on bone loss as induced by OVX. Short-term supplementation (6 weeks) previously reversed OVX induced bone loss, but the longer term feeding had a more pronounced effect as measured in bone $Ca^{2+}$ and bone density.

Regulation on different levels could contribute to these results. Firstly, the EFAs increased the decrease in ATPase activity after OVX back to sham levels. Low ATPase activity after OVX could result in low $Ca^{2+}$ absorption which in turn will upregulate PTH as is reported in this study. Ratio 1:3 reduced PTH levels, though not significantly whilst increasing ATPase activity.

An increase in PTH may be accompanied by an increase in $1,25(OH)_2D_3$, which upregulates its receptor levels. Alternately, as OVX inhibits $Ca^{2+}$ ATPase, VDR are upregulated trying to compensate for the lowering in $Ca^{2+}$ absorption. As the EFAs increased ATPase activity, the VDR number decreased back to sham levels. Clearly EFAs reduced affinity of the VDR.

Inhibition of receptor binding by EFAs is also shown in this study which according to the literature is non-competitive. To prove the kind of inhibition

further *in vivo* studies need to be done that include: Lineweaver-Burke curves and measurements of 1/vmax and -1/km. EFAs may while decreasing VDR binding, upregulate $Ca^{2+}$ absorption, PTH levels and bone calcium by using mechanisms such as changes in the unsaturation of membranes, modulation of second messenger systems or prostaglandin synthesis.

With time and increasing complexity of cell organisation, the role of the fatty acids may have evolved so that they can now be lipid second messengers and co-regulators of steroid hormone-sensitive gene transcription, helping to link membrane signal transduction with the intracellular steroid hormone signalling pathway. [177]

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