Universidade do Algarve

Faculdade de Ciências e Tecnologia

The role of vitamin K in osteoporosis

Nicoleta Cristafovici

Dissertação para obtenção do grau de Mestre em Ciências Farmacêuticas

Trabalho efetuado sob orientação da Professora Doutora Dina Cristina Fernandes Rodrigues da Costa Simes e coorientação da Professora Doutora Carla Alexandra São Bento Viegas

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Declaração de Autoria de trabalho

Declaro ser a autora deste trabalho, que é original e inédito. Autores e trabalhos consultados estão devidamente citados no texto e constam da listagem de referências incluída.

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(Nicoleta Cristafovici)

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Abstract

According to the World Health Organization (WHO), osteoporosis is a “progressive systemic skeletal disease characterized by low bone mass and microarchitectural deterioration of bone tissue, with a consequent increase in bone fragility and susceptibility to fracture." The consequences of these fractures can be serious, sometimes life-threatening; the economic costs of treating fractures are also considerable.

Alternative methods of treating osteoporosis have been researched in recent decades, and vitamin K (VK) has been found to be a viable therapeutic. This dissertation investigates scientific evidence on the role of VK in osteoporosis.

VK is a fat-soluble vitamin required for blood clotting. VK has two main structures: vitamin K1 (VK1) and vitamin K2 (VK2). Different absorption rates, tissue distribution, and bioavailability reflect structural differences between VK1 and VK2. Although they have structural differences, both function as co-factor for the γ-glutamyl carboxylase enzyme (GGCX) in the conversion of glutamic acid (Glu) residues into γ-carboxyglutamic acid (Gla) residues in vitamin K-dependent proteins (VKDPs), involved in hepatic and extrahepatic activity. VKDPs involved in bone metabolism include osteocalcin (OC), matrix G1a protein (MGP), Gla Rich Protein (GRP), Growth Arrest Specific Protein 6 (Gas6), and protein S. OC is the most abundant VKDP in bone. During bone mineralization, osteoblasts produce OC, which binds to calcium ions and hydroxyapatite crystals to modulate bone size and structure.

According to studies, VK2, especially menaquinone-7, has more positive results than VK1 in terms of improving bone mineral density (BMD) and decreasing the risk of fractures. In addition, VK2 suppresses IKB phosphorylation and reduces NF-κB activation, resulting in a decrease in the generation of pro-inflammatory cytokines implicated in osteoporosis physiopathology. VK2 also acts as a ligand for the steroid and xenobiotic receptor (SXR) or pregnane X receptor (PXR), increasing the transcription of genes for extracellular matrix proteins to preserve bone structure.

Resumo

Segundo a Organização Mundial de Saúde (OMS), a osteoporose é uma "doença sistémica progressiva caracterizada por baixa massa óssea e deterioração microarquitectónica do tecido ósseo, com um consequente aumento da fragilidade óssea e da suscetibilidade à fratura". As consequências destas fraturas podem ser graves, por vezes com risco de vida; os custos económicos do tratamento das fraturas são também consideráveis.

Métodos alternativos de tratamento da osteoporose têm sido pesquisados nas últimas décadas, e a vitamina K (VK) tem sido considerada uma terapêutica viável. Esta dissertação investiga provas científicas sobre o papel da VK na osteoporose.

A VK é uma vitamina lipossolúvel necessária para a coagulação do sangue. A VK tem duas estruturas principais: vitamina K1 (VK1) e vitamina K2 (VK2). Diferentes taxas de absorção, distribuição dos tecidos e biodisponibilidade refletem diferenças estruturais entre VK1 e VK2. Embora tenham diferenças estruturais, ambas funcionam como cofator da enzima γ-glutamil carboxilase (GGCX) na conversão de resíduos de ácido glutâmico (Glu) em resíduos de γ-carboxiglutâmico (Gla) em proteínas dependentes de vitamina K (VKDPs), envolvidas em atividade hepática e extra-hepática. Os VKDPs envolvidos no metabolismo ósseo incluem osteocalcina (OC), *Matrix G1a Protein* (MGP), *Gla Rich Protein* (GRP), *Growth Arrest Specific Protein 6* (Gas6), e proteína S. OC é o VKDP mais abundante em osso. Durante a mineralização óssea, os osteoblastos produzem OC que se liga aos iões de cálcio e cristais de hidroxiapatite para alterar tamanho e estrutura óssea.

De acordo com estudos, VK2 especialmente menaquinona-7, tem resultados mais positivos do que VK1 em termos de melhoria da BMD e de diminuição do risco de fraturas. VK2 funciona como um cofator na atividade de GGCX. Além disso, VK2 suprime a fosforilação IKB e reduz a ativação de NF-κB, resultando numa diminuição da geração de citocinas pró-inflamatórias implicadas na fisiopatologia da osteoporose. VK2 atua também como um ligante para o recetor esteroide e xenobiótico (SXR) ou recetor X pregnante (PXR), aumentando a transcrição de genes das proteínas de matriz extracelular para preservar a estrutura óssea.

Resumo alargado

O osso é formado pela matriz óssea e pelas células diferenciadas. A matriz óssea é composta por componentes orgânicos, principalmente colagénio tipo I e componentes inorgânicos, tais como fosfato, bicarbonato de cálcio, magnésio, citrato de potássio, e sódio. As células diferenciadas são importantes no metabolismo ósseo e têm o potencial para a regeneração óssea. Estas células são divididas em 3 categorias: os osteoblastos que são responsáveis pela formação óssea; os osteoclastos que são responsáveis pela reabsorção óssea durante a modelação e remodelação óssea; e os osteócitos que são células maduras desenvolvidas a partir de osteoblastos fazendo parte da matriz óssea e com um papel importante na homeostase óssea. O equilíbrio das funções de cada tipo de célula permite a absorção do tecido ósseo antigo e reposição de tecido ósseo novo. Na presença de desequilíbrio entre a reabsorção óssea e a formação óssea inicia-se o desenvolvimento da osteoporose que está associado a diminuída atividade dos osteoblastos e a elevada atividade dos osteoclastos.

Segundo a Organização Mundial de Saúde (OMS), a osteoporose é uma "doença sistémica progressiva caracterizada por massa óssea diminuída e deterioração microarquitectónica do tecido ósseo, com o consequente aumento da fragilidade óssea e suscetibilidade à fratura". O principal mecanismo patogénico da osteoporose em mulheres na fase da perimenopausa é a diminuição gradual de estrogénio, que aumenta a síntese de citocinas desregulando o eixo RANK/RANKL/OPG. Também a desregulação da PTH, da calcitonina e da vitamina D influenciam no aumento da atividade osteoclástica, diminuindo a massa óssea. Para além disso, o efeito do stress oxidativo afeta o osso, diminuindo a massa óssea, a formação óssea, o número e a disfunção dos osteoblastos através do desequilíbrio existente entre a produção de espécies reativas de oxigénio (ROS) e a defesa antioxidante.

De modo a cobrir as necessidades dos utentes diagnosticados com osteoporose, foram desenvolvidas terapias farmacológicas eficazes na sua prevenção e no seu tratamento, tais como bisfosfonatos, raloxifeno, denosumab, teriparatide, abalopateride, romosozumab, cálcio e vitamina D. O cálcio e a vitamina D são frequentemente considerados tratamentos de primeira linha, quer isoladamente quer em combinação com outros medicamentos.

Nas últimas décadas, a vitamina K (VK) foi investigada no sentido de diminuir os impactos provocados pela osteoporose na saúde óssea. Em 1936, a VK foi identificada, pela

primeira vez, como sendo um componente crucial no processo de coagulação do sangue. Posteriormente, foi descoberto que a VK também opera como cofator da enzima GGCX na conversão dos resíduos de ácido glutâmico (Glu) em resíduos de γ-carboxiglutâmico (Gla) nas proteínas dependentes de vitamina K (VKDPs). VK atua principalmente como cofator da enzima GGCX na reação da γ-carboxilação, embora também tem efeitos independentes da atividade do GGCX, nomeadamente atua como agente antioxidante diminuindo a ROS intracelular, como agente anti-inflamatório na via NF-κB e, mais recentemente, como agente anti-tumoral. Para além destas funções, a VK também interage com o recetor SXR/PXR para manter a homeostase óssea através da produção de proteínas da matriz extracelular. O processo de γ-carboxilação é fundamental para o funcionamento das VKDPs, e, ocorre tanto em VKDPs hepáticas como extra-hepáticas. Portanto, os resíduos de Gla resultantes da γ-carboxilação podem ligar-se aos iões de cálcio, induzindo alteração conformacional da proteína desempenhando a sua função fisiológica.

As VKDP extra-hepáticas implicadas na formação do osso incluem osteocalcina (OC), a VKDP mais importante associado à homeostase óssea, embora a *Matrix G1a Protein* (MGP), *Gla Rich Protein* (GRP), *Growth Arrest Specific Protein 6* (Gas6), e proteína S também podem ter funções importantes no metabolismo ósseo. OC é a proteína não colagénica mais abundante na matriz óssea extracelular produzida pelos osteoblastos. Esta proteína não colagenica funciona como marcador de *turnover* ósseo envolvido na mineralização óssea uma vez que se liga e incorpora iões de cálcio em cristais de hidroxiapatite na matriz óssea. A forma não carboxilada da osteocalcina (ucOC) é considerada fator de risco de fratura na anca. Níveis baixos de ucOC estão associados a uma elevada densidade mineral óssea (BMD) e a um reduzido risco de fratura.

Relativamente a função da VK independente da atividade GCCX, esta atua na via NF-κB. Esta função foi observada em vários estudos *in vitro* e *in vivo*, demonstrando que a VK reduz a ativação da NF-κB e inibe a fosforilação da IkappaB kinase (IKB). Esta sua atuação promove a diminuição da produção das citocinas pro-inflamatórias que posteriormente normaliza o funcionamento do eixo RANK/RANKL/OPG, que aumenta atividade osteoblástica e diminui a atividade osteoclástica, contribuindo assim para a homeostase óssea.

Uma outra função da VK é a comunicação com o recetor SXR/PXR através da interação ligando-substrato sendo MK2, MK3, ou MK-4 o ligando e SXR/PXR o substrato. Esta interação regula e aumenta a transcrição de genes que codificam proteínas da matriz

extracelular, tais como *matrilin-2* (Matn2), *tsukushi* (Tsk), and *cluster-determinant 14* (CD14), resultando num aumento da acumulação de colagénio nas células osteoblásticas, que posteriormente vai ser depositado na matriz óssea.

Ao longo dos anos, vários estudos têm sido realizados na investigação dos efeitos da suplementação com VK1 e VK2 na osteoporose. Os estudos demonstram que a suplementação com VK1 não ajuda no aumento da densidade mineral óssea, no entanto pode diminuir ligeiramente o risco de fratura. Sendo assim, VK2 tem apresentado a redução do risco de osteoporose, uma vez que melhorar a qualidade óssea. Este efeito deve-se certamente a sua ação nos processos fisiopatológicos.

Os farmacêuticos desempenham um papel importante no sistema de saúde, ajudando os utentes na gestão de doenças crónicas, uma das quais é a osteoporose. Em primeiro lugar, os farmacêuticos serão capazes de identificar os utentes de alto risco devido ao histórico de administração prolongada de glucocorticoides. Em segundo lugar, os farmacêuticos são essenciais no aconselhamento e educação dos utentes sobre a administração da medicação, do cálcio, da vitamina D, da VK, a prevenção de quedas, a importância do exercício físico e promovendo a adesão ao tratamento para osteoporose.

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Abbreviations

12-LOX	12-lipoxygenase
(LRP)-5/6	Lipoprotein receptor-related protein 5/6
1, 25-(OH)2D	1, 25-dihydroxyvitamin D
aa	Amino acids
ABC	ATP-binding cassette
ApoA	Apolipoprotein-A
ApoB-48	Apolipoprotein-B48
ApoC	Apolipoprotein-C
ApoE	Apolipoprotein-E
ATP	Adenosine triphosphate
BGLAP	Bone γ-carboxyglutamic acid-containing protein
BHT	Butylated hydroxytoluene
BMI	Body Mass index
BMPs	Bone morphogenetic proteins
Ca^{2+}	Calcium ion
CD 14	Cluster-determinant 14
CD 36	Cluster-determinant 36
CM	Chylomicron
cOC	Carboxylated osteocalcin
DKK1	Dickkopf-related protein 1
dp-ucMGP	Desphospho-uncarboxylated matrix gla protein
DRIs	Dietary reference intakes
DXA	Dual-energy X-ray absorptiometry
FAO	Food and Agriculture Organization
Frz	Frizzled
FT-IRM	Fourier transform infrared microspectroscopy
Gas6	Growth arrest specific protein 6
GGCX	γ-glutamyl carboxylase
GGpp	Geranylgeranyl pyrophosphate
GI	Gastrointestinal

GIO	Glucocorticoid-induced osteoporosis
Gla	γ-carboxyglutamic acid
Glu	Glutamic acid
GRP	Gla-rich protein
HSCs	Hematopoietic stem cells
IGF	Insulin-like growth factor
IKB	IkappaB kinase
IL-1	Interleukin-1
IL-11	Interleukin-11
IL-6	Interleukin-6
KH2	Vitamin k hydroquinone
KO	Vitamin K 2,3 epoxide
LDL	Low-density lipoprotein
LDLR	Low-density lipoprotein receptor
LPL	Lipoprotein lipase
LPS	Liposaccharide
LRP1	Low-density lipoprotein receptor-related protein 1
Matn2	Matrilin-2
M-CSF	Monocyte/macrophage colony-stimulating factor
MDR1	Multidrug resistance 1
MGP	Matrix Gla protein
MK-4	Menaquinone-4
MKn	Menaquinone
MMP-9	Matrix metallopeptidase 9
MSCs	Mesenchymal stem cells
MTP	Mitochondrial trifunctional protein
NAM	National Academy of Medicine
NFATc1	Nuclear Factor of Activated T-cells cytoplasmic 1
NF-κB	Nuclear factor kappa B
NPC1L1	Niemann-Pick C1-Like 1
OC	Osteocalcin
OPG	Osteoprotegerin

OST	Osteoporosis Self-Assessment Tool
Osx	Osterix
p-cMGP	Phospho-carboxylated matrix gla protein
PK	Phylloquinone
PTH	Parathyroid hormone
PXR	Pregnane X receptor
QR	Quinone reductase
RANK	Receptor activator of nuclear factor kappaB
RANKL	Receptor activator of nuclear factor kappaB ligand
RCTs	Randomized controlled trials
RDA	Recommended dietary allowance
ROS	Reactive oxygen species
Runx2	Runt-related transcription factor 2
RXR	9-cis retinoic acid receptor
SD	Standard deviations
SFRP	Secreted frizzled-related protein
SR-BI	Scavenger receptor class B-type I
SXR	Steroid and xenobiotic receptor
SXRE	SXR-responsive elements
TCF	T-cell factor
TNF	Tumor necrosis factor
TNF-α	Tumor necrosis-alpha
Tsk	Tsukushi
UBIAD1	UbiA prenyltransferase containing 1
UCMA	Upper zone of growth plate and cartilage matrix associated protein
ucOC	Uncarboxylated osteocalcin
ucVKDPs	Uncarboxylated VKDPs
USPSTF	United States Preventive Services Task Force
VDR	Vitamin D receptor
VK	Vitamin K
VK1	Vitamin K1
VK2	Vitamin K2

VK3	Vitamin K3
VKDPs	Vitamin K dependent proteins
VKOR	Vitamin K epoxide reductase
VKORC1	Vitamin K epoxide reductase complex subunit 1
VKORC1L1	Vitamin K epoxide reductase complex subunit 1-like 1
VLDL	Very low-density lipoprotein
VSMCs	Vascular smooth muscle cells
WHO	World Health Organization
Wnt	Wingless-type and integrase 1

Chapter 1: Overview

1.1 Introduction

Osteoporosis is a prevalent disease that affects millions of patients worldwide and is most predominant in postmenopausal women. Low bone density and an increased risk of fractures as a result of deterioration of the bone architecture, are two of the most prominent features of osteoporosis (1). Osteoporotic fractures most commonly occur in the hip, spine, and wrist. The consequences of these fractures can be severe, even fatal; the economic costs of treating fractures are also significant, and this can have an impact on patients health-related quality of life (1–3).

Clearly, in the coming years, the prevention and treatment of this pervasive and debilitating disease must become a top priority in the healthcare system. Effective pharmacological therapies for the prevention and treatment of osteoporosis have been developed, such as bisphosphonates, raloxifene, denosumab, teriparatide, abalopateride, romosozumab, calcium, and vitamin D. Calcium and vitamin D are frequently considered first-line treatments, either alone or in combination with other medications (1,4–6).

In the last decades, scientists investigated that vitamin K (VK) can have an important role in prevention and treatment of osteoporosis. VK describes a class of fat-soluble vitamers, considered essential for activation of several proteins that are involved in coagulation homeostasis and calcium homeostasis (7), functioning as a cofactor for the γ-glutamyl carboxylase enzyme (GGCX) in the γ-carboxylation reaction (7,8). This vitamin also operates as anti-inflammatory agent independently of GGCX activity and can interact with the steroid and xenobiotic receptor (SXR), and its murine ortholog, the pregnane X receptor (PXR), to reduce bone resorption and enhance bone formation. Many studies have been conducted over the last two decades to investigate the effects of VK on both physiological and therapeutic levels (9). These studies support that VK is involved in multiple cellular and physiological processes such as oxidative stress (10), immune response, inflammation (11,12), cancer progression (13) and associated with protective and supporting roles in diverse organs or tissues, such as bone (14,15), cardiovascular system (16,17), brain (18), intestine (19), liver (13,20), pancreas (21) and, fat tissues (22). VK naturally occurs as two biologically active vitamers derived from diet: phylloquinone (PK) (vitamin K1 (VK1), and menaquinone (MKn) (vitamin K2 (VK2)) (9,23–25).

VK series has long been recognized as a therapeutic agent, particularly VK2 , which has been used in clinical trials around the world to treat bone brittleness, and evidence suggests its role as a transcriptional regulator and hormone (26). The importance of VK has grown dramatically in recent years as a result of the discovery of VK involvement in vascular calcifications, cardiovascular and bone diseases.

1.2 Objectives

The main goal of this integrated master dissertation in pharmaceutical sciences is to clarify the role of VK, specifically VK2, in bone homeostasis in order to understand its beneficial effect on osteoporosis. It is essential to know the bone physiology, to characterize osteoporosis as a bone disease, comprehend its pathogenesis, the physiological role of VK, and its pharmacokinetic profile to understand how VK might reduce the risk of fractures, i.e., the early onset of osteoporosis.

1.3 Methodology

This dissertation comprises of a literature review intended to synthesize the most recent and important data on the effects of VK on osteoporosis. The dissertation is comprised of a survey conducted between February and October 2022 and the analysis of a wide range of materials, including scientific publications, books, a description of the drugs properties, periodicals, and scientific journals. The majority of the materials used were acquired from databases such as PubMed, Google Scholar, EMA, UpToDate, Web of Science, and B-On, as well as worldwide osteoporosis treatment recommendations. The most common search keywords were "osteoporosis", "vitamin K", "vitamin K2", "bone health", "physiopathology", "bone cells", "supplementation" and "osteocalcin".

Chapter 2: Bone architecture and physiology

2.1 Bone matrix

Bone is composed of bone matrix and differentiated cells. The matrix of the bone contains organic and inorganic components. Phosphate, calcium bicarbonate, magnesium, potassium citrate, and sodium are the inorganic compounds that comprise 67% of the bone (27). Phosphate and calcium are the most abundant of the inorganic compounds found in the bone, and when combined, they form the hydroxyapatite crystals that give the bone its rigid structure. Organic components constitute about 33% of the bone structure and are mostly composed of type I collagen (27,28). This particular form of collagen contributes to the flexibility of bone by linking the inorganic structure of the bone to make it more resistant. The type I collagen aids bone flexibility by linking the inorganic structure to make the bone more robust, lowering the probability of bone shattering. In addition to collagen, bone matrix includes a variety of specific noncollagenous proteins, including osteocalcin (OC), osteonectin, and osteopontin (29). OC and its function will be discussed in the Chapter 6.1.1. Osteonectin connects collagen to hydroxyapatite, functions as a nucleus for mineralization, and regulates the synthesis and proliferation of hydroxyapatite crystals (30), whereas osteopontin reduces mineralization by inhibiting hydroxyapatite production and stimulating osteoclast activity for bone resorption (31). Regarding specialized cells, it can be inferred that they play an important role in bone metabolism and have the potential to regenerate the bone. These cells are osteoblasts, which are responsible for bone formation; and osteoclasts, which are responsible for bone resorption during bone modeling and remodeling. Osteocytes are mature cells that develop from osteoblasts and become part of bone matrix. This metabolism consists of absorbing old bone tissue and recomposing it with new bone tissue(32,33).

2.2 Bone cells

Osteoclasts are multinucleated cells derived from hematopoietic stem cells (HSCs) that differentiate into osteoclasts in response to transcription factors including nuclear factor kappa B (NF-κB) and Nuclear Factor of Activated T-cells cytoplasmic 1 (NFATc1), and growth factors including Macrophage Stimulating Factor (M-CSF) and Receptor Activator of NF-κB Ligand (RANKL). These factors promote the differentiation of monocyte/macrophage progenitors into osteoclast, which are involved in bone resorption (34–36).

Briefly, osteoclast activity begins when RANKL expressed on the surface of osteoblasts binds to receptor activator of nuclear factor kappa B (RANK) expressed on mature osteoclasts. Nonetheless, also can start as a result of parathyroid hormone (PTH) and calcitonin homeostasis (5). The activation of osteoclasts induces cytoskeletal and membrane reorganization, which results in cellular polarization and the formation of a sealing zone, followed by the formation of two new membrane domains: the ruffled border membrane within the sealing zone and the functional secretory domain on the opposite side of the bone (32,35,37). The sealing zone at the bone-cell interface is required for osteoclast adherence and bone degradation (32). When the osteoclasts adhere to bone, bone degradation begins with the production of hydrochloric acid to dissolve hydroxyapatite, and proteolytic enzymes to digest the organic content of bone through the ruffled border membrane (1,5,32,35,37). The two major proteolytic enzymes involved in bone resorption are lysosomal enzymes (e.g., cathepsin K) and matrix metallopeptidase 9 (MMP-9) released by osteoclasts (1,5). The digested bone components leave the functional secretory domain through the transcytosis pathway (37). Osteoclast activity is required for bone modeling, which modifies the structure of bones throughout growing, as well as bone remodeling, which preserves the adult skeleton integrity (32).

Osteoblasts are bone-forming cells that are produced from mesenchymal stem cells (MSCs) (1), bone lining cells, and potentially chondrocytes. During their lifetime, osteoblasts may transform into bone lining cells or osteocytes, or they can suffer apoptosis (28). These bone-forming cells are mostly found in the periosteum and the endosteum, although they may also be found inside compact bone, more specifically in areas of remodeling (38).

Approximately six weeks after conception, the embryo develops its first osteoblasts, which subsequently promote the formation of new bone (39). MSCs develop into osteoblasts through Wingless-type and integrase 1 (Wnt) and bone morphogenic protein (BMP) pathways, in collaboration with Runt-related transcription factor 2 (Runx2), insulin-like growth factor (IGF) and PTH. BMP signaling activation enhances Runx2 expression in stromal cells, resulting in further differentiation to osteoprogenitors. Runx2 then promotes the production of the transcription factor osterix (Osx), which leads to osteoblast development. As they mature, they get embedded in the bone matrix as osteocytes (40).

Runx2 is an important transcription factor in osteoblast differentiation regulated by wingless-type and integrase 1 (Wnt)/β-catenin axis (39). The activation of this axis begins

with the binding of Wnt3a or Wnt10b to Frizzled (Frz) related protein, which causes phosphorylation of lipoprotein receptor-related protein 5 and 6 ((LRP)5/6). This phosphorylation displaces the destruction complex close to LRP5/6, permitting a rise in β-catenin levels in the cytosol while preserving its active state. Following that, β-catenin will connect to T-cell factor (TCF) to initiate Runx2 transcription (39).

Additionally, osteoblasts modulate osteoclast recruitment and activity by expressing RANKL and osteoprotegerin (OPG). RANKL, a tumor necrosis factor (TNF) family member, is expressed on the surfaces of osteoblasts, osteocytes, macrophages, bone marrow stem cells, and activated T lymphocytes. In the presence of M-CSF, RANKL binds to its receptor RANK and stimulates the osteoclastogenesis process (41). OPG, a TNF receptor superfamily glycoprotein, competes with RANKL for RANK binding, hence blocking RANK/RANKL interaction. Thus, the RANK/RANKL/OPG axis allows for the coupling of osteoblast and osteoclast activity as well as the regulation of bone production and resorption. The OPG/RANKL ratio maintains bone density and strength, and OPG deficiency may lead to osteoporosis (39).

Osteocytes make up more than 90% of all mature bone cells, and live in the bone matrix for more than 10 years, therefore they play an important role in bone remodeling. These mature bone cells are produced by MSC-derived osteoblasts and may be found in specific spaces of mature bones known as lacunae. Osteocytes may regulate bone formation by secreting Wnt signaling pathway stimulators such nitric oxide and adenosine triphosphate (ATP), as well as inhibitors like sclerostin and Dickkopf-related protein 1 (DKK1) (1,39,42,43).

2.3 Bone growth

Bone formation starts in the embryo, either via a cartilaginous intermediate, as in the case of long bones, or through a membranous intermediate, as in the case of skull flat bones. Longitudinal postnatal growth is caused by the continued synthesis of cartilage at specific places on the long bones known as growth plates, and the eventual conversion of this cartilage into bone. Genetic, mechanical, and hormonal processes all influence skeletal growth and development. In general, genetic factors determine the fundamental structure of the skeleton, whereas mechanical loading responses alter the strength of specific bones to their functional context. Simultaneously, hormonal processes coordinate calcium and phosphate migration to

and from the skeleton, allowing bone to function as a repository of these minerals under calcium stress (e.g., pregnancy and lactation). At the molecular level, bone formation is orchestrated by a variety of cytokines and growth factors that regulate bone cell division, maturation, and activity (29).

The mechanism of bone development is divided into two types: endochondral ossification and intramembranous ossification. Endochondral ossification is required for the development and growth of long bones, the healing of bone fractures, and the synthesis of cartilage by chondrocytes, while intramembranous ossification is required for rudimentary bone formation and bone fracture regeneration (1). During intramembranous bone development, MSCs multiply and differentiate into osteoblasts, which generate bone by coupling with extracellular matrix proteins, the most prevalent of which is type I collagen. Once coupled, the extracellular matrix is mineralized by the deposition of calcium phosphate as hydroxyapatite (Ca10(PO4)6(OH)2) (39).

2.4 Bone remodeling

The renewal and repair of bones occurs through a process known as bone remodeling. Bone remodeling is required to preserve structural integrity, bone volume, and calcium and phosphate homeostasis (44). The remodeling cycle is highly controlled and regimented, with five overlapping phases of activation, resorption, reversal, formation, and termination, happening over the period of 120-200 days in cortical and trabecular bone, respectively (28). Bone cells are in control of maintaining bone density and mineral homeostasis by balancing bone resorption and bone formation (39). The process of resorbing old bone and activating osteoblasts to produce collagen and construct new bone is a spatially and temporally coordinated process by multiple progenitor and mature bone cells (45,46). However, bone modeling/remodeling occurs largely during skeletal development and growth, but continues to a lesser amount into adulthood and is responsible for periosteal bone expansion throughout aging (5). Regulation of osteoclast and osteoblast activity during bone remodeling is influenced by growth factors, hormones (PTH), cytokines and RANK/RANKL/OPG axis that play important roles in bone remodeling, precisely because regulate osteoclast activity, differentiation, and survival (1).

2.5 Calcium and vitamin D role in bone formation

Calcium and vitamin D are necessary for bone health maintenance. Therefore, deficits in calcium and vitamin D are among the most significant risk factors for the development of osteoporosis (47). Calcium is a chemical element that, in the form of a positively charged ion (Ca^{2+}), is a component of the mineral that makes up bone, along with phosphate. It also plays key functions in intracellular signaling, coagulation, and the function of nerves and muscles; as a result, maintaining steady extracellular concentrations is a homeostatic priority. Vitamin D is an organic substance that may be taken as part of the food or synthesized by ultraviolet-B irradiation in the skin (42). Its structure is based on steroid hormones and functions as a precursor of calcitriol, the primary hormone that regulates intestinal calcium absorption (48).

PTH is a hormone controlled by 1, 25-dihydroxyvitamin D (1, 25-(OH)2D), the active structure of vitamin D and, calcium levels. In case of insufficient calcium from the diet, vitamin D actives the PTH production to mobilize calcium from the skeleton into the bloodstream (42). Increased PTH levels stimulate bone metabolism, resulting in bone resorption and bone loss and consequently the release of Ca^{2+} into the blood. This process allows PTH to maintain calcium homeostasis, normocalcaemia and mediates bone remodeling. When bone resorption occurs at a rapid rate, fracture risk is raised independent of a person bone mineral density (BMD), since bone erosion weakens the skeleton (6).

Vitamin D and calcium supplements are advised for osteoporotic individuals with inadequate calcium intake or absorption, vitamin D deficiency, or undergoing pharmaceutical therapy for osteoporosis (49). Some meta-analysis studies reported a relative risk reduction for hip fracture of 0.81-0.87, which translates to a decrease of 13-19%, when calcium is taken with vitamin D (50,51). The next **Table 2.1** shows the recommended dietary allowance (RDA) of these supplements according to United States Preventive Services Task Force (USPSTF) to prevent osteoporosis (52).

Table 2.1 Recommended dietary allowance of calcium and vitamin D to prevent osteoporosis. (52)

Recommended dietary allowance (RDA)			
	Gender	Age (years old)	RDA
Calcium	Women	19-50	1000 mg/d
		+50	1200 mg/d
	Men	19-70	1000 mg/d
		+70	1200 mg/d
Vitamin D	Women and Men	19-70	600 IU/d
		+ 70	800 IU/d

Calcium and vitamin D supplements also have side effects that are related with a higher incidence of renal calculi and gastrointestinal (GI) discomfort. GI side effects include constipation, excessive abdominal cramping, bloating, and, importantly, upper GI symptoms (49).

Chapter 3: Osteoporosis

3.1 Definition and classification

According to the definition provided by the World Health Organization (WHO), osteoporosis is a "progressive systemic skeletal disease characterized by low bone mass and microarchitectural deterioration of bone tissue, with a consequent increase in bone fragility and susceptibility to fracture" (3,39). Osteoporosis is the most common bone disease (2), and it happens when there is an imbalance between bone resorption and bone formation (1,39). The consequence of this imbalance causes an increase in bone resorption, which decreases BMD (1). In other words, bone breakdown is superior to bone formation (14).

Osteoporosis could be classified as primary or secondary depending on its etiology. Primary osteoporosis (type 1) is defined by a decline in estrogen production, which causes bone loss, and it is a natural part of the aging process. It is most frequent in women after menopause, although it may also be seen in elderly men. Secondary osteoporosis (type 2) is a bone loss that occurs as a consequence of chronic predisposing medical conditions such as hypogonadism, hyperparathyroidism, disorders as malabsorption, or the continued use of medicines such as glucocorticoids, and excessive alcohol consumption (1,2).

Fractures caused by osteoporosis are most prevalent in the spine, hip, and wrist. Hip and vertebral fractures are the most debilitating effects of osteoporosis and are linked to an increase in morbidity and mortality (16,17). These fractures may also result in full recovery. Osteoporotic fractures occur in both male and female old adults; however, they are more common in postmenopausal women (2).

Dual-energy X-ray absorptiometry (DXA) is the most frequent approach for diagnosing osteoporosis recommended by the USPSTF (3) and other international standards (53); it identifies a decrease in BMD, represented as a T-score (1). T-score quantifies the number of standard deviations (SD) by which an individual BMD deviates from the predicted mean value for young, healthy individuals. Osteoporosis is operationally defined as a BMD value at least 2.5 SD below the young female adult mean (T-score less than or equal to –2.5 SD) (54). The more negative the number, the weaker are the bones and more probable they will shatter (55). The different disease states are shown in the table below (**Table 3.1**).

Table 3.1 Osteoporosis states according to bone mineral density (BMD).

Status	Bone mineral density (T- score) – standard deviations (SD)
Normal	-1 or above
Osteopenia	Between -1 and -2,5
Osteoporosis	Below -2,5
Severe osteoporosis	Below -2,5 with one or more fractures

Another approach is to utilize the Osteoporosis Self-Assessment Tool (OST), a simple calculation that uses age and weight to identify those at risk of having low BMD (3).

OST → Score = [weight (kg) - age (years)] × 0.2

Younger postmenopausal women with an OST score below 2 should be tested for BMD. Using an OST cutoff less than 2 to select younger postmenopausal women for BMD testing is appropriate if pharmacological therapy will be initiated for a BMD T-score of –2.5. In order to prevent osteoporosis, screening should be done on all women aged 65 or older, and women younger than 65 if they have specific risk factors, such as low weight, smoking habits, and a history of bone fractures (3,56).

3.2 Epidemiology: how common is osteoporosis?

According to estimates based on the WHO definition of osteoporosis, the disease affects roughly 6.3% of men over the age of 50, and 21.2% of women over the same age range worldwide. This suggests that, depending on the number of men and women in the world, around 500 million individuals may be affected by this bone condition (1,39). Osteoporosis-related fractures are a severe public health issue because they have a significant negative impact on the quality of life. According to the International Osteoporosis Foundation, one in every three women and one in every five males over the age of 50 suffer from osteoporosis. Furthermore, osteoporosis is responsible for about 8.9 million fractures each year, meaning that an osteoporotic fracture occurs every three seconds. Hip fractures impact around one-third of patients, and up to 20% of patients die within a year of the fracture, primarily owing to pre-existing conditions (39).

Based on statistics from the National Health and Nutrition Examination Survey III, the National Osteoporosis Foundation estimates that more than 9.9 million Americans have osteoporosis and another 43.1 million have low bone density (2,57).

Osteoporosis was estimated to affect 32 million Europeans in 2019 (European Union, plus Switzerland and the United Kingdom), representing 5.6% of the total European population aged +50. This comprises roughly 25.5 million women (22.1% of women aged 50+) and 6.5 million men (6.6% of men aged 50+) (54).

The epidemiological study performed in Portugal in 2019, estimated 681,000 (5.6% of the total population) Portuguese suffer from osteoporosis. Also in 2019, approximately 70,700 new fragility fractures were reported, with a 28.9% rise expected in 2034, reaching 91 200 fractures. Furthermore, the proportion of women at high fracture risk who didn´t receive treatment (treatment gap) was 75% in 2019 (up from 37% in 2010) (58).

According to research, osteoporosis and fragility fractures are a costly human and economic burden in all parts of the globe. In 2019, the total direct cost in the EU27+2 (excluding the value of lost quality-adjusted life year) was €56.9 billion. Hip fractures were expected to account for 57% of total expenses, vertebral fractures 10%, distal forearm fractures 2%, and others 32%. The economic cost of new and prior fractures in Portugal was estimated to be €1.0 billion in 2019, representing 5.6% of total national healthcare expenditure. This was an increase of €423 million when compared to the amount estimated in 2010 (€577 million) (54,58,59).

3.3 Etiology and Risk factors

In addition to postmenopausal and old age, osteoporosis is impacted by multiple factors, such as personal and environmental risk factors, as shown in **Table 3.2** (1,56,57,60). Regardless of the type of risk factor, their consequence is to decrease BMD by accelerating bone absorption, hence increasing the risk of bone fracture (39). The most prominent factors are hormones such as estrogen, testosterone and PTH, and the long-term use of glucocorticoids. Estrogen, testosterone, and PTH, all play important roles in bone remodeling by suppressing bone breakdown and stimulating bone growth. In women, peak bone mass occurs between the ages of 25 and 30 (61,62). After this age, estrogen gradually decreases in concentration over time. On postmenopausal women, a reduced estrogen level is noticed,

which causes significant bone loss (63). Low estrogen levels increase osteoclastogenic activity, which leads to increased bone resorption (64,65).

Long-term glucocorticoid usage may lead to problems such glucocorticoid-induced osteoporosis (GIO). Glucocorticoids promote osteoclast development and maturation while suppressing osteoblastogenesis by accelerating osteoblast and osteocyte apoptosis, resulting in increased bone resorption and reduced bone formation (39,66). Within three to six months of starting glucocorticoid medication, there was a fast reduction in BMD in GIO patients (67).

Regular exercise is one of the best ways to improve bone mineral density that works as a protective factor. The impact of these variables on bone health from an early age should be emphasized via education and specific preventative actions (56,68).

Table 3.2 Risk factors of Osteoporosis. Adapted from (1,34,48,49)

Individual risk factors (Nonmodifiable risk factors)	**Environmental risk factors (Modifiable risk factors)**
Age	Excessive alcohol consumption
Female gender	Smoking
Family history	Body Mass index (BMI) less than 20 kg/m2 (56)
Previous facture	Poor nutrition
Ethnicity	Vitamin D deficiency
Menopause or hysterectomy	Nutrition disorders
Long term glucocorticoid therapy	Estrogen deficiency
Rheumatoid arthritis	Insufficient exercise
Primary or secondary hypogonadism in men	Calcium levels
Hematopoietic disorders	Lifestyle habits
Chronic disease	Body weight

3.4 Physiopathology

Osteoporosis is a multifactorial disease with a complex pathophysiology, potentially caused by genetic, endocrine disorders, and nutritional factors (4). The pathological process of osteoporosis is a condition in which bone absorption exceeds bone formation. The imbalance between osteoclast and osteoblast activity causes deep, large resorption cavities, making bones fragile and susceptible to fracture. The relative lack of osteocytes compromises the mechanosensory network necessary for the repair of bone micro-damages. The increase in micro-damages also increases the likelihood of bone fragility and fractures. The **Figure 3.1** shows schematically the base of physiopathology of osteoporosis (69).

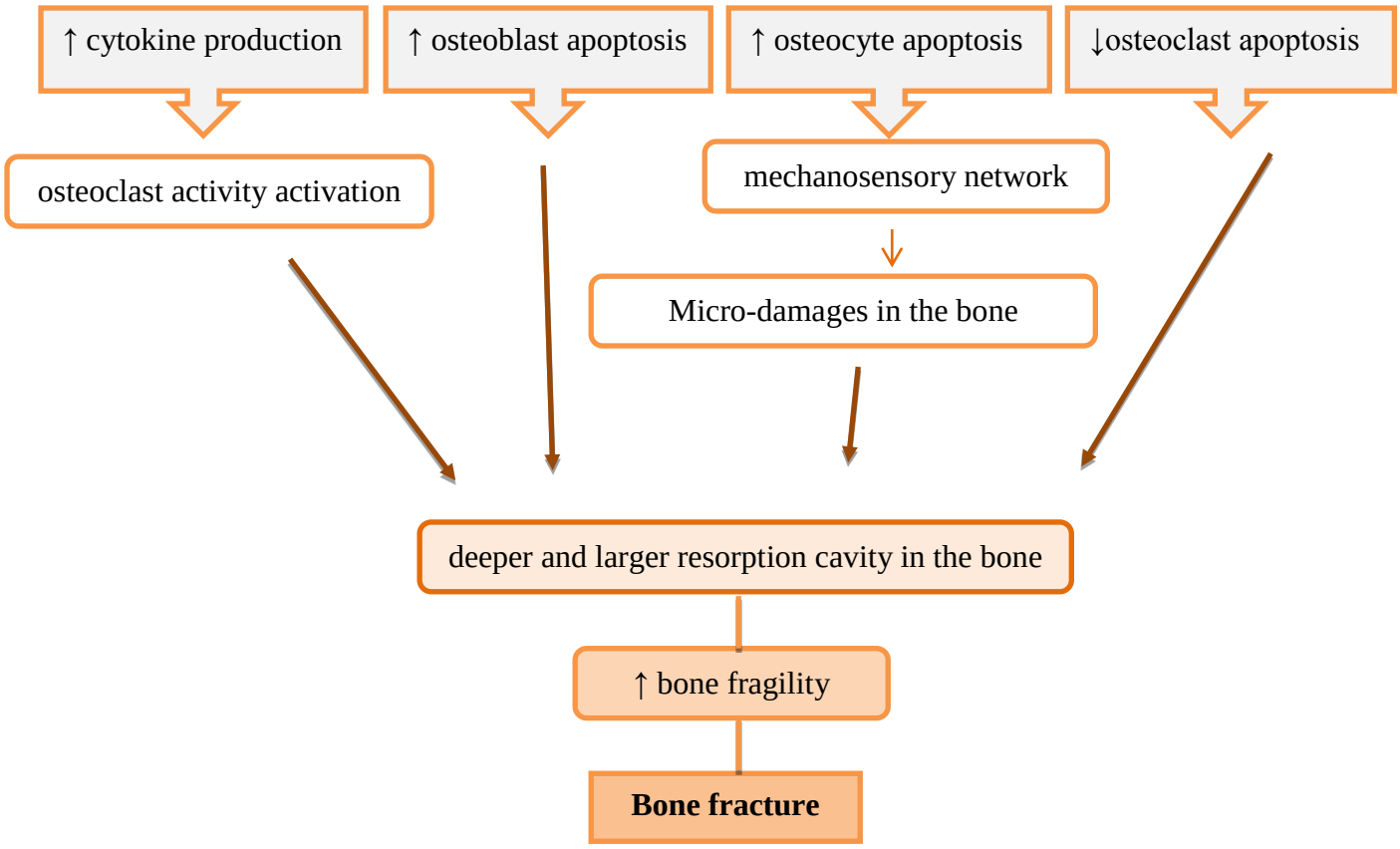

Figure 3.1. Resumed schematic representation of osteoporosis physiopathology. Adapted from (69).

The main pathogenic mechanism of osteoporosis in perimenopausal women is estrogen deprivation. Low estrogen levels induce the synthesis of interleukin-1 (IL-1), interleukin-6 (IL-6) and tumor necrosis-alpha (TNF-α), which increases RANKL expression, decreases OPG expression, and causes RANK/RANKL interaction, that increases osteoclastic activity. The lack of estrogen also promotes apoptosis of osteoblasts and osteocytes, while increases the functional survival time of osteoclasts (64,65,69).

Other hormones, such as PTH, calcitonin, and vitamin D, play a role in bone development, but when their homeostasis is affected, bone fragility results. Continuous high dose of PTH release indirectly increases bone resorption by increasing RANKL/MCSF expression and decreasing OPG expression. In the meanwhile, modest intermittent PTH release promotes bone formation by increasing the survival, proliferation, and differentiation of osteoblasts (62,70). While PTH increase the production of active form of vitamin D (1, 25-(OH)2D or Calcidiol), calcitonin can directly and indirectly inhibit PTH effects, resulting in decreased calcium resorption from the kidneys, increased calcium uptake from the intestine, and suppression of bone resorption. Calcitonin inhibits the function of osteoclasts by binding to its receptor (4,71).

Another pathogenic pathway is related to RANK/RANKL/OPG axis, since PTH, PTH-related protein (PTHrP), cytokines, and prostaglandins can promote osteoclastogenesis by increasing RANKL expression and decreasing OPG expression (71). To regulate osteoblast function, bone remodeling needs the activation of both the Wnt and BMP pathways. Nonetheless, the Wnt signaling pathway persists to be inert, which enables β-catenin to be phosphorylated, ubiquitylated, and ultimately degraded by the proteasome. Since β-catenin is essential for gene transcription, unstable or decreased levels of β-catenin leads to lack of transcription gene activation, such as the Runx2 that controls osteoblastic differentiation (72).

The importance of oxidative stress in the pathogenesis of osteoporosis has also been established. The imbalance that occurs between the production of reactive oxygen species (ROS) and the antioxidative defense is the basis of oxidative stress. The effect of oxidative stress on bone is manifested as a decrease in bone mass, bone formation, osteoblast numbers, and osteoblast dysfunction (4,73).

3.5 Clinical manifestations

In general, osteoporosis don´t present clinical manifestations until a fracture appears, which is often the consequence of a fall. Sometimes, many people without symptoms falsely conclude that they don´t have osteoporosis, whereas many people with hip or foot pain wrongly conclude that their symptoms are caused by osteoporosis. Fractures of the ribs, proximal and distal humerus, and other bones in the upper and lower arms are also common manifestation of osteoporosis. Most of the common fractures, which account for around two-thirds of the total, don´t cause any symptoms and are discovered by accident during chest or abdominal radiography. Fractures may result in a variety of complications, including pain, deformity, incapacity, and even a loss of height (74,75).

Chapter 4: Pharmacological treatment of osteoporosis

There are a variety of pharmacological therapies for osteoporosis that interact with different bone processes. These medicines are classified into three big pharmacological classes: antiresorptive agent, bone-forming agent, and dual action agent. The antiresorptive drug may cause osteoclast apoptosis or inhibit osteoclast recruitment in order to decrease bone resorption, whereas the bone-forming drug operates on osteoblasts to increase bone production. The dual-acting drug suppresses osteoclastic activity and enhances osteoblastic activity (1,5). **Table 4.1** describes the medicines in terms of mechanism of action, side effects, pharmacokinetics, and posology.

4.1 Antiresorptive

The antiresorptive medicines that acts to decrease bone resorption are bisphosphonates, selective estrogen receptor modulators (SERMs), RANK inhibitor, and calcitonin. **Bisphosphonates** are the first-line osteoporosis therapy. Its mechanism of action is to attach to the hydroxyapatite crystals on resorbing bone surfaces. When osteoclasts begin to resorb bisphosphonate-impregnated bone, the released bisphosphonate inhibits the osteoclasts ability to create the ruffled border, attach to the bony surface, and generate the protons required for ongoing bone resorption (76). The drug molecule has two phosphonate ($PO3^-$) groups connected by a carbon atom, allowing for a wide range of potential modifications (18). First-generation bisphosphonates (clodronate; etidronate) are distinguished by their alkyl or halide side chains, while second-generation derivatives (tiludronate; pamidronate) contain either a sulfur-side chain or an amino terminal group, and third-generation bisphosphonates (ibandronate; zoledronate) have an imidazole group instead of aliphatic side chain. The potency of bisphosphonates is reduced in the following order: etidronate < tiludronate < clodronate < pamidronate < alendronate < risedronate (2). Alendronate and risedronate are the first-choice bisphosphonates due to their effectiveness in preventing vertebral and hip fractures and their cost-effectiveness (20). Bisphosphonates accumulate in bone, which is why they may be administered weekly, monthly, annually, or even longer intervals and still suppress bone turnover, and why discontinuing therapy may be done in certain individuals. Due to their low lipophilicity, bisphosphonates have a natural tendency for poor absorption via the GI epithelium, limiting the transcellular diffusion (2,5).

Oral bisphosphonates are normally taken in the morning, before eating or drinking, with a full glass of plain water. After taking bisphosphonates, patients must wait at least 30 minutes before eating, drinking or taking other drugs (2). Oral administration of bisphosphonates can cause upper GI adverse effects which are considered as the main problem of oral N-containing preparations and the most common reason for treatment discontinuation.

SERMs are a class of drugs that act as agonists or antagonists of intracellular estrogen receptors in distinct tissues. Raloxifene is the only SERMs that has been approved worldwide for the prevention and treatment of postmenopausal osteoporosis (2). Raloxifene interacts with the RANKL/RANK/OPG axis, limiting osteoclast recruitment and activation by decreasing RANKL release, and hence reduces bone resorption (5). Furthermore, it promotes bone mineral density while lowering the risk of endometrial cancer. A disadvantage of raloxifene is that it has a bioavailability less than 2% owing to extensive first pass glucuronidation (2).

Calcitonin is a synthetic polypeptide hormone having natural calcitonin characteristics found in mammals, birds, and fish (39). This drug binds to receptors mostly found on the surface of osteoclasts, inhibiting bone resorption, and increasing bone growth. Calcitonin has been used to treat osteoporosis and has been determined to be safe with no major or long-term negative effects. It is, however, less effective than bisphosphonates (2). Calcitonin also has analgesic properties (39).

The **RANKL inhibitor**, denosumab, is a monoclonal antibody that reduces osteoclast development, activity, and survival in humans. This monoclonal antibody has been approved for the treatment of postmenopausal osteoporosis, bone metastases from breast cancer, and bone loss in males with prostate cancer (39). In GIO, denosumab was shown to increase BMD more effectively than risedronate (77). Denosumab binds competitively to RANKL, preventing RANK binding and consequently inhibiting osteoclasts activation. In this mode, denosumab inhibits bone resorption and turnover potently, resulting in BMD increase. This medication is contraindicated for hypocalcaemic people (2). The discontinuation of denosumab causes a rebound stimulation of bone turnover to levels higher than before therapy. This causes fast bone loss and a possible increase in the risk for vertebral fractures (5).

4.2 Bone-forming agent

Bone‐forming drugs include PTH and PTHrP. Teriparatide is a recombinant human PTH analogue that contains the first 34 amino acids (aa) of PTH N-terminus, while abaloparatide is a synthetic 34-aa analogue of PTHrP that is similar to PTHrP at 1-20 aa (5). Teriparatide and abaloparatide treatment increases bone turnover and stimulates new bone formation by increasing osteoblast activity more than osteoclast activity. The resorption is less with abaloparatide (39).

Teriparatide is a potent anabolic drug that is used to treat postmenopausal women and men with severe osteoporosis, as well as those with established GIO who are on long-term glucocorticoids. Teriparatide has been found to minimize fracture risk and is now a therapy option for people with osteoporosis who are at high risk for fracture (2). Abaloparatide (PTHrP1-34) is expected to be more anabolic than teriparatide (78). Over an 18-month period, abaloparatide decreased the incidence of new vertebral fracture by 86% and nonvertebral fracture by 43% in a phase III clinical study (79). The use of this drug is restricted to two years. Furthermore, abaloparatide is less expensive to utilize than teriparatide (39).

4.3 Dual-acting agent

Romosozumab is a humanized anti-sclerostin antibody that is administered as a monthly subcutaneous injection. Sclerostin is a protein released by osteoblasts and osteoclasts that inhibits osteoblast differentiation, proliferation, and activity. It binds to LRP-5/6 on osteoblasts competitively and inhibits the Wnt/β-catenin pathway, limiting osteoblast differentiation (80). The administration of romosozumab produces significant and rapid improvements in bone formation markers. Despite continued treatment, the increase in bone production return to baseline levels after 6 months (81). Bone resorption indicators fall fast following treatment initiation and stay below baseline throughout the treatment period. The alterations in bone turnover indicators show that romosozumab has a dual impact of stimulating bone production while inhibiting bone resorption (5).In phase III clinical studies, romosozumab increased BMD more than alendronate and teriparatide. Furthermore, compared to placebo, it demonstrated a 73% decreased incidence of new vertebral fracture after 12 months (82). The FDA approved this anti sclerostin antibody notwithstanding the fact that it had significant cardiovascular events in clinical trials (83). Other anti-sclerostin

monoclonal antibodies, such as blosozumab (84) and setrusumab (85), are in the process of the drug development in phase II and phase III respectively (86).

Table 4.1 Resume of pharmacological treatment in osteoporosis.

Pharmacological class	Drug	Mechanism of action	Side effects	Pharmacokinetics	Posology
Antiresorptives					
RANKL inhibitor	Denosumab (Prolia®) (87)	IgG2 prevents RANKL/RANK interaction, ↓osteoclast numbers and function and ↓ cancer-induced bone destruction.	Hypocalcaemia, Diarrhoea.	BA - 62%. $t_{1/2}$: 28 days; T_{max}= 10 days	SC, every 6 months
Bisphosphonates	Alendronate (Fosamax®) (88)	Inhibiting farnesyl diphosphate synthase (FDPS) in the mevalonate pathway, resulting in the prevention of prenylation and loss of function of the small GTPases (regulate osteoclast function) (2)	Upper GI adverse reactions as abdominal pain, acid regurgitation, constipation, diarrhea.	Low oral BA, $t_{1/2}$: 10 years. CLR: 71mL/min	oral, daily, or weekly
	Ibandronate (Boniva®) (89)		Anaphylactic reaction/shock, atypical fractures of the femur, osteonecrosis of the jaw, GI irritation.	Low oral BA, $t_{1/2}$: 10- 72 hrs. CLR: 84-160 ml/min	oral, daily, or monthly; IV, Q3M
	Zoledronic acid (Zometa®) (90)		bone pain, fever, fatigue, arthralgia, myalgia, rigors, and arthritis.	Terminal $t_{1/2}$: 146 hours.	IV, once yearly
	Risedronate (Actonel®) (91)	binds to bone hydroxyapatite and inhibits osteoclast-mediated bone resorption	Upper GI adverse reactions.	Low oral BA, Terminal $t_{1/2}$: 480 hr.	oral, daily, weekly, or monthly
SERMs	Raloxifene (Evista®) (92)	raloxifene has selective agonist or antagonist activities on tissues responsive to estrogen. It acts as an agonist on bone and partially on cholesterol metabolism	venous thromboembolic events, which occurred in less than 1% of treated patients.	BA: <2% Excretion primary in feces. $t_{1/2}$: 27.7 hours	Oral, once daily.
	Calcitonin (93)	↓ bone resorption by a direct action on osteoclasts via its specific receptors.	GI tract disorder, skin flushes.	low oral BA $t_{1/2}$: 50 to 80 min	SC or IM, once daily.
Bone-forming Agents					
PTH and PTH-related protein analogs	Teriparatide (Movymia®) (94)	↑ bone formation by direct effects on bone forming cells, ↑ intestinal/ tubular absorption of Ca2+ and ↑ excretion of phosphate by the kidney.	Nausea, pain in limb, headache, and dizziness.	$t_{1/2}$: 1 hr. CLR (women): 62 L/hr. CRL (men): 94 L/hr.	SC, daily
	Abaloparatide (Eladynos ®) (95,96)	an agonist PTH type 1 receptor (PTH1R) and activates both G protein–mediated cAMP signaling and β-arrestin-mediated ERK-1/2 signaling pathways with similar potency.	Blood in the urine, depression, constipation.	$t_{1/2}$: 1.7 hr.	SC, daily
Dual-acting agent					
Anti-human sclerostin	Romosozumab (Evenity®) (97)	(IgG2) binds and inhibits sclerostin, ↑ bone formation by activating bone lining cells, ↑ bone matrix synthesis by osteoblasts, and recruiting osteoprogenitor cells, ↓bone resorption by altering osteoclast mediator expression.	Nasopharyngitis, arthralgia and Hypocalcemia, common cold, and joint pain.	BA: 81% T_{max}: 5 days $t_{1/2}$: 12.8 days	SC, Monthly for 12 months.

Tmax=time to peak drug concentration; BA=bioavailability; $t_{1/2}$= half-time; CRL=Clearance; IV =intravenous; PTH = parathyroid hormone; Q3M = every 3 months; SC=subcutaneous

Chapter 5: All about Vitamin K

5.1 Discovery

The discovery of VK opened the door for scientific progress in several health-related areas. Carl Peter Henrik Dam started his research on cholesterol metabolism in 1928 by observing the effects of a low-fat diet on hens. This was the beginning of VK discovery and application (**Figure 5.1**). This Danish scientist discovered that chicks fed cholesterol- and fat-free chicken feed for more than two to three weeks, had a natural tendency to bleed spontaneously (8,25,98–100). Years later, Dam collaborated with Paul Karrer and identified a previously undiscovered antihemorrhagic component after examining the lipid portion of the diet. This lipid-soluble component was assigned the first letter of the vitamin alphabet available, which corresponded to the first letter of the German term "Koagulation" and was thought to be important for its anti-hemorrhagic property. In 1936, VK was identified, for the first time, to be a crucial component in the process of blood clotting (25,99).

In the United States at the time, Edward Albert Doisy isolated antihemorrhagic VK and characterized its naphthoquinone ring structure (98). In 1939, scientists successfully isolated VK1 from alfalfa and VK2 from fermented fishmeal. The two vitamers were identified within a year, enabling for industrial production. Soon after its discovery, VK became crucial in the treatment of liver diseases and the prevention of newborn hemorrhage (99).

Later, the Nobel Committee for Physiology or Medicine acknowledged their pioneering work and its importance. Henrik Carl Peter Dam and Edward Adelbert Doisy were awarded the Nobel Prize in Physiology and Medicine in 1943 for discovering and explaining the molecular structure of VK (24,98,99).

The biological importance of VK was not uncovered until the 1970s. During this decade, it was discovered that a novel amino acid termed γ-carboxyglutamic acid (Gla) was present in all vitamin K dependent protein (VKDP). Also, during this time period, the first bone VKDP, OC, was found, which was a significant advance in the understanding of the VK cycle. VK is required as a co-factor for the GGCX enzyme for the conversion of glutamic acid (Glu) residues into Gla residues in VKDPs. The resulting Gla residues may bind calcium ions, inducing a protein conformational change required for physiological function (98,100). Other VKDPs with broad tissue distribution and functional capacity were found in the

decades that followed. In the 1990s and 2000s, epidemiological and intervention studies investigated the impact of VK discoveries on bone and cardiovascular health (100).

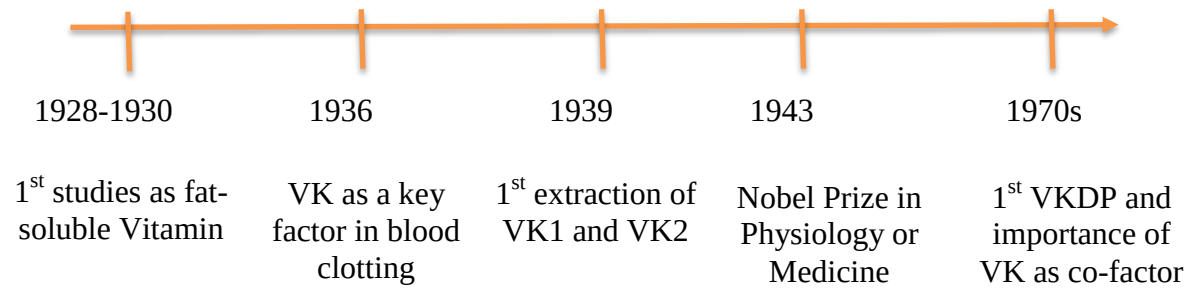

Figure 5.1. Chronology of vitamin K discovery and its application.

5.2 Classification

Chemically, VK is a quinone, which consists of a cyclic organic compound with two carbonyl groups (C = O), either adjacent to or separated by a vinylene group (CH = CH) in a six-membered unsaturated ring (101) that is present in the electron transport chains of a wide variety of organisms (102). VK occurs naturally as two biologically active vitamers obtained from food: PK, commonly known as VK1, and MKn, also known as VK2 (9,23–25). These vitamers may be differentiated from one another based on the level of saturation as well as the length of the aliphatic lipophilic side chain that is attached to the 3-position (**Figure 5.2**) (9,23). VK is a series of fat-soluble molecules distinguished by the presence of a 2-methyl-1,4-naphthoquinone ring structure known colloquially as menadione or vitamin K3 (VK3) (9,23–25,103). VK3 is a synthetic, hydrophilic form of VK that vertebrates may convert into MKn by adding a prenyl side chain (9,24). This form of VK should be referred to as a pro-vitamin since it is not obtained by food but is a product of VK1 catabolism and a circulating precursor of tissue MK-4 (104–106).

PK contains a phytyl side chain consisting of four prenyl units (9,25,107) while MKn comprise a polyunsaturated aliphatic side chain with 4 to 13 isoprenyl units. MKn are categorized according to the number of prenyl units that menadione contain. VK2 is classified into two types: short-chain (menaquinone-4 (MK-4)) and long-chain (i.e., MK-7, MK-8, and MK-9) (25,107). MK-4 is the predominant form of VK (>90%) in tissues and generally can be converted from PK/menadione through two possible mechanisms. The first one is systemic conversion which occurs when menadione is prenylated in tissues after being generated from

PK in the colon and liver; the second is local conversion which happens when menadione is cleaved and prenylated in the brain (26). The next figure (**Figure 5.2**) shows the chemical structure of the most common vitamer of VK.

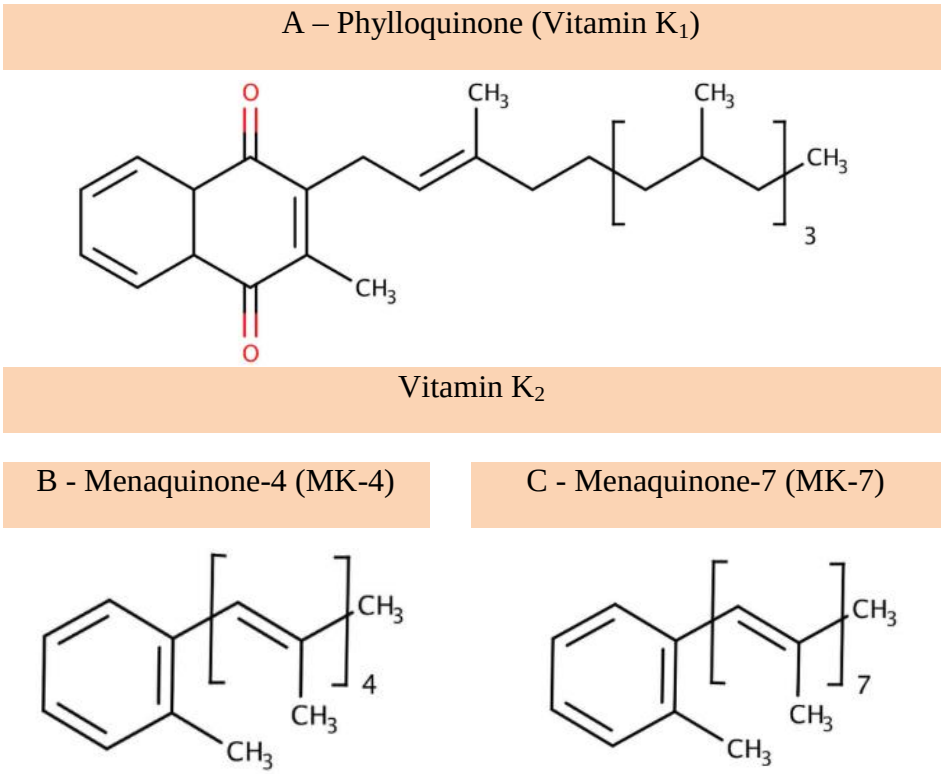

Figure 5.2. The most common vitamers of vitamin K.

Vitamin K4 commonly known as menadiol, is a synthetic water-soluble connected in the body to other synthetic VK forms, such as pro-vitamin. Its biosynthesis might be by reduction of pro-vitamin or one of its ester forms (e.g., diacetate vitamin K3) (24,108).

5.3 Sources

VK vitamers may derive from either natural sources or chemical synthesis, although VK2 also has the biosynthetic pathways (23,104). The predominant dietary form of VK is VK1, a vitamer found mostly in photosynthetic organisms such as cyanobacteria, algae, and green plants because is the result of the shikimate pathway in the photosynthesis process (23,24,102). Green plants high in VK1 include kale, romaine lettuce, broccoli, cabbage, and spinach (24). Moreover, fruits as avocado, kiwi, and grapes contain VK1 as well (23–25). Soybean oil is the most major source of PK, followed by rapeseed oil and olive oil, which contain an average of 180, 130, and 55 µg/100 g, respectively (24).

MKn, the other form of VK, have the best sources in fermented foods such as Natto, sauerkraut, cheese, curd cheese, and butter (26). Additionally, VK2 may be found in the yolk of eggs, as well as in beef, liver, and salmon (23,25,26). Except for the MK-4 form, VK2 may be biosynthesized by anaerobic bacteria prevalent in the gut flora, such as Bacteroides and Enterobacteria. Nonetheless, MK-4 is produced from VK1 in human and animal tissues via a specific conversion that occurs in the pancreas, testes, cerebrum, and vessel wall and is mediated by UbiA prenyltransferase containing 1 (UBIAD1) (9,24,25). This enzyme, which is the human homologue of the prenyltransferase found in Escherichia coli transfers the geranylgeranyl group from geranylgeranyl pyrophosphate (GGpp) to menadione, thus catalyzing the biotransformation of PK into MK-4 (24,109).

VK1 is chemically synthesized for commercial use as supplements, pharmaceuticals and fortified food by condensing naphthoquinone with isoprenoid precursors (25,102). In the chemical manufacturing of VK1, the emphasis has been on reducing the biologically inactive form (*trans*(Z)-isomer) and the toxicity of the chemicals utilized in the synthesis (104,110). Furthermore, it wagers on biotechnological manufacturing to improve the quality of the final active product (*trans*(E)-isomer) and minimize supplement production expenses (111).

Traditional chemical production of VK2 is costly and produces a limited yield of the all *trans* MK-7 form (active form) (112). Therefore, other methods of industrial manufacturing, such as the biosynthetic production of natural VK2 by bacterial fermentation, have been examined. This is owing to microorganisms selective all-trans isomer synthesis and the ease of manipulating and optimizing growth conditions of various bacterial strains (104).

Estimating an acceptable intake is challenging since the current requirement is an estimate based on geographical consumption. The current recommended daily reference intakes (DRIs) for VK is solely based on ingestion of VK1, which is the quantity of VK1 required for blood coagulation (25). The recommended intake of VK varies by age and gender, and various big organizations report DRIs of VK, as indicated in **Table 5.1**. DRIs are a set of reference values that are used for dietary recommendations and nutritional status assessments of healthy individuals (25,113).

Table 5.1. Vitamin K daily reference intakes (DRIs). (25).

	Man	**Woman**
World Health Organization (WHO) / Food and Agriculture Organization (FAO)	65 µg/day	55 µg/day*
National Academy of Medicine (NAM)	120 µg/day	90 µg/day*
Commission of the European Communities	75 µg/day	75 µg/day

*Based on 1 µg/day/kg vitamin K

Although 90–120 g/day is recommended for adults, subclinical VK deficiency is frequent, since studies *in vivo* show this is inadequate to induce complete carboxylation of VKPDs. In fact, the DRIs only guarantees the carboxylation of hepatic VKDPs and does not account for the full carboxylation of extra-hepatic VKDPs (114,115). The low DRIs for VK are mainly due to the existence of a VK recycling cycle (114). At this time, there are no official DRIs that have been established for VK2 (25).

5.4 Vitamin K cycle

VK is a fat-soluble vitamin, nevertheless, the human body only stores extremely little levels and without a regular diet it is quickly depleted. Due to this limited VK storage capacity, the body recycles VK through the VK oxidation-reduction cycle (**Figure 5.3**) to reuse it several times. As a result of the VK cycle, nutritional need is reduced while guaranteeing VK availability for the crucial activity of γ-carboxylation (26,116–119).

The VK cycle is the metabolic pathway by which oxidized vitamin, produced by γ-carboxylation of VKDPs in the endoplasmic reticulum, is converted back to its reduced state (120). This redox cycle occurs in several tissues, most notably in the liver and bone. The integral membrane enzymes GGCX and vitamin K epoxide reductase (VKOR) are required for this process, and they work on membrane-bound VK. For VKDP carboxylation, either VK1 or VK2 may serve as a cofactor (104,107).

VK cycle contains two phases: reduction and carboxylation (121). The cycle begins with the diet-derived vitamin in the form of quinone, which is then reduced to its active form hydroquinone (KH2) by the quinone reductase (QR) at the cost of NADPH. The next reaction leads to the oxidation of KH2 to epoxide (KO). The presence of the KH2 is necessary for the activity of the GGCX, which is responsible for converting the uncarboxylated VKDPs into γ-glutamyl carboxylated proteins. Carbon dioxide and oxygen are also required for the process

to proceed. The last step involves restoring the quinone molecule by the VKOR activity. Anticoagulants like warfarin, which are known as VK antagonists, block the VKOR action and in this way the levels of active KH2 decrease (104,118,119,122,123).

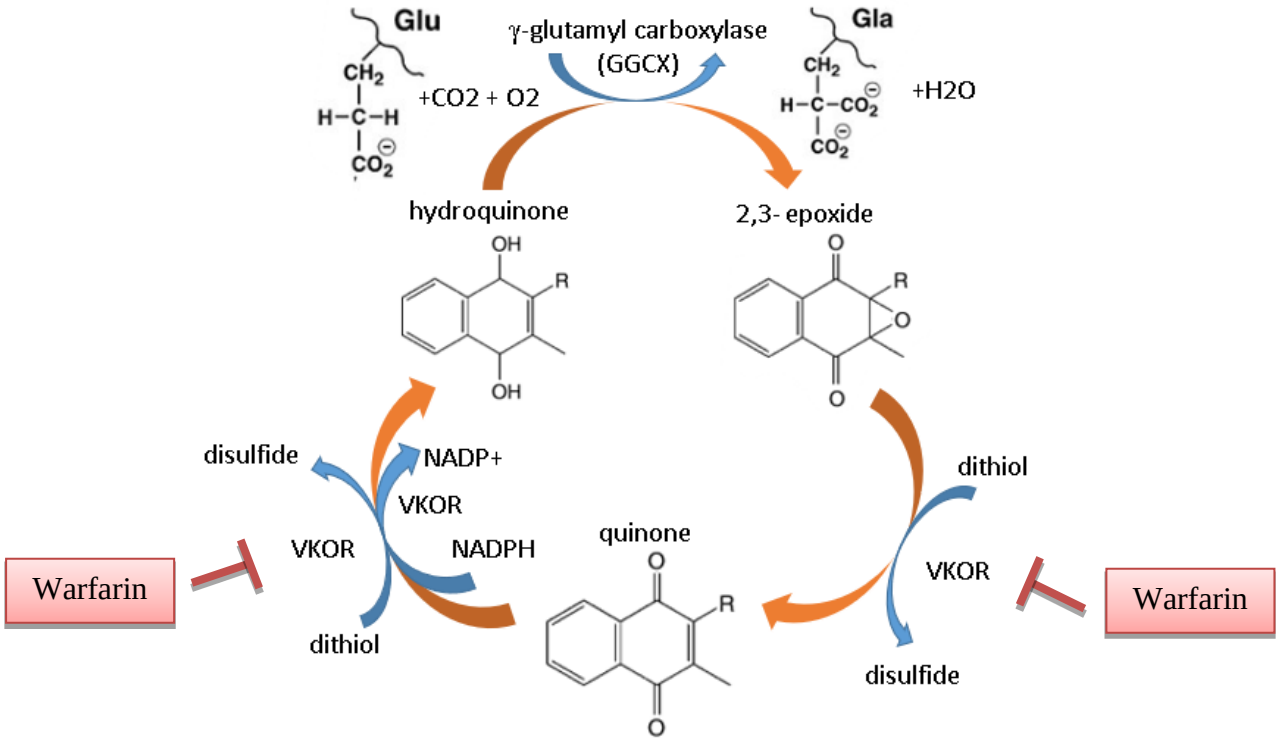

Figure 5.3. The Vitamin K cycle. Adapted from (86).

5.5 Pharmacokinetic profile of Vitamin K

Every consumed substance has a route to be digested and subsequently eliminated. Pharmacokinetics is the study of how molecules are absorbed, distributed, metabolized, and eliminated in the body, and it is used to analyze the whole period of VK exposure (**Figure 5.4**) (124). All natural forms of VK are very lipophilic and so incompatible with blood. VK, unlike other fat-soluble vitamins, is only linked to lipoproteins in the blood (125).

After consuming VK, whether via dietary or supplements, the **absorption** process starts in the intestines. VK absorption in the intestine is mediated by bile salt and pancreatic solubilization. After reaching the intestinal lumen, VK is absorbed through a mixture of micelles composed of bile salts, pancreatic lipolysis products, and other dietary lipids (23). Micelles are absorbed by small-intestinal enterocytes and integrated into specialized lipoproteins associated with apolipoprotein-A (apoA) and apolipoprotein-B48 (apoB-48) on the cell surface, known as chylomicron (CM), which transport the molecule to the basolateral site of the endothelium (23,125,126). CMs are secreted by exocytosis from gut villi into lymphatic capillaries (lacteals) and enter the circulation through the thoracic duct, where apolipoprotein-C (ApoC) and apolipoprotein-E (ApoE) are added (23,125). In capillaries, lipoprotein lipase (LPL) removes chylomicrons from triglycerides, leaving cholesterol-rich remnants in circulation. Remnant of chylomicrons, upon reaching the liver, are converted to very low-density lipoprotein (VLDL), which, together with Apolipoprotein B-100, C, and E, penetrates osteoblasts through receptors as low-density lipoprotein (LDL) interacting with low-density lipoprotein receptor (LDLR) and low-density lipoprotein receptor-related protein 1 (LRP1) (127,128). It is considered that osteoblasts acquire VK1 mostly from CM remnants and MK-7 mainly from LDL (129). Endocytosis is the process through which VK enters bone. ApoE is particularly important since it was discovered that ApoE polymorphism influences VK levels (127,128).

VK1 is absorbed in the small intestine by an energy-dependent process, while VK2 is mostly absorbed in the colon via a passive diffusion mechanism, but it may also be absorbed in the small intestine (103,127). Due to their high lipophilicity, the intestinal absorption of long-chain MKn is superior compared to MK-4 and PK. In addition, VK2 has a longer half-life than VK1 (26). A research that compared VK1 with MK-7 reveals that MK-7 has a half-life period of 68 hours compared to just 1-2 hours for VK1 (130). Various types of VK are carried by distinct carriers (23).

VK is absorbed in the small intestine by Niemann-Pick C1-Like 1 (NPC1L1), a highly expressed transporter in this part of the gut (131,132). NPC1L1 is an important modulator of extracellular cholesterol transport from the surface of the intestinal epithelium to the apical membrane of hepatocytes in order to prevent excessive biliary cholesterol loss. Consequently, the administration of ezetimibe, an NPC1L1 inhibitor, may cause VK malabsorption (133). Also, the scavenger receptor class B-type I (SR-BI) and cluster-determinant 36 (CD 36) are two membrane proteins that enhance the transfer of lipids and are implicated in VK absorption (125).

VK deficiency may be caused not only by insufficient food intake but also by a variety of health conditions, such as liver disease, biliopancreatic disorders, cystic fibrosis, alcoholism, and enteric disease that may induce malabsorption (inflammatory bowel disease, short bowel syndrome, etc.) (103,126) Furthermore, the following disorders and drugs have been shown to interfere with VK absorption:

(i) Use of **antibiotics** for more than 10 days, such as rifampicin, may reduce VK absorption by stimulating metabolism;

(ii) The use of **phenytoin** during pregnancy or breastfeeding may decrease VK in newborns;

(iii) Low-fat diets and fat-blocking medications

(iv) Bile acid sequestrants such as **cholestyramine, colestipol, and colesevelam**, which restrict fat absorption, might impair VK absorption owing to the binding of bile acids;

(v) **Olestra,** and **orlistat** are medications that drive patients to reduce their fat intake since it is associated with unpleasant GI tract side effects;

(vii) Preservative butylated hydroxytoluene (BHT);

(vi) Mineral oil;

(viii) GI tract disorders, liver diseases, and estrogen medications (7,24).

The **distribution** of VK in bodily organ tissue is inconsistent; VK1 was discovered to be mostly distributed in the liver, but VK2 was shown to be more abundant in extrahepatic tissues (23). The **metabolic** transformation of PK into MK-4 involves the use of pro-vitamin as an intermediary (107). Both VK1 and VK2 are quickly metabolized *in vitro* into a

combination of quinone, KH2, and KO (103). In humans, VK1 and MKn are metabolized in the liver via similar mechanisms, beginning with initial ώ-hydroxylation mediated by CYP4F2, followed by shortening of the polyisoprene side chain via β-oxidation by mitochondrial trifunctional protein (MTP) to carboxylic acids (5 C, 7 C, or 10 C metabolites), which are glucoronate by UDP-glucuronosyltransferase in the endoplasmatic reticulum. The gluconate metabolites are **excreted** in urine and bile and may be used as indicators of VK status (24,127).

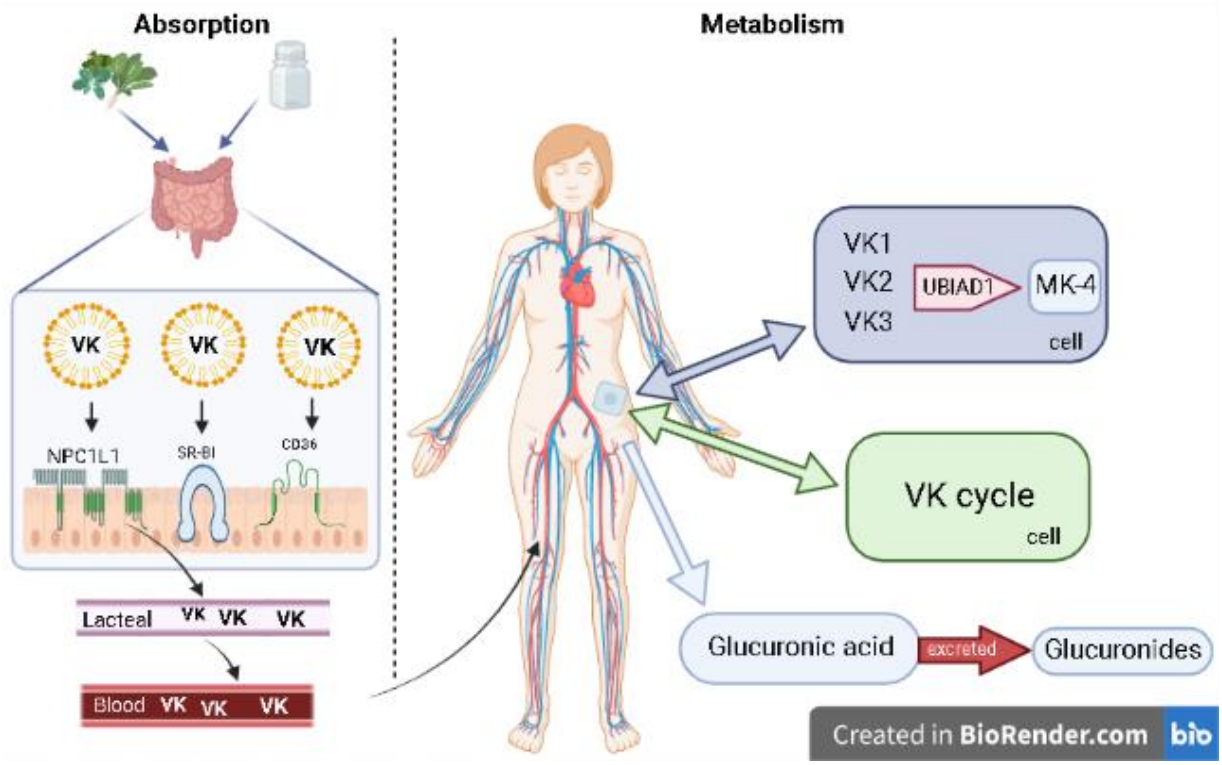

Figure 5.4. Pharmacokinetic profile of Vitamin K. Adapted from (23).

Chapter 6: Functions of vitamin K in bone linked to osteoporosis

The principal function of VK is to act as a co-factor for the GGCX enzyme in the γ-carboxylation reaction, although it also has effects that are independent of GGCX activity, as an antioxidant agent decreasing intracellular ROS, anti-inflammatory agent in the NF-κB pathway and, most recently, as anti-tumor agent (Figure 6.1) (104,134,135). Besides those functions, VK also interacts with the SXR/PXR receptor to maintain bone homeostasis (136). The γ-carboxylation process occurs on both hepatic and extra-hepatic VKDPs, and is required for functionality of VKDPs (134). Gla residues from VKDPs allow high affinity binding with calcium ions and calcium in the mineral form (122). VKDPs are involved in physiological and pathological processes namely, tissue mineralization, inflammation, energy metabolism (102), neuroprotection and cellular growth and survival (104). The hepatic VKDPs is a group of 7 proteins involved in the coagulative cascade, that include coagulation factors II (prothrombin), VII, IX, X and anticoagulation proteins C, S, and Z. These proteins are synthesized in the liver and depend on VK1 dependent γ-carboxylation (9,25,122). Extra-hepatic VKDPs that are implicated in the bone and vascular mineralization include OC, matrix Gla protein (MGP), growth arrest-specific protein 6 (Gas6), and Gla-rich protein (GRP) (9,25).

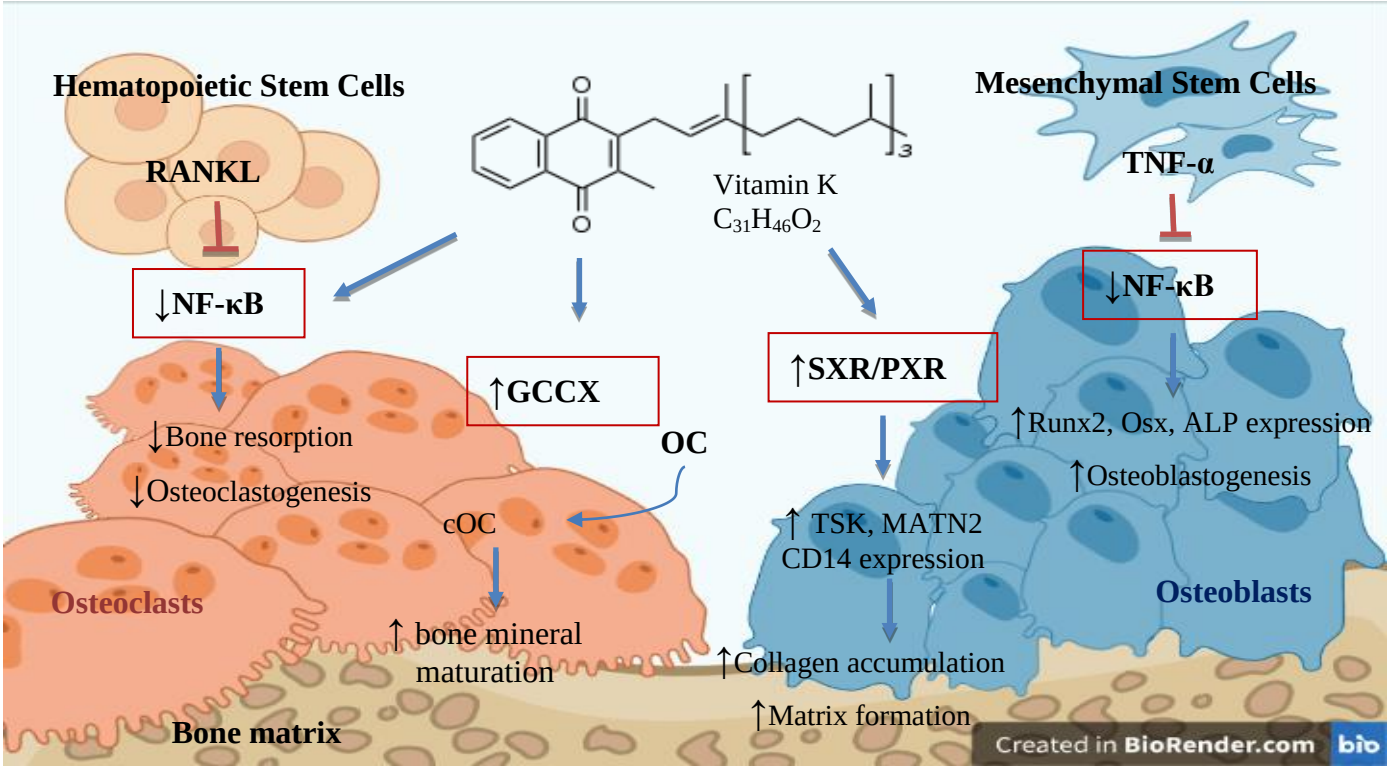

Figure 6.1. Mechanism of action of Vitamin K in bone homeostasis. Adapted from (117).

6.1 Vitamin K – γ-carboxylation of VKDPs involved in bone homeostasis

OC is probably the most important VKDP associated with bone homeostasis, although MGP, GRP, Gas 6 and protein S might also have important functions in bone metabolism (127,137). VK status for bone metabolism may be determined indirectly using a VKDP activity parameter measuring uncarboxylated proteins (127). The uncarboxylated VKDPs (ucVKDPs) are released into the blood circulation by the many tissues in the body that are responsible for VKDPs production. The most significant ucVKDP for bone health is uncarboxylated osteocalcin (ucOC) and its blood levels may be an effective indication of VK insufficiency to maintain bone homeostasis (14). The scientific evidence on VK benefits and VKDPs functions suggest that it may soon be acknowledged as a super micronutrient used in clinical practice as a dietary supplement, not only for osteoporosis, but also for other pathologies (134).

6.1.1 Osteocalcin

OC was discovered in the late 1970s as the most abundant non-collagenous protein in the bone extracellular matrix. In humans contains 49 amino acids (aa) with Gla residues at positions 17, 21, and 24. OC is produced during bone formation by osteoblasts and to lesser extent odontoblasts under vitamin D control on osteoblastic differentiation and osteoid formation (**Figure 6.2)**. This protein is primarily involved in bone mineralization because it binds and incorporates calcium ions into hydroxyapatite crystals in the bone matrix (9,26,138). The capacity of osteocalcin to bind calcium is reliant on the γ-carboxylation of three glutamic acid residues by VK (139).

Vitamin D receptor (VDR) is a transcription factor that activates the bone γ-carboxyglutamic acid-containing protein (BGLAP) gene at the promoter region, thereby stimulating OC transcription (140). BGLAP gene is inactive during osteoblast proliferation, however highly expressed during osteoblast differentiation. After gene transcription, proprotein convertase furin, which is responsible for pro-OC maturation *in vitro* and *in vivo*, proteolyzes the pre-pro-OC peptide (141). Thus, the proteolytic phase begins by transforming pre-pro-OC into a prepeptide (23 amino acids), then into pro-OC peptide (75 amino acids), and lastly cleaves pro-Gla/Glu OC into Gla/Glu OC (141,142). VK stimulates the pro-OC peptide carboxylation of Glu residues 17, 21, and 24 to produce Gla residues. Since not all OC is carboxylated, a certain quantity of ucOC can be detected (142). Glu carboxylation at

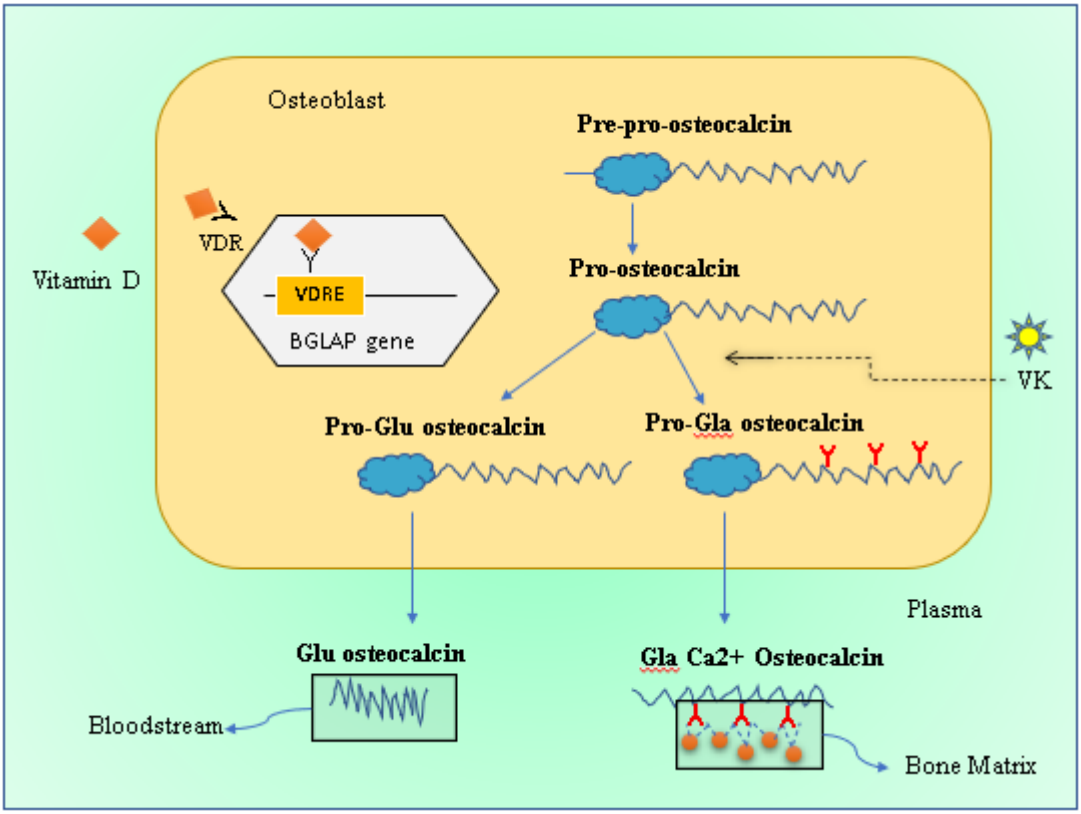

Figure 6.2. Synthesis of osteocalcin and its γ-carboxylation. Adapted from(115).

position 17 is probably crucial for OC spatial and structural conformation, allowing carboxylated osteocalcin (cOC) to interact properly with hydroxyapatite crystals (44,138). ucOC and cOC forms are released by osteoblasts in a calcium-dependent mechanism, but cOC is the only form interacting with calcium. cOC has two major domains: one calcium-dependent "Gla helix" that binds to hydroxyapatite, the main component of bone matrix, and another "-COOH-terminal beta sheet" that attracts monocytes, the presumed progenitor of osteoclasts (26). ucOC, on the other hand, has a low affinity for hydroxyapatite and is more readily released into circulation (142). Only 10-30% of produced OC enters the bloodstream, while the remainder stays bound to the bone matrix. 1/3 of total osteocalcin is ucOC (143). It is considered that ucOC is a risk factor for hip fracture (7). Low levels of ucOC are associated with higher BMD and a lower risk of fracture (138).

OC is a bone turnover marker that plays an important role in the production and regulation of bone matrix (9). An *in vitro* study explains osteocalcin bio-mineralization mechanism. During osteogenic differentiation of MSCs with knockdown OC, analysis of the mineral species through Raman spectra, revealed delayed maturation of mineral species and lower hydroxyapatite levels, relative to the control MSCs. Overall, the mineral-to-matrix ratio

was decreased after three weeks of osteogenic induction. Also, OC knockdown resulted in downregulation of Runx2, ALP, osteonectin, and type I collagen, but upregulation of Osx. This study supports the role of osteocalcin in mineral maturation and modulation of MSCs osteogenic differentiation. In addition, molecular evidence suggest that osteocalcin is not just a late protein product of osteogenesis, but also a signal to control transcription factor expression for osteogenic differentiation (144).

An *in vivo* study shows that osteocalcin decreases osteoblast activation. Von Kossa staining and mineral apposition rates in tetracycline-labeled bones showed no alterations in osteocalcin knockout mice. Thin slices of femora from 4-week-, 6-month- (intact and ovariectomized), and 9-month-old wild-type and osteocalcin-knockout mice were analyzed by Fourier transform infrared microspectroscopy (FT-IRM). FT-IRM spectra detected mineral, carbonate, and apatite concentrations, size, and perfection. The mineralization process was unaffected in 4-week-old knockout and wild-type mice. Six-month-old wild-type animals had more mineral in cortical than trabecular bones; knockout and wild-type bone mineral levels were similar. At each age, carbonate:phosphate ratios were greater in wild-type animals. Cortical bones of 6-month-old wild animals contain larger/flawless phosphate crystals. In 6-month-old ovariectomized wild-type animals, mineral content increased from periosteum to endosteum, but stayed constant in knockout animals' bones. Knockout cortices had lower carbonate:phosphate ratios than wild-type and similar crystallite size and perfection. Spatial resolution shows osteocalcin accelerates bone mineral maturation (145).

The amount of ucOC in the blood indicates bone turnover and VK status. A lack of VK elevates serum ucOC. Serum OC concentration may help monitor follow-up changes that BMD cannot detect, and increased levels may be a better tool to identify persons with rapid bone turnover after menopause (146). An ELISA detection study showed that women with osteopenia and osteoporosis had significantly greater levels of OC than those with normal BMD ($p<.0001$). After three months of treatment, OC levels showed significant changes in women monitored on therapy (147).

6.1.2 Matrix Gla Protein

MGP is an extrahepatic VKDP with 84 aa and a molecular weight of 10 kDa. MGP is synthesized and secreted primarily by vascular smooth muscle cells (VSMCs) and chondrocytes, but is mainly found in bone, dentine, and cartilage (148). This protein has five Gla residues for γ-carboxylation and three serine sites to be phosphorylated. As a result,

phosphorylation and γ-carboxylation determines the activity status: phospho-carboxylated matrix gla protein (p-cMGP) is the fully active form, whereas desphospho-uncarboxylated matrix gla protein (dp-ucMGP) is the completely inactive form (9,138). The p-cMGP function as an inhibitor of pathological calcification with high affinity for calcium ions, hydroxyapatite and BMP (148).

The following *in vitro* studies give insight on the participation of MGP in bone metabolism with its particular significance of VK-dependent γ-carboxylation (149). Fractures and osteoporosis have been also associated to uncarboxylated MGP (150). According to a recent study, MGP has a positive effect *in vitro* on osteoblast proliferation, differentiation, and osteogenesis. Overexpression of MGP increased the osteogenic and differentiation properties of MG63 cells, but knockdown decreased them (151). Expression of MGP mRNA was increased in ovariectomized Sprague-Dawley rats and primary osteoblasts by low-dose estradiol, VK, and PTH in a dose-dependent manner (152). PTH stimulates the MGP and Wnt/β-catenin proteins controlling osteogenesis (153). MGP affects osteoblast activity through Wnt/β-catenin pathway helping on bone formation (151). It suggests that MGP modulates bone development, but more research is necessary to confirm the action of MGP in bone metabolism.

6.1.3 Other VKDPs associated to osteoporosis

GRP, also known as upper zone of growth plate and cartilage matrix associated protein (UCMA), is a VKDP initially discovered in the calcified cartilage of the *Acipenser nacarri* (Adriatic sturgeon) (154). In humans, GRP is a circulating protein with 74 aa and has 15 possible Gla residues, the highest density of Gla residues of all known VKDPs (9,155). GRP mRNA was detected in trabecular bone from rat rib, tail, vertebra, and femur, being specifically expressed in osteocytes and osteoblasts (156). The protein was shown to function as an inhibitor of mineralization in the vascular and articular system, both at tissue and systemic level, and shown to possess anti-inflammatory properties independently of its γ-carboxylation status (155). Currently, the function of GRP in bone is mostly unclear, and more studies are required in this area.

The Gas6, a 75 kDa protein, and Protein S, a protein cofactor for the anticoagulant actions of protein C, are VKDPs involved in several cellular and non-cellular processes, including apoptosis, cell proliferation, inflammation, and myelination (9,12,44,157). Gas6 exerts its effect by interacting Gla-dependently with tyrosine kinase receptors of the TAM

family (Tyro3, Axl, and MerTK). These receptors cannot be activated without the γ-carboxylation of Gas6. In terms of bone metabolism, Gas6 has been found to promote osteoclast bone resorbing activity by binding Tyro3 produced in osteoclasts. Protein S was initially identified as a fibrinolysis-related protein. Posteriorly, Protein S, a GAS6 homolog and Tyro3 ligand, was found in chondrocytes and modulates directly osteoclast activity (158,159). GAS6 suppresses chondrogenic differentiation in cartilage metabolism (44,160).

6.2 Vitamin K – ligand of steroid and xenobiotic receptor/pregnane X receptor

In 2003, a novel process of VK2 activity was revealed (160), which comprises the direct interaction of ligands MK2, MK3, or MK-4 with the substrate nuclear receptor, SXR, and its murine ortholog, PXR (119,160). This ligand-substrate interaction regulates and augments the transcription of genes encoding extracellular matrix proteins (161), such as matrilin-2 (Matn2), tsukushi (Tsk), and cluster-determinant 14 (CD14), resulting in an increase in collagen buildup in osteoblastic cells (9,162). Tsk encodes a protein with a collagen-accumulating function, Matn2 is a protein involved in the formation of filamentous networks in bone matrix, and CD14 modulates osteoblastogenesis and osteoclastogenesis via stimulating B cell differentiation (127). Activation of SXR/PXR by a ligand result in the formation of a heterodimer with the 9-cis retinoic acid receptor (RXR), which binds to SXR-responsive elements (SXRE) in the regulatory regions of typical SXR/PXR-responsive genes. Typical SXR/PXR-responsive genes include the drug-metabolizing enzyme CYP3A4 and the ATP-binding cassette (ABC) family transporter multidrug resistance 1 (MDR1), which are implicated in drug excretion and detoxification (103,160).

SXR also plays a role in bone metabolism via its influence on vitamin D metabolism. The activation of SXR may have two possible effects. On one hand, certain drugs may activate SXR, resulting in CYP3A4 expression, activation of 24- and 25-hydroxylases, and consequently vitamin D insufficiency. Considering that PXR contains roughly 60% of the aa sequence in the VDR´s DNA binding domain, it was hypothesized that PXR may act on the vitamin D-responsive element (VDRE) in osteoblasts, catabolizing 25-hydroxyvitamin D and 1, 25-(OH)2D. As a result, PXR causes vitamin D insufficiency and bone damage (163). SXR activation, on the other hand, may inhibit CYP24A1 (24-hydroxylase activity) in the kidney, raising 1,25(OH)D levels (164). In conclusion, SXR/PXR inhibits bone resorption and stimulates bone formation. This suggests that SXR/PXR may be an important regulator in the maintenance of bone homeostasis (127).

6.3 Vitamin K – anti-inflammatory agent in NF- κB pathway

Evidence from the scientific community reveals that VK also has an anti-inflammatory effect, which is an essential component against a range of diseases associated with aging, including osteoporosis. Several *in vitro* and animal studies have shown that VK reduces the activation of NF-κB and inhibits IkappaB kinase (IKB) phosphorylation, which leads to a reduction in the production of pro-inflammatory cytokines (165–168). Since both vitamers of VK can suppress a liposaccharide (LPS)-induced inflammatory state *in vitro* and *in vivo* in the mouse model, the anti-inflammatory activity of VK was suggested to be mediated via the naphthoquinone ring (166,167). *In vivo*, the presence of VK has been associated with lower levels of inflammatory markers (104).

For osteoclast development and resorption, activation of NF-κB signal transduction pathway is crucial. Its activation, on the other hand, strongly inhibits osteoblast development and function. VK was shown to have a mechanism in bone homeostasis by blocking the NF-κB signal independently of the GCCX activity. The main mechanism that regulates p65 NF-κB subunit activation is its interaction with IκB. Phosphorylation of IκB, followed by ubiquitination and proteosomal degradation, results in the release and nuclear translocation of active NF-κB species. VK2, particularly MK-7, inhibits osteoblast and osteoclast formation by inhibiting basal and cytokine-induced NF-κB activation and raising IκB mRNA in a γ-carboxylation-independent manner (168).

According to a research, MK-4 suppresses NF-κB transcriptional activity and decreases IL-6 gene expression in LPS-treated cells. LPS stimulation causes a protein phosphorylation cascade, which leads in NF-κB activation. In this case, VK therapy inhibited the phosphorylation of IKB which is necessary for LPS-induced activation of NF-κB (166). Basically, VK has a direct inhibitory action of NF-κB on bone formation *in vivo*, where time- and stage-specific inhibition of the inhibitor of IKB in differentiated osteoblasts increased trabecular bone mass and BMD, ameliorating ovariectomy-induced bone loss by promoting a compensatory increase in bone formation (169).

Furthermore, research found that the NF-κB p65 subunit (TNF-α-activated transcription factor) is reported to inhibit vitamin D-stimulated osteocalcin transcription in osteoblastic cells. The p65 NF-κB subunit effectively integrates into the VDR transcription complex in TNF-α-treated bone cells. Through this mechanism, the rise of TNF-α during menopause may contribute to the development of vitamin D resistance (170).

6.4 Vitamin K2 Supplementation in osteoporosis

Several studies investigating the effects of VK1 and VK2 supplementation on osteoporosis have been conducted throughout the years. A modest dosage of VK1 (500 µm/day) supplementation for three years did not enhance bone density in the treatment group (171). Another trial using VK1 (5mg) for two years found no significant difference in BMD compared to placebo, indicating that it does not protect against age-related loss in BMD. However, the therapy group had considerably fewer fractures (50% reduction) (172). VK1 supplementation don't have scientific proves that can help inhibiting osteoporosis proliferation.

However, VK2 has been shown to lower the risk of osteoporosis. VK2 seems to improve bone quality, which leads to a significant reduction in fractures; although, in certain trials, bone density was not necessarily changed (7).

Several studies have demonstrated a notable positive effect of VK2 in bone loss and BMD (particularly lumbar) in osteoporosis patients (173,174). VK2 in combination with calcium and vitamin D3 improves BMD and reduces fracture risk in postmenopausal women and rats following ovariectomy, suggesting its potential to augment calcium and vitamin D3 therapy (175,176). In Japan, where most of these trials were conducted, VK2 is indicated as the standard of treatment. Supplementation with vitamin D, calcium, and VK2 lowers ucOC and increases lumbar BMD (177). A systematic review revealed that VK2 reduced vertebral fractures by 60%, hip fractures by 77%, and nonvertebral fractures by 81% in Japanese patients (173).

A study of 241 osteoporotic individuals who were given VK2 (45 µg/day) combined with calcium, reported a maintenance on BMD, but those who received calcium and a placebo, lost 2.5% of their lumbar BMD. Furthermore, the therapy group had 65% fewer fractures than the control group (178). A three-year randomized control trial study revealed that supplementing VK2 at 180 µg/day decreased the typical age-related reduction in BMD in the lumbar spine and femoral neck, but not in the whole hip. VK2 (MK-7) significantly reduced vertebral height loss in the lower thoracic spine (179). Another study investigated the effect of MK-7 (375 µg) for 12 months on BMD with calcium and vitamin D supplementation. The results demonstrate that ucOC serum levels were lowered by more than 70%, the ucOC/OC ratio was reduced by more than 60%, ALP was raised by 5%, and

microarchitecture in trabecular bone in the tibia was preserved, despite no significant impacts on BMD. This suggests that vitamin MK-7 helps in the maintenance of trabecular bone formation in the tibia(180).

According to the findings above, the best strategy to prevent and treat osteoporosis is to take VK2, calcium, and Vitamin D supplements. The appropriate amounts must yet be studied.

Chapter 7: Pharmacist role in osteoporosis

Pharmacists play an important role in many healthcare systems by assisting patients in managing chronic disorders, one of which is osteoporosis. In this context, pharmacists may first assist in identifying high-risk patients, such as those on prolonged glucocorticoid medication, who may then be targeted for BMD testing and treatment initiation. Second, pharmacists may advise and educate patients on drug consumption, fall prevention, calcium, vitamin D, exercise, and adherence to treatment (181). Besides, is also very important the literacy in general public about functions of VK that pharmacist can help to give the right information.

A systematic review of the literature revealed three randomized controlled trials (RCTs) (182–184) that investigated the effect of pharmacy interventions on two gaps in osteoporosis management: identifying at-risk individuals and improving therapy adherence. RCTs were carried out in the United States (Eastern Iowa), Australia (New South Wales), and Canada (Alberta) using various methods.

In Eastern Iowa, participated 15 community pharmacies and 96 adults on chronic glucocorticoid therapy. Patients in the control group got standard medical attention. Patients in the treatment got information and an informative booklet regarding glucocorticoid-induced osteoporosis. Pharmacists in the treatment group supervised drug therapy to detect and manage drug-related problems. At baseline and after 9 months of monitoring, a Web-based survey at the pharmacy collected glucocorticoid, medication, and osteoporosis risk variables. Using an intent-to-treat strategy, pre–post frequency changes were compared with contrasts for bisphosphonate medication, estrogen therapy, calcium supplement, discussion of GIO risk, bone density test, bone mineral density test, inactivity, and low calcium diet (183).

In New South Wales, 12 community pharmacists gave 217 participants advice or referrals on preventing osteoporosis after screening with a BMD test plus risk-assessment questionnaire or a risk-assessment questionnaire (184).

In Alberta, 262 patients satisfying BMD testing standards. These standards include men or women 65 years or 50–64 years with one significant risk factor including prior fracture, family history of osteoporosis, glucocorticoids for > 3 months, or early menopause. The patients were randomly allocated to either intervention or control groups. The

intervention comprised printed materials, education, and ultrasonography. Primary outcome was BMD or osteoporosis treatment within 4 months (182).

All these RCT results converge to the same conclusion: pharmacists are in a unique position to assist lessen the burden of osteoporosis by increasing the identification of high-risk patients for treatment, particularly those on corticosteroid therapy. Furthermore, pharmacist identification and counseling of patients at risk for osteoporosis leads in greater DXA testing and increases in calcium intake (181).

In practice, pharmacists should pay attention to patient quality of life in order to maintain a healthy body weight (BMI >20 kg/m2) and an acceptable dietary protein consumption (0.8 g/kg of body weight per day). Furthermore, patients should be instructed to avoid smoking, excessive alcohol use, and to enhance physical exercise. Walking 30 minutes each day and doing modest resistance workouts are frequently suggested to promote bone health and to prevent osteoporosis (3). To avoid falls in community-dwelling older persons at risk, the USPSTF advises individualized decision making, exercise or physical therapy, and vitamin D supplementation (800 IU/d) (3). Since VK2 is typically present in low quantities in the diet, oral supplementation may enhance VK2 status, particularly in postmenopausal women, a population at risk for osteoporosis (129).

Chapter 8: Conclusion

Osteoblastic activity initiates bone formation during the earliest stages of embryogenesis. Over time, bone turnover is necessary, requiring osteoclasts to resorb old bone and osteoblasts to produce new bone, hence keeping BMD within the healthy limits. After the age of 30, BMD will drop progressively owing to a variety of factors, including individual and environmental variables. When there is an imbalance between bone formation and resorption, BMD decreases, and the risk of fractures rises. BMD below -2.5 SD indicates osteoporosis.

Osteoporosis is the most prevalent chronic metabolic bone disease, and it is estimated to affected globally more than 500 million individuals. As the aging population in many countries grows, osteoporosis may become a global problem, affecting quality of life. Osteoporosis is a disorder characterized by reduced bone density and an increased risk of fracture owing to degeneration of bone architecture. Thus, the primary objective of therapy is to lower the risk of fractures. There are several treatment options, mostly medications that assist postmenopausal women and older men to prevent the disease. Bisphosphonates, raloxifene, denosumab, teriparatide, abalopateride, romosozumab, calcium, and vitamin D are examples of effective pharmacological therapy for the prevention and treatment of osteoporosis. Due to the side effects of these medicines, others potential treatment was investigated, namely VK that don't have any known side effects or toxicity, even when VK is administrated in high doses.

The significance of VK in osteoporosis has been studied throughout the past decade. VK functions as a cofactor for the enzyme GCCX, which carboxylates both hepatic and extrahepatic VKDPs. It may, however, act independently of this enzymatic role to lessen the inflammation generated by NF-kB in osteoporosis. VK can also interact directly with SXR to increase the production of extracellular matrix proteins.

Extra-hepatic VKDPs that are implicated in the bone mineralization include OC, MGP, Gas6, protein S and GRP. OC is probably the most important VKDP associated with bone homeostasis because after its γ-carboxylation, it binds and incorporates calcium ions into hydroxyapatite crystals in the bone matrix. Studies show that VK reduces the activation of NF-κB and inhibits IKB phosphorylation, which leads to a reduction in the production of pro-inflammatory cytokines involved in osteoporosis physiopathology. Several trials with VK1 and VK2 supplementation were made, but VK2 shows more positive results associated

with increasing BMD and lowering risk fractures. The identified activity of VK2 in osteoporosis must be solidified by the conduct of more research.

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