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the role of vitamin K in the bone-vascular axis

Grzegorz Wasilewski

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The calcium paradox – the role of vitamin K in the bone-vascular axis

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Content

CHAPTER

General introduction

General introduction

Cardiovascular disease (CVD) is a collective term for diseases affecting the circulatory system, including heart and vasculature. It is the number one cause of death globally, despite numerous attempts to raise awareness about CVD worldwide. Within CVD we distinguish - but not limited to - coronary heart disease (CHD), stroke and aneurysm. CHD, also known as coronary artery disease (CAD) and stroke are the most common and lethal types of CVD[1]. Revealing risk factors for developing CVD and identification of biomarkers for early diagnosis of the disease are of crucial importance to fighting CVD successfully.

For over a decade it is appreciated that soft tissue calcification of arterial walls (vascular calcification; VC) is a risk factor for mortality and cardiovascular events[2]. VC can occur in any arterial wall (i.e. carotid arteries, coronary arteries, aorta, peripheral arteries) and is considered to be a major cause of arterial stiffness[3,4]. Coronary Artery Calcification (CAC) is associated with the presence and extent of coronary atherosclerosis[5,6]. The amount and progression of CAC, as quantified by computed tomography (CT), are associated with a risk for future cardiovascular events[7].

It is known that individuals who have an increased propensity to develop VC are more prone to experience cardiovascular events. Postmenopausal women, for example, have increased risk for cardiovascular events which is explained, at least in part, by a decline of bone calcium and an increase in VC. The association between bone loss and increase of VC is also known as the 'calcium paradox'[8]. Several studies described the correlation between bone loss and VC [9,10]. Similar paradigms have been observed within the chronic kidney disease (CKD) population. Bone deterioration and VC share similar pathophysiological characteristics in which both calcium and phosphate play a major role[11].

Vascular Calcification

VC does not discriminate between the rich and the poor and appears to be less a consequence of our modern Western lifestyle as expected. In fact, the first described cases of VC date back as early as 1520BC. Citizens of Ancient Peru and Southwest America, and also Pharaohs of ancient Egypt had VC[12,13]. To date, the real courses of VC are still poorly understood. Bone formation, a physiological deposition of calcium minerals, is well described as an organized multicellular process mediated by endochondral ossification or mineralization of chondrocytes. VC, on the other hand, has long been regarded as a stochastic and passive process that is associated with aging, inflammation and renal dysfunction. This view has changed dramatically over the past decade primarily driven by the increase of fundamental knowledge about genotypes, phenotypes and functions of the vascular cells in arterial walls. Currently, we appreciate VC as an actively regulated process.

We distinguish multiple cell types in the arterial vasculature of which vascular smooth muscle cells (VSMCs) are the most abundant. VSMCs play an important role in our current concept of active regulation of VC. The phenotypic plasticity of VSMCs is key to this concept and is associated with a variety of proteins and cellular structures that inhibit or accelerate VC (see below). Among these proteins are matrix Gla protein (MGP), bone morphogenic proteins (BMPs), Fetuin-A, pyrophosphate, Klotho, and osteopontin but also larger cellular structures such as extracellular vesicles[14–17].

VC is clinically relevant at two sites of the vascular wall: the intimal site (in association with atherosclerosis) and the medial site (in association with ageing, CKD and T2DM).

Intimal calcification

Intimal calcification is associated with atherosclerosis, which is a chronic, inflammatory disease of the arterial wall. The classic view of atherogenesis starts with the infiltration of lipids into the sub-endothelial layer, in regions where endothelial integrity is compromised[18]. Often, this occurs at regions with disturbed haemodynamics and high shear stress such as carotid and coronary arteries. Monocytes infiltrate and become macrophages that phagocytose oxidised lipids until they render into foam cells that secrete pro-inflammatory cytokines. Medial VSMCs respond to these cytokines by switching into migratory phenotypes that infiltrate the pre-atheroma lesion of the intima. Within the atheroma plaque environment, VSMCs will switch into synthetic phenotypes that have enhanced Extracellular Vesicle (EV) secretion[19]. Plaque VSMCs can also execute apoptosis, generating apoptotic bodies. The apoptotic bodies and the EVs, when not phagocytosed properly, will induce microcalcification[20]. These spotty, small mineral deposits are thought to destabilize the plaque and make it more prone to rupture[21]. Microcalcifications can grow further and become macrocalcifications. In contrast to microcalcifications, macrocalcifications are thought to have a stabilizing effect on atherosclerotic plaques[22]. The macrocalcification is detectable by CT and is used in the clinic as a measure of atherosclerotic burden. It is noteworthy that microcalcifications escape the detection by CT. Recent imaging techniques such as IVUS and NaF18 PET imaging make it possible to detect microcalcifications. Detection of microcalcifications is paramount since our perspective on intimal calcification has changed from an end-stage process of atherosclerosis into an early process contributing to atherogenesis and atherosclerotic plaque vulnerability[23]. This new perspective also urges the need of understanding the molecular mechanisms of intimal calcification.

Medial calcification

Medial calcification, also known as Mönckenberg's sclerosis, is the deposition of calcium minerals in the medial layer of the arterial vessel wall. It preferentially occurs at sites where elastin or collagen fibers are damaged, for example in Marfan disease. Medial calcification can underly arterial stiffness and aneurysm formation. As in intimal calcification, phenotypic switching of VSMCs triggers osteocalcin (OC) and alkaline phosphatase synthesis along with upregulation of osteoblast-like genes and become incapable of performing their physiological role of regulating vascular tone leading to increased stiffness, eventually resulting in ventricular hypertrophy[24–26]. Besides mineralizing elastin, de-differentiation of contractile VSMCs into osteoblast-like cells warrants emphasis on medial calcification. Moreover, unfavourable haemodynamic conditions such as hypertension and mechanical stress among VSCMs synthetic phenotype leads to dissociation of the medial layer of the arteries known as thoracic aortic auneryusm[27]. Cellular senescence is another factor contributing to medial calcification. Senescent VSMCs are less proliferative than contractile compatriots and has been shown to express osteogenic markers including RUNX2 and Col1a1[28]. This calcification subtype manifests greatly in the ageing population and patients suffering from chronic kidney disease (CKD) and diabetes mellitus (DM)[3,29]. In opposition to the development of intimal calcification, the presence of inflammatory cells and lipids might not be found within the medial layer. Additionally, it has been shown that medial calcification is a risk factor for the development of atherosclerosis and intimal calcification[30]. This is likely due to the interaction of mechanisms involved in both processes.

Calcium-paradox: bone loss and vascular calcification

VC per se is an inorganic process depending on Ca^{2+}- and phosphate- including other ions as is bone mineralization. Both VC and bone mineralization have been shown to be steered by similar cellular processes. Yet, bone demineralization as for example in postmenopausal women and CKD patients with osteoporosis is often accompanied by an increase in VC. This co-existence has been designated

as the calcium paradox[8]. Osteoporosis and CVD have long been regarded as pathologies with independent aetiologies[31,32] . Recently, it became accepted that osteoporosis has a relationship with the onset and development of CVD. Calcium supplementation is one of the main current treatments for postmenopausal bone loss. It was reported that calcium supplementation is associated with increased myocardial infarction indicating detrimental effects on the coronary arteries[33,34]. Furthermore, calcium deposits in the abdominal aorta and carotid arteries have been linked to low bone mineral density[35,36].

CKD is defined as impaired kidney function with glomerular filtration rate (GFR) <50ml/min/1.73m^2 for 3 months irrespective of the cause[37]. The prevalence of CKD worldwide varies between 12 to 15% of the world population[38]. CKD patients frequently develop CAC and arterial stiffening, both strong risk factors for CVD morbidity and mortality[39,40]. Patients with CKD are also far more likely to die from CVD than progress to end-stage CKD[41,42]. In patients with end-stage CKD, arterial stiffness is associated with a further decline in kidney function and increased mortality[43,44]. One of the main complications of CKD is a disturbance in mineral metabolism that is characterized by increased circulating levels of phosphate and calcium[45]. These patients also frequently show bone abnormalities[46]. In advanced CKD both dietary interventions and phosphate binders (PBs) are used to target hyperphosphatemia. Interestingly, clinical studies with PBs consistently show a slower progression of VC if non-calcium-containing PBs were used[47,48] and faster progression of VC if calcium-containing PBs were administered[49,50].

Calcium metabolism and vascular calcification

A key process of both bone and vascular mineralization is calcium metabolism, which is regulated by the hormonal system. Endocrine regulation of calcium is mediated by several hormones, namely parathyroid hormone (PTH), calcitonin and vitamin D (Figure 1). PTH is an 84 amino acid peptide hormone released by the parathyroid glands (PTGs). PTGs are nodular structures residing at the thyroid gland separated by a fibrous capsule. The main function of the PTGs is to react to small changes in calcium concentrations in the blood circulation. The PTGs use the calcium-sensing receptor (CaSR) to sense changes in plasma Ca^{2+}-ion levels and to regulate the secretion of PTH[51,52]. When levels of calcium ions fall below the physiological level, PTH is released into the bloodstream where it is transported to the kidney. In the kidney, PTH increases tubular reabsorption of calcium and minimising calcium loss via urine. Further, PTH stimulates the synthesis of 1α-hydroxylase in the kidney, catalysing the formation of active vitamin D, 1,25-dihydroxycholecalciferol from its precursor 25-dihydroxycholecalciferol. The active form of vitamin D enhances intestinal calcium absorption via basolateral extrusion of calcium by the intestinal plasma membrane pump and stimulates bone mineralisation[53]. Calcitonin exerts its action in response to hypercalcemia in opposition to PTH and vitamin D. Calcitonin is a 32 amino acid peptide hormone synthesized and released by C-cells of the thyroid gland. Upon release, calcitonin reduces circulating levels of calcium in two ways. Firstly, it inhibits the renal tubular calcium reabsorption and secondly suppresses the action of bone-resorbing osteoclasts and their differentiation via the calcitonin-osteoclast receptor causing contraction and reduction in motility[52].

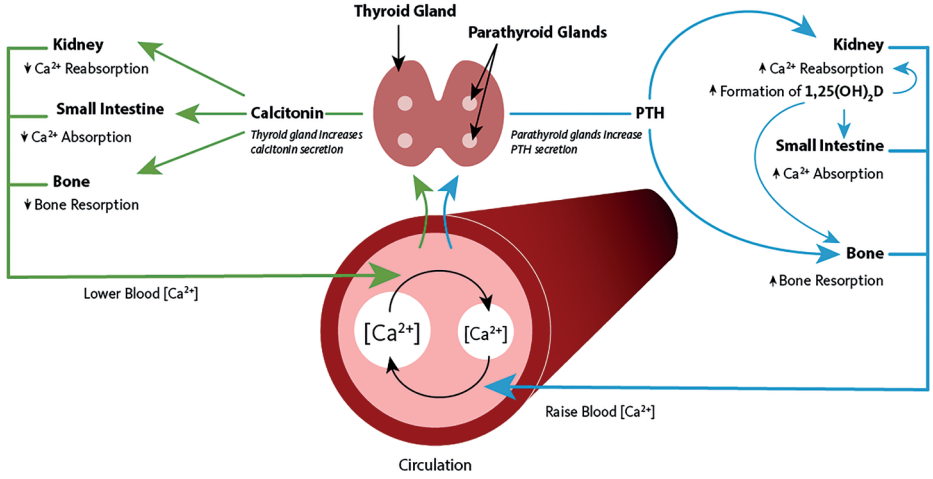

Figure 1. Calcium Homeostasis.

Source: adapted from https://lpi.oregonstate.edu/mic/minerals/calcium#references[54]

The detrimental side of the calcium paradox is yet poorly understood. Both VC and osteoporosis share the common pathophysiology of dysregulated calcium metabolism. Many treatments to combat osteoporosis involve calcium supplementation as a building block to support bone formation. Multiple meta-analyses analysed the effects of calcium supplementation on bone mineral density, many of which showed little or no benefit[55,56]. Although supplementing calcium to combat osteoporosis seems intuitively correct, little is known about the deleterious effects on the cardiovascular system. Meta-analyses suggest that calcium supplementation increases the risk of MI and stroke and potentially mortality, yet some show no associations[33,56–60]. Moreover, calcium excretion is facilitated by the kidney of which elevated urinary levels contribute to the formation of kidney stones[61]. It is important to note that Ca^{2+}-supplements, depending on the formulation, can increase circulating levels of calcium in serum[62,63]. Another common problem in the elderly is bone fracture risk. Vitamin D is known to impact bone mineralization and is therefore often given in combination with calcium to limit bone loss which is a causal link with fractures. A recent meta-analysis of 33 trials showed that the use of supplements that included calcium, vitamin D or both was not associated with a decrease in the risk of bone fracture among elderly[64].

In addition to calcium and vitamin D, vitamin K supplementation has been investigated as a treatment of VC and bone demineralization in preclinical and clinical studies[65]. Vitamin K is an essential cofactor for the posttranslational modification (PTM) of MGP, an inhibitor of calcification that is synthesized by VSMCs. In this thesis, I will explore the topic of vitamin K and VSMCs further in greater detail.

Phenotypic switching of vascular smooth muscle cells

Under physiological conditions, VSMCs regulate the tonality of the vascular system by their ability to withstand elastic recoil and to modulate the diameter of arteries and arterioles[66]. It has now been widely recognised that VSMCs are cells of remarkably high plasticity. It allows them to respond and adapt to extracellular environmental changes such as biochemical or mechanical stressors.

The first report of VSMCs plasticity dates to 1961 where observations of "thickened intima in a doubly ligated segment of an artery" had been noted in the treatment of arterial ligature[67]. VSMCs are embedded within the medial scaffold of elastin and collagen fibers of arteries. Healthy contractile VSMCs express a panel of contractile proteins including alpha-smooth muscle actin, calponin, and myosin heavy chain. In atherosclerosis, VSMCs can undergo phenotypic switching from contractile to synthetic phenotypes[68]. This phenotypic switch is accompanied by a release of pro-inflammatory cytokines, downregulation of contractile proteins and acquisition of pro-synthetic markers including KLF4, S100A4 and NOX5[69–72]. After vascular repair, VSMCs possess the ability to de-differentiate back to contractile VSMCs[69,73]. The phenotypic plasticity enables VSMCs to migrate towards sites of injury and inflammation to perform reparative actions. The migration is regulated by a variety of stimuli such as Platelet Derived Growth Factor (PDGF), thrombin, Fibroblast Growth Factor 23 (FGF23) and pro-inflammatory interleukins[74–77]. Synthetic VSMCs synthesize and deposit elastin and collagen fibers into the extracellular matrix. In addition, they release considerable amounts of EVs such as exosomes that may serve as nucleation sites of calcification and are strongly linked to the initiation of calcification[69,78]. Among inhibitory molecules that reduce the progression of atherosclerosis, we can distinguish heparin sulphates, anti-inflammatory interleukins and vitamin K[79–82].

Persistent stress signals can cause VSMCs to switch to an osteo/chondrogenic phenotype. This phenotypic switching is accompanied by downregulation of contractile proteins such as calponin, α-SMA, sm22a[83–85] and upregulation of bone and cartilage-specific genes including RUNX2, BMP-2, SOX9, Col1A1, Col10A1 and increased ALP activity[71,86,87]. BMP-2 and to a lesser extent phosphate have been documented as factors responsible for the osteo/chondrogenic phenotypic switching of VSMCs[71,88]. High phosphate levels further upregulate the phosphate channel transporter PiT-2, which is a crucial regulator of phosphate-mediated VC *in vitro* and *in vivo*[89]. Moreover, high phosphate levels can cause mitochondrial dysfunction[90], elevated ROS production and apoptosis with the release of apoptotic bodies[84,91]. In addition to this, osteo/chondrogenic VSMCs are prone to release elevated levels of EVs directly facilitating VC[86,92]. Collectively, the osteo/chondrogenic phenotype of VSMCs puts a strong pro-calcifying pressure on its surroundings. The calcification process by osteo/chondrogenic VSMCs bears similarities with ossification such as regulation by BMP-2[86] and EVs[93,94]. Both processes also have their unique features. Apoptosis, elevated reactive oxygen species (ROS) and inflammation are observed during VC but not during bone formation[93,95].

Vitamin K and Vitamin K dependent proteins

Vitamin K has been known since 1929 when its biological role was discovered in chickens as an anti-hemorrhagic factor[96]. Vitamin K stands for a group of fat-soluble compounds with a common 2-methyl-1, 4-naphthoquinone ring, that differ in side-chain at the 3-position. Vitamin K comes in two flavours and can be classified into phylloquinone (K1) and menaquinones (K2; MKn) depending on the side-chain structure (Figure 2). K1 is found mainly in leafy green plants, whereas K2 is synthesised by the gut bacteria (Figure 3). There is now substantial evidence that after absorption, vitamin K1 can be converted to K2, and more specifically into MK-4[97,98]. Vitamin K is thought to have anti-oxidative properties and to support the electron transport of the mitochondrial oxidative phosphorylation[99,100]. Another well-established role of vitamin K concerns its cofactor activity for the enzyme gamma-glutamylcarboxylase which catalyses the conversion of protein-bound glutamic acid residues (Glu) into gamma-carboxyglutamic acid residues (Gla) during PTM[101]. Proteins undergoing this type of PTM are termed vitamin K-dependent proteins (VKDPs) or Gla-proteins. The gamma-carboxylation is essential for the biological activity of VKDPs.

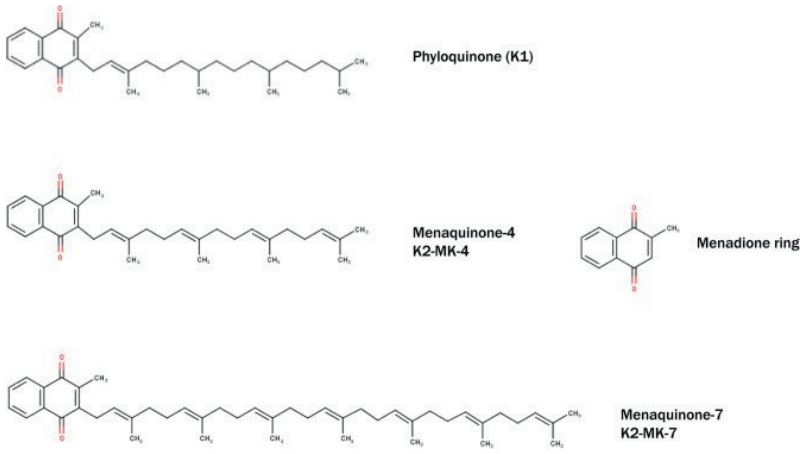

Figure 2. Structures of Vitamin K

We distinguish two groups of VKDPs on basis of their site of synthesis: i) the hepatic VKDPs, including the coagulation factors II, VII, IX, X and the anticoagulant proteins C, S and Z, ii) the extrahepatic VKDPs, encompassing osteocalcin (OC), MGP, growth arrest-specific gene-6 protein (Gas6) and Gla Rich Protein (GRP). The biological significance of gamma-carboxylation in pathophysiology has been underscored by the linkage between reduced carboxylation status of MGP and increased risk of CVD [102–104]. Moreover, counteracting gamma-carboxylation by vitamin K antagonists is an established therapeutic strategy to reduce coagulation in patients at risk for thrombosis[105].

The role of vitamin K-dependent proteins as calcification inhibitors

MGP is a small 15kDa VKDP produced predominantly by VSMCs and chondrocytes. Its function was first discovered in MGP knockout mice which showed extensive calcification of aorta and cartilage[107]. MGP is a strong endogenous inhibitor of VC and it needs to be gamma-carboxylated in order to express this inhibitory activity[108]. The mechanisms through which MGP inhibits VC are still not fully understood. Carboxylated MGP (cMGP) can bind hydroxyapatite crystals with high affinity and can halt further progression of crystal growth[109,110]. cMGP can bind BMP-2 and prevent direct binding of BMP-2 to its receptor[111]. Activation of the BMP-2 receptor is involved in endochondral ossification, bone formation and transdifferentiation of VSMCs into osteo/chondrogenic-like cells[87,88,112]. Uncarboxylated MGP (ucMGP), the inactive fraction of MGP, is now widely accepted as a biological marker for vitamin K deficiency and its circulating levels correlate with the CAC score and cardiovascular morbidity and mortality[113–117]. UcMGP is also present at sites of calcification in atherosclerotic plaques suggesting a local deficiency of vitamin K that contributes to VC[118,119].

GRP, also known as upper cartilage growth matrix protein (UCMA), is another VKDP that inhibits calcification. It contains 16 Gla residues within the 74 amino acid sequence and is found at sites of pathological calcification[120,121]. GRP is synthesized in bone, cartilage, and vasculature[120,122]. Interestingly, GRP knockout mice do not present pathologies of bone or cartilage[123]. GRP inhibits calcification directly via shielding EVs and CPPs[16] and indirectly via regulating osteo/chondrogenic differentiation and phosphate-induced mineralisation of VSMCs[86]. The impaired carboxylation status of GRP has been linked to pathologies in cartilage, including osteoarthritis[124].

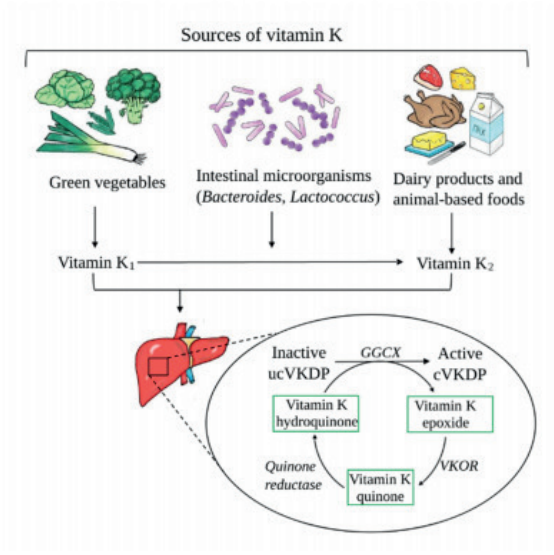

Figure 3. Adapted from: Vitamin K and D Supplementation and Bone Health in Chronic Kidney Disease—Apart or Together?[106].

The beneficial role of Vitamin K in CKD and osteoporosis

An increasing number of studies demonstrate the association between low vitamin K status in CKD and bone health. It is reported that circa 70-90% of CKD patients are vitamin K deficient due to dialysis and poor nutritional status[125–128]. Circulating levels of the fully inactive dephosphorylated ucMGP are associated with abdominal aorta calcification in CKD patients[128]. Administration of vitamin K2 (MK7) to CKD patients on haemodialysis reduces the circulating level of vitamin K deficiency markers[129,130]. Poor vitamin K status is also a strong independent risk factor for vertebral fractures in patients on hemodialysis[130,131]. Restoring the vitamin K levels significantly improve bone turnover markers[130,131]. Several clinical trials have tested the efficacy of vitamin K in reducing VC and improving bone quality. Vitamin K has been shown to retard bone loss and improve arterial stiffness in postmenopausal women as well as prevent fractures while sustaining lumbar density[132–135].

Osteoporosis research has demonstrated that vitamin K supplementation reduces loss of bone mass density and decreases the risk for hip fractures[133,136]. Supplementation of vitamin K reduces the levels of circulating uncarboxylated osteocalcin (ucOC) in osteoporotic women. ucOC levels directly correlate with increased risk of fracture[126,136].

Use of stem cell models in research of bone and cardiovascular disease

Despite the technological advancements in *in vitro* and *in vivo* models for VC and bone formation, the standard techniques still lack translation potential into clinical practice. Mesenchymal stem cells (MSCs) have been considered to fill this translational gap in bone research. MSCs have been used as a source for pluripotent stem cells that can be differentiated into bone cells. This source, however, possesses several limitations, including a short passage number, a limited number of cells per donor and low donor availability. Recent advancement in stem cell science has introduced the induced pluripotent stem cells (iPSCs) as promising alternatives to MSCs, and, hence, to research bridging the translational gap to clinical applications. iPSCs are highly similar to embryonic-derived stem cells. They can be prepared by reprogramming somatic cells through overexpression of the Yamanaka factors Oct3/4, Sox2, Klf4 and c-Myc[137,138]. iPSCs can be expanded almost endlessly and can be differentiated into any cell type of the body making them a robust source for any cell type to *in vitro* research. The iPSC technology also opens up the possibility to study genetic pathologies on an individual basis. With only a small amount of patient-derived somatic cells, for example, circulating white blood cells, patient-specific iPSCs can be generated and studied. Despite its young existence, the iPSC technology has already been used extensively to create models for cardiovascular and bone research. IPSCs derived VSMCs have been successfully generated to study ectopic calcification[138]. Using iPSCs to generate MSCs is the next step in combining the best from both stem cell technologies[139]. iPSC-derived mesenchymal stem cells have already been studied in bone regenerative therapies successfully[140,141]. Last but not least, differentiating cells from pluripotency into mesenchymal and osteogenic lineages will expand our research possibilities to investigate similarities and differences between VC and bone formation at the molecular level. Such knowledge is imperative to successfully design targeted therapies to treat VC and bone demineralization.

Outline of the thesis

My thesis contributes to further understanding of the roles of VSMCs and vitamin K in VC. In **chapter 2,** I provide an up-to-date review of current literature on the interaction between the bone-vascular axis and the role of vitamin K and VKDPs. I specifically focus on CKD and osteoporosis. **Chapter 3** describes an *in vivo* study studying the combination of PBs with vitamin K2. PBs are effective in reducing phosphate but also reduce the absorption of vitamin K. We tested the combination of vitamin K2, MK7 and PBs in a newly developed rodent model of CKD. In **chapter 4** I provide evidence that *in vitro* vitamin K acts as an inducer and enhancer of osteogenesis in induced pluripotent stem cell derived mesenchymal cells. We present a novel role for vitamin K2, MK-7 in anti-inflammatory and pro-mineralisation pathways In **Chapter 5** I elaborate on the mechanisms that induce calcification of VSMCs and chondrocytes. I have investigated the role of intracellular calcium influx, oxidative stress, vitamin K and calcium channel antagonists in VC. **Chapter 6** summarises the key findings of this thesis, discusses its relevance to current published literature and presents a future perspective on VC**.**

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CHAPTER

2

The Bone—Vasculature Axis: Calcium Supplementation and the Role of Vitamin K

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Abstract

Calcium supplements are broadly prescribed to treat osteoporosis either as monotherapy or together with vitamin D to enhance calcium absorption. It is still unclear whether calcium supplementation significantly contributes to the reduction of bone fragility and fracture risk. Data suggest that supplementing post-menopausal women with high doses of calcium has a detrimental impact on cardiovascular morbidity and mortality. Chronic kidney disease (CKD) patients are prone to vascular calcification in part due to impaired phosphate excretion. Calcium-based phosphate binders further increase the risk of vascular calcification progression. In both bone and vascular tissue, vitamin K-dependent processes play an important role in calcium homeostasis and it is tempting to speculate that vitamin K supplementation might protect from the potentially untoward effects of calcium supplementation. This review provides an update on current literature on calcium supplementation among post-menopausal women and CKD patients and discusses underlying molecular mechanisms of vascular calcification. We propose therapeutic strategies with vitamin K2 treatment to prevent or hold the progression of vascular calcification

INTRODUCTION

Calcium is an abundant element in nature and is a major component of sedimentary rock that covers 75 to 80% of the Earth's surface. Calcium is also widely abundant in the human body, primarily in bone, and teeth. Calcium salts are occasionally found outside bone in a variety of tissues; this is broadly termed as extra-skeletal calcification. In these extra-skeletal sites, calcium exists in multiple forms, including amorphous calcium phosphate, hydroxyapatite, and magnesium whitlockite. A remarkable observation is that under several pathological conditions, as will be discussed, the calcium mineral content of bone declines, while it is increasing on these extra-osseous sites. This has been termed the "calcium paradox" and was introduced to describe the paradoxical correlation between lower bone calcium content with parallel increased vascular calcium content[1]. The calcium paradox refers to epidemiological data reporting that postmenopausal women experience bone loss, yet simultaneously screen positive for vascular calcification. This phenomenon is common in osteoporotic women and patients suffering from chronic kidney disease (CKD). Prevalence and morbidity of both cardiovascular disease and osteoporosis are increasing in the global population. Such observations have been noted in several studies, where a correlation of low bone mineral density (BMD) was associated with increased cardiovascular mortality[2–6].

The use of calcium supplements has been widely advised due to their assumed ability to support bone health and BMD[7, 8]. Calcium is an essential element for bone growth during childhood[9], as well as in preserving bone mineral density during adolescence[10]. However, a systematic review and meta-analysis of the effects of calcium supplementation along with vitamin D treatment showed that this treatment was not associated with a lower incidence of fracture risk in adults, questioning whether calcium supplementation contributes to the maintenance of healthy bone[11]. In turn, recent data suggest that calcium supplements increase the prevalence of myocardial infarction[12], and may increase risk of coronary artery calcification (CAC)[13]. Moreover, higher doses of calcium from supplements than calcium obtained from dietary intake might promote cardiovascular calcification[14]. Thus, despite the relative benefit of calcium supplementation for bone, calcium supplements became controversial because of a possibly increased cardiovascular risk. Substantially different from calcium from dietary sources, calcium form supplements induce an acute rise in serum calcium levels that highly oscillates in blood for up to 6 h[15, 16]. The plasma calcium concentration is tightly regulated by vitamin D, parathyroid hormone (PTH), and calcitonin[17, 18].

Vitamin K-dependent proteins (VKDP) also play an important role regulating mineralization both in bone and the vasculature. Osteocalcin (OC) is produced exclusively by osteoblasts and supports the binding of calcium to the bone mineral matrix, whereas matrix Gla-protein (MGP) is synthesized by vascular smooth muscle cells and chondrocytes to prevent ectopic calcification. While hepatically produced coagulation factors are the prototypical VKDP, the extra-hepatic VKDPs also unequivocally need vitamin K as a cofactor to become biologically active. Related to that, vitamin K2 has been shown to prevent bone loss and strength and prevents stiffening of arteries[19, 20]. Western diet does not provide sufficient vitamin K to activate all OC and MGP that is produced[21, 22]. Also in CKD patients, vitamin K deficiency is prevalent, so K2 supplementation has been suggested as treatment option to attenuate vascular calcification[23,24]. In this review we provide the latest insights of the calcium paradox and the potential of using vitamin K to support both bone and vascular health.

BONE METABOLISM

Calcification generally is a physiological process, necessary to build bone and dentin. Bone provides structural support, strength, necessary for locomotion, and protection from the environment. The balance in bone formation and bone resorption is crucial for optimal bone health. A disturbed balance

of this process results in bone loss and is termed osteoporosis. During childhood, bone is formed and bone peak mass is achieved during young adulthood, after which bone mass gradually declines. Bone loss is the consequence of bone resorption outbalancing bone formation[25]. This is accompanied by bone architectural changes including trabecular bone becoming thinner, less abundant, and osteoclastic perforation of cortical bone[26].

Bone Formation

The skeleton is systematically renewed in the process of bone remodeling to maintain strength and rigidity. Bone remodeling can be part of calcium homeostasis system and enables the skeleton to adapt to changes. Bones adapt their structure depending on their function, mechanical strain and need for stability during development. It is mediated on the surface of cortical and trabecular bone, and at anatomically different sites, named basic multicellular subunits[27]. Two pathways of bone formation exist, together termed osteogenesis. The first is known as endochondral ossification and involves differentiation of mesenchymal cells into chondrocytes or osteoblasts[28, 29]. As chondrocytes mature, they expand in size and become hypertrophic and eventually undergo apoptosis, secreting vesicles that initiate mineralization of the extracellular matrix[30]. As they die, with vascular evasion and matrix remodeling (osteoclast-mediated), the calcified cartilage is subsequently replaced by bone. Nestin-positive mesenchymal progenitors associated with the invading vasculature differentiate into bone-forming osteoblasts and deposit a type I collagen-based bone matrix on the degraded cartilage template[31,32]. The second process of bone formation is intramembranous ossification. First, mesenchymal cells directly differentiate into osteoblasts, which are bone-forming cells. Next, the type I collagen matrix is deposited by these cells, which can bind calcium salts, which form hydroxyapatite crystals. This mineralization of the matrix underlies the strength and compactness of the bone. With time, osteoblasts eventually become trapped in the calcified extracellular matrix and transdifferentiate into osteocytes. Osteoblasts are the only bone cell type releasing the vitamin K-dependent protein OC (discussed below). As the newly formed bone is laid, its deposition must be tightly regulated to maintain homeostasis. This balance is achieved by bone-resorbing cells, entering the blood vessels of bone, which are termed osteoclasts and are of macrophage origin. Each osteoclast can secrete hydrogen ions, thereby acidifying the bone surface dissolving mineralized matrix, followed by interactions that enhance the action of osteoblasts[33–35]. Upon resorption, bone-matrix embedded osteocalcin is released contributing to its circulating levels[36].

Bone Loss

Bone loss is most typical in women after reaching the age of 50 years following menopause. The pattern of sex hormonal secretion drastically changes after the menopause, resulting in disbalance in bone turnover markers, making postmenopausal women susceptible to osteoporosis and fractures. Remarkably cardiovascular diseases are also more prevalent in postmenopausal women. Therefore, it is important to understand the molecular mechanisms by which hormonal changes lead to both osteoporosis and cardiovascular disease[37, 38]. The post-menopausal period is accompanied by a substantial reduction of estrogen levels leading to bone resorption, yet simultaneously reducing calcium absorption[39]. It is not the aim of this review to elaborate on the effect of estrogen on the vasculature [reviewed elsewhere([39])]. Instead, we will focus on specific pathways involved in calcium metabolism. PTH is released upon hypocalcemia, indirectly stimulating the release of calcium from bone. In CKD, autonomous production of PTH may occur. Additionally, PTH promotes reabsorption of ultra-filtered calcium in distal tubules and activates vitamin D thereby increasing circulating calcium levels by raising gastrointestinal uptake of calcium[17, 18]. Calcium-sensing receptors (CaR) present on the surface of parathyroid glands enable sensing of circulating calcium concentration[40], contributing to calcium modulation. Vitamin D is a fat-soluble vitamin that can be obtained from diet, sun exposure,

or supplements, and is metabolized by a series of enzymatic reactions in the body producing its active 1,25-dihydroxyvitamin D form[41, 42]. Vitamin D (in inactive form) is often prescribed in combination with calcium supplements. Active 1,25-dihydroxyvitamin D enhances absorption of intestinal calcium and phosphate thus contributing to the regulating of mineral balance[43, 44]. In the absence of vitamin D, only 10–15% of intestinal calcium is absorbed, which can be increased to 30–40% in the presence of active vitamin D[45, 46]. Vitamin D was found to stimulate production of vitamin K-dependent proteins, like osteocalcin[47]. Osteocalcin is a protein involved in bone mineralization [reviewed elsewhere[48]]. Remarkably, inclusion of vitamin K in calcium and vitamin D supplements improved BMD and ucOC when compared with vitamin D and calcium alone[49]. CKD patients often experience deficiency of 1,25-dihydroxyvitamin D as a consequence of lost kidney mass and the effects of fibroblast growth factor 23[50], resulting in declined activity of 1-alpha hydroxylase[51–53]. Reduced serum levels of 1,25-dihydroxyvitamin D result in hypocalcemia on top of positive phosphate balance, both stimulating PTH release and eventually leading to secondary hyperparathyroidism.

VASCULAR CALCIFICATION

Vascular calcification is a pathological process and has been firmly established as a risk factor for cardiovascular events and mortality[54, 55]. Vascular calcification is a process of extraosseous mineral deposition in blood vessels, including large arteries such as aorta, carotid arteries, iliac arteries, and cardiac valves. Bone mineralization and vascular calcification share many similarities, including expression of bone-related proteins in the vasculature and secretion of extracellular vesicles (EVs) both preceding the phase of calcification[56, 57]. Vascular calcification can occur either in the tunica media or tunica intima of the vessel wall. Medial calcification is also known as Möckenberg's sclerosis and involves vascular smooth muscle cells (VSMCs) calcification in the absence of previous local lipid accumulation, and inflammation. Medial calcification is related to CKD, diabetes mellitus, and aging, and results in increased arterial stiffness and risk of cardiovascular events[58, 59]. In contrast, intimal calcification is associated with atherosclerotic plaque formation and the amount of calcification is a measure of atherosclerotic burden[1]. For many years vascular calcification was considered as a clinically irrelevant process reliant of passive deposition of calcium crystals, merely reflecting a passive feature of disease and aging. Recent evidence however suggests otherwise, and vascular calcification appears to be a highly regulated process. SMCs release calcification inhibitors, thus efficiently preventing spontaneous calcification despite supersaturation of extracellular calcium and phosphate levels[60].

Vascular Smooth Muscle Cell Phenotypic Switching

SMCs are the main cellular component of the tunica media in arterial vessels providing structural support and regulating vascular tone and elasticity to alterations in pressure conditions. In physiology VSMCs possess a contractile phenotype and express contractile-specific markers, including alpha-smooth muscle actin, calponin, and SM22alpha, enabling them to perform contraction of the vessel wall [reviewed elsewhere[61, 62]]. VSMCs function is associated with a high level of phenotypic plasticity to perform a variety of functions including production of extracellular matrix and repair[61, 63]. Several factors have been implicated in regulating VSMC phenotype, including mineral imbalance (calcium, magnesium, and phosphate-induced loss of calcification inhibitors and presence of calcification promotors)[64]. Downregulation of contractile markers is a hallmark for VSMC phenotypic switching[65]. It has been shown that phosphate can induce an osteochondrogenic phenotypic switching of VSMC, as will be outlined in more detail below[61, 66–69], whereas elevated calcium levels shift the contractile phenotype toward a synthetic SMC phenotype[57]. Both calcium- and phosphate-induced phenotypic switching are associated with an increase in the secretion of calcifying extracellular vesicles[56, 57].

Elevated Phosphate Levels Promote Osteochondrogenic Differentiation of SMCs

CKD patients often develop medial calcification[70]. In CKD, a strong correlation between serum phosphate levels and vascular calcification is present[71, 72]. In an animal model of CKD, arterial calcification developed after feeding animals a phosphorous-rich diet only[73]. Initiation and progression of calcification in CKD patients correlates with impaired mineral metabolism represented by elevated serum level of phosphate and/or calcium[74]. Moreover, high circulating phosphate levels have been linked to increased cardiovascular morbidity even among young people receiving dialysis[75] and in CKD patients[76]. In vitro, elevated phosphate levels result in upregulation of bone-like markers in SMC including osterix, alkaline phosphatase (ALP), and Runx2, and downregulation of SMC contractility markers[77]. SMC cultured in osteogenic cell culture media differentiate into calcifying SMC resembling osteoblasts[68]. In aortic valves of patients with aortic stenosis, valvular interstitial cells demonstrate similarities with osteoblasts[78], which also exhibit lamellar bone formation[79]. Upon injury or in atherosclerosis, SMCs induce the release of platelet-derived growth factor (PDGF) similarly to platelets[80, 81]. SMC are known to express the PDGF receptor subtypes and the level of expression is greatly increased in connective tissue and in SMCs followed by PDGF stimulation[82].

THE CALCIUM PARADOX

The paradoxical co-existence of declined calcium-mineral content in bone, and parallel increased arterial calcification, as a consequence of impaired calcium metabolism, is termed the calcium paradox. This is most pronounced in post-menopausal women and CKD patients. Many studies have consistently shown a coexistence of osteoporosis in post-menopausal women and increased calcification of either abdominal aorta or carotid arteries[5, 83–90]. Such paradox of decreased bone mineral density and vascular calcification has also been documented in a population study of middle-aged men, suggesting it is not unique to women[91], and pointing to a specific metabolic abnormality. In patients with CKD disturbed calcium and phosphate homeostasis is present and many studies consistently reported bone abnormalities including decreased BMD and fractures and coexistence of increased vascular calcification and all-cause mortality[92–108]. Kidney Disease: Improving Global Outcomes (KDIGO) guidelines recommend the term chronic kidney disease-mineral bone disorder (CKD-MBD) to express this clinical syndrome encompassing mineral (e.g., calcium), bone, and cardiovascular calcification abnormalities that develop as a complication of CKD[109]. In addition to bone disease, patients with CKD are also prone to vascular calcification, bone fragility and fractures. It has been shown that patients on dialysis, which is the end stage of CKD (CKD stage 5D), have an increased risk of fractures[110, 111] and vascular calcification[112], and therefore the calcium paradox also exists in CKD patients. CKD pathological characteristics include biochemical imbalances leading to elevated levels of circulating phosphate[113–115]. In untreated patients, circulating calcium levels are decreased due to vitamin D deficiency, whereas vitamin D supplementation might be beneficial in improving biochemical endpoints in CKD patients[116]. Vitamin D is often used in combination with calcium supplementation therapy. In patients on dialysis, coronary artery calcification is prominent and contributes to high mortality and morbidity. However, this use of both calcium and vitamin D, while being possibly protective for bone disease, may aggravate vascular calcification. Uraemia-related cardiovascular risk factors, including hyperphosphatemia and elevated Ca x P product, correlate with quicker onset of vascular calcification[117]. Circumventing this calcium paradox may be accomplished by VKDP[118, 119], as will be outlined below.

AGENTS THAT ALTER TISSUE MINERALIZATION

In the following sections, we will discuss treatments known to influence bone and vascular mineralization, and how they might impact calcium metabolism.

Calcium salt	Elemental calcium % (w/v)	Bioavailability	Advantages/disadvantages
Carbonate	40	High (comparable with citrate)	Requires acidic stomach conditions before absorption, might cause acidic rebound, cheap provides greatest amount of elemental calcium
Tricalcium phosphate	38	Moderate (found lower absorption than citrate when used in fortified juice)	High calcium content
Citrate	21	High (higher than lactate/tricalcium phosphate)	Not dependent on stomach acidity, many tablets needed
Gluconate	9	High (comparable with calcium carbonate)	Many tablets needed
Lactate	13	High (comparable with calcium carbonate)	Many tablets needed
Acetate	25	High (scarce information on human subjects)	Inexpensive, wide range of intestine pH absorption
Chloride	27	High (intravenous injection for treatment of hypocalcemia)	Not commonly prescribed low amount of elemental calcium
References		(93, 94, 125)	(11, 33–39, 41)

Table 1. Comparison of calcium salts frequently used in calcium supplements. Salts are listed according to elemental calcium content which does not necessarily reflect on bioavailability. Absorption is also influenced by stomach acid due to the salt structure e.g., calcium carbonate is basic and needs hydrochloric acid in stomach to produce calcium chloride which is further absorbed.

Calcium Supplements

Calcium is important for optimal bone health throughout life. Although dietary intake of calcium may suffice to meet the recommended daily intake, calcium supplements may be an option if diet falls short. dose Globally, recommendations for daily calcium intake vary. The Institute of Medicine (IOM) recommends a daily intake of 1,000 mg/day for men aged 19– 70 years and women 19–50 years old, and 1,200 mg/day for older individuals[92] whereas National Osteoporosis Society suggests an intake between 800–1,000mg a day[120]. While calcium intake comes from dietary sources such as dairy products, certain vegetables, and fortified foods, many people do not achieve the recommended intake from diet alone. It is estimated that ∼35% of the adult U.S. population uses calcium supplements[121]. Calcium plays a vital role in various physiological activities, such as nerve conduction, muscle contraction, blood clotting, protein folding, brain function, and regulated cell death (apoptosis)[122, 123]. Such broad function of calcium in the body requires precise regulation, and calcium oscillates between 2.15 and 2.60 mmol/L for total plasma calcium in adults and between 1.17 and 1.33 mmol/L for plasma ionized calcium as free calcium represents some 45% of total circulating calcium levels. This free form is the regulated calcium and accounts for bone mineralization as well as pathological calcification[124].

Calcium Forms, Absorption, and Effects

Several formulations of calcium are available on the market, differing in bioavailability, and elemental calcium content. Calcium carbonate is the most common form available. However, many studies showed superiority of calcium citrate over calcium carbonate, due to higher bioavailability and because it does not require acidic stomach conditions before ingestion[102]. In a study carried out in post-menopausal women supplemented with di-calcium phosphate over a period of 12 months, serum calcium levels did not vary significantly, and only urinary calcium increased progressively in time when compared to the control group. The increased excretion of calcium may indirectly reflect the rise of the renal threshold for excretion and together with the amount of absorbed calcium it may contribute to complications such as deposition in the vasculature[103]. One of the most applied therapeutic intervention for fracture risk is calcium in the form of pills or organic powder. Commercially available calcium is often marketed in combination with vitamin D3 to increase intestinal absorption of calcium (Table 1). It has been proposed that no more than 500 mg of elemental calcium should be taken as single dose to maximize absorption and to avoid side effects, like

gastrointestinal complaints[94]. When calcium supplements are not exceeding the nutritional daily intake of 800 mg, a low cardiovascular risk was observed[104]. Clinical guidelines consider a cumulative calcium intake from foods and supplements that does not exceed 2,000 to 2,500 mg/d, as defined by National Academy of Medicine, as safe for cardiovascular disease outcome[105, 106]. Numerous studies and extensive meta-analyses reported on the efficacy and cost-effectiveness of calcium supplementation (with or without vitamin D), in improving bone mineral density as well as decreasing fracture risk[83–85, 107, 108]. Furthermore, in individuals with inadequate calcium intake, the supplementation plan seems to be beneficial in reducing fragility fractures especially in osteoporotic women[86, 107]. Calcium supplementation was also demonstrated to be effective in preventing reduction in bone loss and turnover in healthy population[87]. A recent double-blind controlled trial also proved the effectiveness of medium and high calcium intake in maximizing bone mineral density in adolescent girls[88]. In addition, many studies described neutral or protective effects of calcium-rich foods on cardiovascular outcomes including atherosclerosis, risk of infarction, stroke, and cardiovascular mortality[89, 90, 126–130]. However, recent data challenge the assumption that calcium supplementation improves bone mineral density. A meta-analysis on the correlation between calcium supplementation alone or with vitamin D and bone mineral density in people over 50 years of age demonstrated little beneficial effect (1–2%) in the first year with nearly no further benefits after 1 year on bone mineral density[8]. With such low effects, it would be challenging to implement calcium supplementation into standard treatment for reduction of fracture risk in the healthy elderly population[131]. A recent review summarizing the use and efficacy of calcium supplementation in treating osteoporosis and fracture risk questions the use of calcium supplements because of the weak beneficiary effect on fracture risk while increasing the risk of gastrointestinal problems, kidney stones, and cardiovascular risk[132]. Despite positive outcomes of calcium supplementation, a risk for cardiovascular risk events may exist in specific population. It was recently shown that women who receive calcium supplementation were at higher risk for increased vascular morbidity and mortality, including myocardial infarction[108, 133–139]. In turn, recent systematic reviews and meta-analyses do not confirm that supplementing calcium (with or without vitamin D) increased prevalence of coronary heart disease, cardiovascular mortality or all-cause mortality, data on which the above-mentioned statement by the National Academy of Medicine is based upon[105, 131]. Rapidly elevated transient calcium levels in blood caused by excessive supplementary calcium have been suggested to promote coagulation when compared with placebo in postmenopausal women, likely due to interaction with platelets expressing calcium-sensing receptor (CaSR)[140, 141]. Hypercoagulability is considered to have a reinforcing effect on atherosclerosis in animal studies, contributing to cardiovascular disease. Also, many coagulation proteins have been described in human atherosclerotic plaques[142]. These findings are in line with the association between high calcium intake and cardiovascular calcification in CKD patients[143]. Reconciling these sometimes opposing details is difficult. There appears to be some protection from fracture risks by calcium supplements, but its safety is still not sufficiently established. Therefore, additional research is still needed. Calcium-based phosphate binders have been used extensively as a first-choice option since 1970 to alleviate hyperphosphatemia associated with CKD patients due to their low cost, availability, and effectiveness. These calcium-containing phosphate binders are given to CKD patients to complex dietary phosphate, thereby reducing phosphate uptake[144, 145]. As with most supplements, also calcium-containing phosphate binders have side effects, which include abdominal cramps, intestinal bloating, and diarrhea[146]. Further, excessive intake of calcium supplements might also result in milk-alkali syndrome and hypercalcemia[92]. In addition, also in patients with CKD, the use of calcium-containing binders are associated with progression of CKD, and the recently updated KDIGO guideline suggest to restrict its use[109].

Vitamin K and Vitamin K-Dependent Proteins

Vitamin K was discovered in 1929 by the Danish biochemist Henrik Dam during his experiments on cholesterol metabolism in chickens. When fed low-fat diets, chickens experienced prolonged clotting time and hemorrhage, which surprisingly could not be rescued when diet was enriched with cholesterol. Dam assumed a deficiency of a vitamin required for coagulation, which he termed "Koagulation vitamin," hence vitamin K[147]. Indeed, vitamin K was shown to be a fat-soluble vitamin, consisting a group of structurally related compounds including vitamin K1 (phylloquinone) and vitamin K2 (menaquinones) (Figure 1).

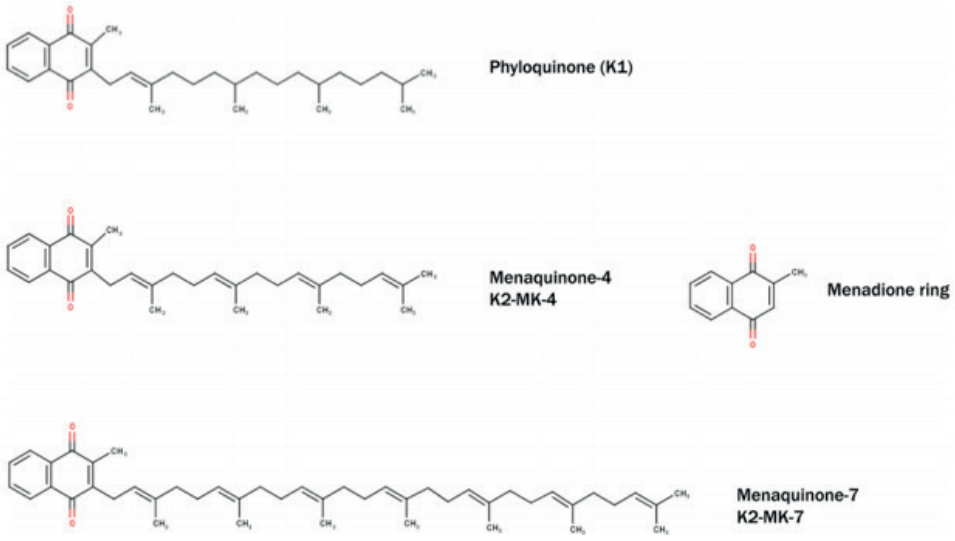

Figure 1. Structural formulae of naturally occurring and biologically active Vitamin K–phylloquinone (K1) and menaquinones (K2-MK-4 and K2-MK-7). All vitamins share common menadione ring (also known as vitamin K3).

Vitamin K1 contains a phytyl chain, whereas K2 is classified according to the length of isoprenoids and indicated as MK-n, where n represents the number of residues. Both vitamins share a common 2-methyl-1,4-naphthoquinone ring, also known as menadione. The main source of vitamin K1 is green vegetables[148], whereas vitamin K2 can be found in fermented foods such as soy beans, cheese, and sauerkraut. The richest source of vitamin K2 (MK-7) is a Japanese dish named Natto, which is produced from fermented soy beans with aid of the Bacillus Subtilis bacteria strain[149]. In addition to nutritional consumption, gut bacteria Lactococcus[150] and Escherischia coli[151] are able to synthesize long chain menaquinones (Figure 1). The primary biological function of both K-vitamins is being an unequivocal cofactor in the post-translational modification of VKDP via carboxylation of glutamic acid residues (Glu) to y-carboxylated-glutamic acid residues[152]. To fulfill this function, vitamin K needs to be reduced to its active cofactor form (KH2) by quinone reductases. The enzyme y-glutamylcarboxylase (GGCX) oxidizes KH2 to vitamin K-epoxide (KO)[153]. Both vitamins K1 and K2 can partake in the activation of VKDP; however, long-chain menaquinones, which are more hydrophobic, have a higher bioavailability and longer half-life and thus bioactivity[154, 155]. VKDP are a group of proteins that require carboxylation of specific protein-bound glutamate-residues, allowing them to bind with high affinity to calcium. This was first demonstrated in coagulation, showing that VKDP of the coagulation cascade need carboxylation to acquire biological activity. This role of vitamin K on coagulation is clinically widely applied by the use of warfarin as anticoagulant treatment. The extra negative charge in VKDP bind via calcium to negatively charged phospholipids to exert their function. In the last three decades, extra-

hepatic VKDP have been discovered, including OC, MGP, and Gla-rich protein (GRP; also termed Upper zone of growth plate and Cartilage Matrix Associated protein, Ucma)[156]. The function of non-hepatic VKDP has recently be discovered and include prevention of vascular calcification[157] and importantly also promotion of bone metabolism[158]. The current knowledge of vascular calcification inhibitors has gained attention of both scientists and clinicians to research their molecular action, aiming to alleviate disease caused by vascular calcification.

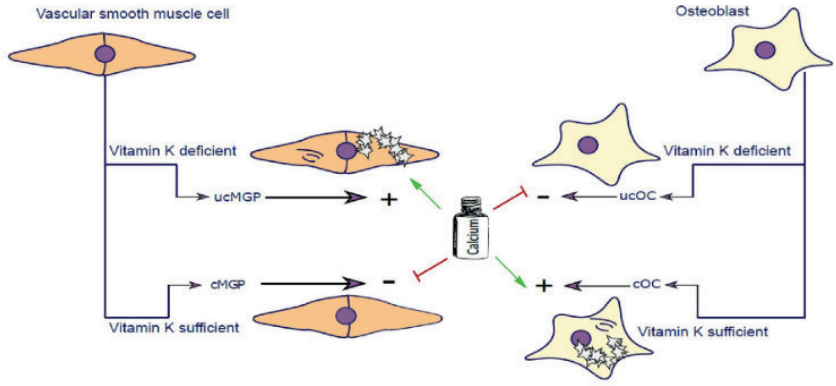

Figure 2. Vascular smooth muscle cells (VSCMC) and osteoblasts are able to synthesize Matrix-Gla-Protein (MGP) and Osteocalcin (OC), respectively. In the presence of vitamin K both proteins are carboxylated (cMGP and cOC) preventing calcification of VSMC and promoting mineralization of Osteoblasts. Vitamin K–dependent carboxylation mechanism keeps extracellular matrix of VSMC free of calcification and simultaneously promotes mineralization of osteoblast matrix. In Chronic Kidney Disease patients, calcium serum levels are elevated further potentiating the calcification of SMCs. Similarly, in post-menopausal women, calcium homeostasis is further impaired contributing to impairment of calcium utilization by osteoblasts. In the event of vitamin K deficiency, both MGP and Osteocalcin are not carboxylated and cannot perform their molecular function.

Osteocalcin

OC is a major non-collagenous protein abundantly present in bone, responsible for management of skeletal mineralization[159, 160]. OC knock-out/null rodents undergo increased bone mineralization, followed by an increase in trabecular thickness, density and bone volume[161–163]. During skeletal development, bone mass increases due to the dominant function of osteoblasts which secrete OC, amongst other proteins, enabling bone to grow. In addition to bone function, OC is implicated in stimulating testosterone synthesis and insulin release[164, 165]. Other roles of OC are not covered in this review and have been reviewed elsewhere[166]. To execute its physiological function, OC needs to be activated by carboxylation, catalyzed by vitamin K. Carboxylated OC (cOC) has a high affinity for calcium ions and aids in forming a hydroxyapatite lattice preceding mineralization of bone[167, 168] (Figure 2). Upon bone degradation, OC, incorporated into mineralized bone, is liberated. Serum OC levels were negatively correlated with bone mineral density (BMD) in postmenopausal women and healthy subjects[169–171]. In a study of healthy girls, plasma phylloquinone was inversely correlated with circulating OC concentrations showing that a better vitamin K status was associated with decreased bone turnover in healthy girls[172].

Matrix Gla Protein

The discovery of MGP dates back to 1983 where it was first purified from bovine bone matrix and named after the presence of gamma-carboxyglutamate residues on MGP[173]. Shortly thereafter MGP was confirmed to be present in cartilage, lung heart, kidney, and vasculature, with the highest protein expression in SMCs and chondrocytes[174–176]. Knocking out MGP in mice induced advanced medial calcification and subsequent vessel rupture followed by death in the majority of mice within 6 weeks after birth. This animal model resembles the human Keutel syndrome which is caused by a mutation in the MGP gene[177, 178], which impairs carboxylation of MGP thereby inducing intimal and medial

calcification (179). MGP is also dependent on carboxylation of gla-residues, catalyzed by vitamin K, to execute its function as an inhibitor of vascular calcification (Figure 2)[180, 181]. Uncarboxylated MGP (ucMGP) is associated with increased risk of vascular calcification, and therefore some researchers advocate that vitamin K status in CKD patients should be carefully monitored[182]. Another mode of action of MGP, besides being an inhibitor of arterial calcification, is inhibition of bone morphogenic protein2/4 (BMP2/4)[183, 184]. BMP2 was found to be present in human atherosclerotic lesions[185], acting as a downstream signal for osteogenic phenotype switching of SMC by increasing the influx of phosphate into cells[186]. In MGP-deficient SMCs, upregulation of osteogenic-specific proteins was notified[187] and it can be speculated that MGP prevents osteogenic transition of SMC by interacting with BMP-2[188].

Gla Rich Protein

GRP, also known as Ucma, is a vitamin K-dependent protein secreted by chondrocytes[189, 190] and present in cartilage, bone[191], and vasculature[192, 193]. Despite the creation of GRP knockout mice its precise molecular action remains to be elucidated, because these animals had no manifest deficits in cartilage or bone development[194]. So far, the role of GRP has been implicated in calcium regulation in extracellular matrix[156, 192], and thus being an inhibitor of ectopic calcification[192, 193]. Indeed, GRP inhibits calcification of aortic tissue by promoting a contractile SMC phenotype via increasing expression of α-smooth muscle actin[193]. Moreover, GRP was found to be directly associated with calcium-phosphate crystals suggesting that this protein-crystal interaction modulates calcification[156]. Also, in CKD stage 5D, GRP inhibits EV and calcifying protein particles (CPP) induced vascular calcification[195]. In addition, GRP was found to promote osteoblast[196] and chondrocyte differentiation[189, 190]. More recently, it was shown that GRP inhibited phosphate-induced SMC calcification via BMP-dependent signaling suggesting its role in regulating osteochondrogenic differentiation of SMCs[69]. As mentioned above, MGP also inhibits calcification via a BMP-dependent mechanisms[57, 197] and this novel function of GRP function via a BMP-dependent mechanism suggests that both MGP and GRP deficiency contribute to phosphate-induced vascular calcification and cardiovascular risk. Table 2 summarizes vitamin-K dependent proteins involved in calcification.

	Bone	Vasculature	Cartilage
MGP	✓	✓	✓
Gla rich protein (UCMA)	✓	✓	✓
Osteocalcin	✓	?	?
Reference	(173, 191, 198, 199)	(192, 193, 197)	(189–191, 200, 201)

Table 2. Occurrence of selected vitamin K dependent proteins in different tissue compartments.

Phosphate Binders and Vitamin K

Despite many years of research there is no definite proof that phosphate binders improve outcome, despite their capacity to control phosphate. Although direct studies suggest superiority of non-calcium containing binders over calcium containing binders, it is still unclear if this is due to an advantage of non-calcium containing binders or added risks from calcium containing binders[143, 202–204]. Even more striking is that the use of any phosphate binders in earlier CKD, despite lowering phosphate, did not reduce the progression of coronary calcification[71]. This conundrum may be explained by the recently demonstrated ability of phosphate binders to also bind vitamin K (Table 3). The advantage of lowering phosphate concentrations is thus offset by aggravation vitamin K deficiency. The lack of difference in this CKD patient subgroup could be explained by effective inherent protection in these patients or by

simultaneous undesired binding of vitamin K by some phosphate binders resulting in vitamin K deficiency which serves as co-factor for enzymes that activate calcification inhibitors[218, 219] (Figure 3). More recently, it was shown that CKD patients on dialysis treated with the phosphate binder sevelamer revealed higher circulating levels of dpucMGP, the inactive form of MGP[221]. These findings support the in vitro notion and hypothesis that phosphate binders induce a vitamin K-deficiency. Besides the above-mentioned phosphate binders, new forms have recently been developed such as iron-based phosphate binders. Iron oxyhydroxide have been proven to be as potent as sevelamer in decreasing phosphatemia[222], while not interfering with vitamin K-metabolism[218].

Phosphate lowering agent	Binding mechanism	Generic name	Calcium based	Effect on phosphate	Effect on Ca x P product	Effect on calcium/ hypercalcemia	Interaction with vitamin K
Calcium acetate/magnesium carbonate	Ionic		Yes	↓	↓	↑	Yes
Calcium acetate	Ionic		Yes	↓	↓	↑	?
Calcium carbonate	Ionic	CaCO	Yes	↓	↓	↑	Yes
Lanthanum carbonate	Forms insoluble phosphate complexes	LanCO	No	↓	↓	↓	Yes
Aluminum hydroxide	Ionic	Al salts	No	↓	?	↑	?
Sucroferric oxyhydroxide	Covalent binding	FeSa	No	↓	↓	NS change	No
Sevelamer hydrochloride	Ionic	Sevelamer HCl	No	↓	↓	↓	?
Sevelamer carbonate	Ionic	Sevelamer CO_3	No	↓	↓	NS change	No
Colestilan	Ionic		No	↓	↓	NS change	?
Bixalomer	?		No	NS change	NS change	NS change	?
Nicotinamide	inhibition of sodium/phosphorus co-transporter	Vitamin B3	No	↓	↓	NS change	?
Ferric citrate	Ionic		No	↓	NS change	NS change	?
Reference	(205, 206)			(207–217)			(218–220)

Table 3. Summary of selected features and effects of available phosphate binders. Ca, Calcium; P, Phosphorous.

Vitamin K to Escape the Calcium Paradox

As outlined, vitamin K has a role in healthy bone formation, while at the same time it provides protection against ectopic calcification, especially in the cardiovascular system. Therefore, it is tempting to speculate that the calcium paradox in fact reflects vitamin K deficiency. It has been shown that patients with CKD frequently are vitamin K-deficient, which is likely attributable to dietary advice to limit their potassium intake (i.e., intake of green leafy vegetables and thus vitamin K1) and to lower phosphate intake (i.e., intake of dairy products and thus vitamin K2). Besides, these dietary restrictions, especially patients on dialysis frequently suffer loss of appetite, further affecting the intake of essential nutrients, including vitamin K. Another risk for vitamin K deficiency is the use of phosphate binders as outlined above. Finally, use of anticoagulant therapy with vitamin K antagonists in CKD patients will propel this deficiency even further[223]. Although novel direct oral anticoagulants are available, these are often considered unsuitable for patients with a glomerular filtration rate below 30 ml/min/1.73m^2 . Also, in healthy subjects it was shown that the majority has subclinical vitamin K deficiency as deduced from the presence of increased concentrations of uncarboxylated VKDP in the circulation[22, 180, 224]. Recent evidence, as outlined in detail above, suggests that vitamin K is an important factor in bone and vasculature in CKD patients and post-menopausal women, and that its role may be overlooked. It creates a window of opportunity to supplement vitamin K in the abovementioned subgroups including CKD patients and post/perimenopausal women frequently deficient in vitamin K. Although supplementation with vitamin K2 (MK-4) daily for 3 years did not

improve bone mineral content or bone mineral density, it did maintain bone strength at femoral neck site in post-menopausal women[19], thus indicating a beneficial effect on post-menopausal bone strength loss. Aside from MK-4's known function for gamma carboxylation, and thereby preventing ectopic calcification to occur, it was shown to also promote maturation of osteoblasts[225] and to suppress osteoclast maturation while promoting their apoptosis[226, 227]. MK-7, a long-chain menaquinone, was found to have more beneficial effect on bone and facilitates bone mineralization, including cortical bone structure as compared to MK-4[228]. In support to in vivo evidence, several trials assessed the feasibility of MK-7 as treatment for CKD and post-menopausal osteoporotic patients. It was shown that MK-7 (MenaQ7) improves bone strength at the femoral neck via increasing bone mineral content (BMC) and bone mineral density (BMD)[19, 229, 230]. In addition, hemodialysis patients supplemented with MK-7 showed a substantial decrease in dp-ucMGP along with ucOC and PIVKA-II (protein induced by vitamin K absence or antagonism–II) in a dose-dependent manner, implicating that MK-7 improves vitamin K-status in liver, bone, and vasculature[24, 231]. In osteoporotic patients, vitamin K2 resulted in elevated levels of cOC and prevented fractures when compared with placebo-treated osteoporotic patients[232]. Moreover, both MK-4 and MK-7 supplementation resulted in an increase of cOC and a decrease of ucOC and improved BMD[229, 233– 238].

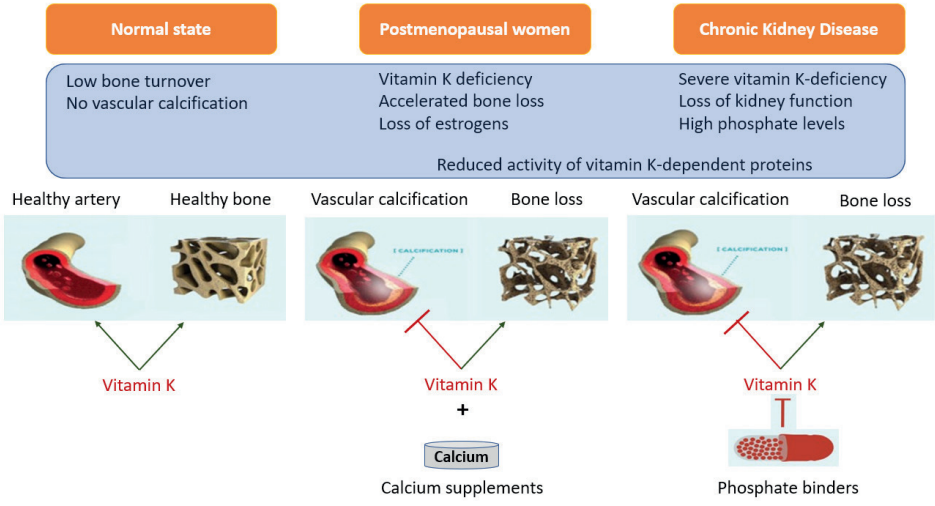

Figure 3. Representation of systemic action of vitamin K on bone and vasculature in the calcium presence. Calcium based phosphate binders are known to reduce the levels of adsorbed phosphate by directly coupling reaction in the gastro intestinal tract. Phosphate binders were also shown to bind Vitamin K suggesting it might affect its free circulating form. When coupled with phosphate binders, vitamin K is unable to perform its biological function of positively utilizing calcium into the bone and simultaneously acting as calcification inhibitor.

Besides its beneficial effects on bone health, high intake of MK-7 successfully blocked age-related vascular stiffening[239] in post-menopausal women. Moreover, MK-7 was better than placebo at reducing severe aortic calcification and relative risk of coronary heart disease[208, 240]. Ongoing clinical trials will evaluate its effectiveness in reducing vascular calcification in patients with coronary artery disease[241]. In a crosssectional study, nutritional long-chain menaquinone intake was associated with decreased coronary calcification in postmenopausal women[240, 242]. Moreover, MK-7 improved arterial stiffness and elastic properties of the carotid artery in a healthy postmenopausal woman[20] and improved vitamin K status in dialysis patients by decreasing inactive levels of MGP by daily supplementation[24]. In another randomized clinical study, K1 supplementation slowed the progression

of CAC in healthy older adults with preexisting CAC, demonstrating the potential efficacy of vitamin K treatment for vascular calcification. Inactive MGP (dp-ucMGP) has been correlated with severity of CKD and is positively associated with amount of vascular calcification[24, 224, 243, 244]. MK-7 (MenaQ7) supplementation in patients with CKD 3-5 significantly reduced circulating levels of dp-ucMGP[24]. Collectively, these data imply that vitamin K could serve as complementary nutrient to calcium (and vitamin D to protect from increased risk for vascular calcification thereby allowing more safe treatment of osteoporosis. Vitamin K supplementation in post-menopausal patients appeared beneficial in combination with calcium and vitamin D3 for bone health and vasculature[239]. The combination of vitamin K and calcium could reduce risk on post-menopausal bone and simultaneously prevent vascular calcification, thereby aiding the beneficial effects of calcium in bone and preventing the negatively associated vascular effects of supplemental calcium intake.

CONCLUSIONS

To date, calcium supplements are the most commonly used non-prescription drug to treat age-related bone loss. Also, in patients suffering from chronic kidney disease, calcium-based phosphate binders are commonly prescribed. However, the rising concern of side-effects from calcium supplementation illustrates a clinical dilemma: supplementation of calcium—either with or without vitamin D—comes at the price of increased risk of vascular calcification. Clinical studies demonstrate that increased intake of vitamin K could be a promising complementary nutrient in supporting both bone health and protecting vascular calcification. Thereby it can increase safety of current treatments of osteoporosis and provide an escape from the calcium paradox. Future clinical trials should be carried out to confirm the feasibility of such combination.

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CHAPTER

3

Combining phosphate binder therapy with vitamin K2 inhibits vascular calcification in an experimental animal model of kidney failure

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Abstract:

Background: Hyperphosphatemia is strongly associated with cardiovascular disease and mortality. Recently, phosphate binders (PBs), which are used to bind intestinal phosphate have been shown also to bind vitamin K, thereby potentially aggravating vitamin K deficiency. This vitamin K-binding by PBs may offset beneficial effects of phosphate level reduction on reducing vascular calcification (VC). Here we assessed whether combining PBs with vitamin K2 supplementation inhibits VC. Methods: We performed 3/4Nx in rats, after which warfarin was given for three weeks to induce vitamin K-deficiency. Next, animals were fed a high phosphate diet in the presence of low or high vitamin K2 and were randomized to either control or one of four different PBs for eight weeks. The primary outcome was the amount of thoracic and abdominal aorta VC measured by high-resolution micro-Computed Tomography (*μ-CT)*. Vitamin K status was measured by plasma MK7 levels and immunohistochemically analyzed in the vasculature using ucMGP specific antibodies. Results: Combination of high vitamin K2 diet and PB treatment significantly reduced VC as measured by *μ-CT,* for both thoracic ($p=0.026$) and abdominal aorta ($p=0.023$), compared to MK7 or PB treatment alone. UcMGP stain was significantly more present in the low vitamin K2 treated groups in both thoracic ($p<0.01$) and abdominal aorta ($p<0.01$) as compared to high vitamin K2 treated groups. Moreover, high vitamin K diet and PBs led to reduced vascular oxidative stress. Conclusion: In an animal model of kidney failure with vitamin K-deficiency, neither PB therapy nor vitamin K2 supplementation alone prevented VC. However, the combination of high vitamin K2 with PB treatment significantly attenuated VC.

Translational statement

Phosphate binders bind vitamin K *in vitro* and thus might interfere with the carboxylation of matrix Gla-protein in the vessel wall causing vascular calcification. This has so far not been substantiated *in vivo*. Moreover, CKD patients very often suffer from vitamin K deficiency. We investigated the effect of co-administration of phosphate binders and vitamin K on vascular calcification in a novel CKD model with vitamin K deficiency and high phosphate intake. In our model, the combination of vitamin K2 with PB treatment increased vitamin K levels and attenuated vascular calcification (VC) development. Our findings might provide a combination therapy to combat VC in chronic kidney disease. These finding should be translated to human research.

Keywords

Phosphate binders, vitamin K2, vascular calcification, chronic kidney disease, matrix Gla protein

Introduction

Cardiovascular mortality increases progressively with advancing chronic kidney disease (CKD). Traditional risk factors for cardiovascular disease only partially explain these observations, pointing to a role of non-traditional risk factors such as uremia, hyperphosphatemia, oxidative stress and possibly vitamin K deficiency[1]. Many of these risk factors contribute to vascular calcification (VC), which is an important contributor to morbidity and mortality especially in late-stage CKD[2]. One major established contributing factor for VC and mortality is a high serum phosphate concentration[3]. In late-stage CKD both dietary interventions and phosphate binders (PBs) are used to target hyperphosphatemia. Generally, phosphate binder studies consistently show a slower progression of VC if non-calcium-containing PBs are used[4–7]. This is in line with *in vitro* data, showing that a slight increase of calcium on top of increased phosphate levels induces calcification of the arterial medial layer[8]. Lowering phosphate levels with non-calcium-containing PBs such as lanthanum carbonate and sevelamer carbonate has been shown to attenuate VC progression[4, 6, 7, 9]. However, even with the use of non-calcium-containing PBs, VC frequently progresses[10]. One explanation for this observation may be the overlooked effect of PBs on vitamin K status. Vitamin K is a key player in protection against VC, as it is a mandatory cofactor for the activation of matrix Gla protein (MGP). MGP is a vitamin K-dependent protein produced and secreted by vascular smooth muscle cells and an important local inhibitor for calcification of the vessel wall[11]. In late-stage CKD, the prevalence of vitamin K deficiency is high[12]. This is in part due to dietary restrictions in potassium-rich dietary products (leafy green vegetables rich in K1) and phosphate-containing food (dairy products such as cheeses rich in vitamin K2)[13]. Vitamin K deficiency is aggravated in patients using vitamin K antagonists[14]. Additionally, it has been established that vitamin K is bound by several PBs *in vitro*[15, 16] as well as *in vivo*[17], with different affinities. Recently, it was demonstrated that clinical use of PB therapy was associated with increased serum levels of dephosphorylated uncarboxylated MGP (dp-ucMGP), a biomarker indicative of vitamin K deficiency[18]. Increased serum levels of dp-ucMGP have been associated with increased VC and mortality[19–21]. Since in vitro and in vivo PB-related differences in vitamin K binding have been described we tested a variety of PBs, both calcium containing and calcium free PB's. We hypothesized that combining PB therapy with vitamin K2 supplementation will abolish the impact of PBs on vitamin K status, thereby preventing vitamin K deficiency and providing optimal therapy to reduce or hold progression of VC.

Methods

Ethical statement

The study was approved by the Animal Experiments Committee of the VU University of Amsterdam and Maastricht animal care committee. All relevant licenses were obtained, and the study was performed according to national guidelines for the care and use of animals.

Animals

Ninety male Sprague Dawley (Charles River, Ecully, France) weighing 220-250 gram at start of the experiment were used. Animals were maintained under conventional laboratory conditions, allowed to acclimatize one week before the experiments started and had full access to water and food (Teklad Diets, Madison WI, USA).

3/4 nephrectomy

Renal insufficiency was induced by a 3/4 nephrectomy. At least 24 hours prior to surgery, blood was drawn from the tail vein for serum reference levels. During a single surgical procedure, rats were anesthetized with Isoflurane® (2,5%, with 40% oxygen) and received a s.c. injection of Temgesic® (0,03 mg/kg buprenorfinehydrochloride) 30 min before surgery to ensure analgesia during the procedure.

The left kidney was exposed through an abdominal incision after which half of the arterial branches were ligated, directly followed by full ligation and removal of the right kidney. 6-8 hours after the first injection, administration of Temgesic® was repeated to prolong analgesia during recovery. Animals were kept individually for the first 24 hours post-surgery and daily checked to monitor recovery.

Experimental design

One week after 3/4 nephrectomy, rats received a purified diet for three weeks (Altromin, Lage, Germany), containing calcium 0,76%, phosphate 0,45%, 3 mg/g warfarin (Sigma) and 1,5 mg/g vitamin K1 (Sigma). Our rat model consists of concomitant administration of vitamin K1 and warfarin to overcome vitamin K antagonism in liver but not in extrahepatic tissues[22]. This enables us to study VC yet prevents major bleeding in the animals. We used warfarin in our experiment to mimic the condition of CKD patients that are often subclinically deficient in vitamin K[12]. Next, the diet was switched to a purified diet, calcium 1,34%, phosphate 1,2%, either with or without PB. The following PBs were used: calcium carbonate, lanthanum carbonate, sevelamer carbonate and sucroferric oxyhydroxide. The diets contained 1000 mg PB per kilogram body weight per day. The 1000 mg dose was based on the dry weight and not on the active substance[23]. Each group was subsequently divided into a high vitamin K2 (100 µg/gram; Nattopharma ASA, Oslo, Norway) and low vitamin K2 (5 µg/gram) subgroup for another 8 weeks (Figure 1). After the treatment period, rats were sacrificed and blood was collected from the portal vein into 105 mM trisodium citrate. Plasma was prepared and aliquots were frozen at -80o C until analysis. After bleeding, the rat vasculature was washed by injecting a sterile isotonic buffer (40 mM Hepes, 150 mM NaCl, 5 mM KCl, 1 mM MgCl2 and 2.5 mM CaCl2, pH 7.3) via the left ventricle. Aortas were harvested, dissected and fixed overnight into 1% (v/v) Hepes-buffered paraformaldehyde, containing 150 mM saline at 4˚C, before embedding into paraffin.

Biochemical measurements

Before surgery and before sacrifice the following data were collected: serum creatinine, urea, calcium and phosphate levels. Measurements were conducted by standard laboratory techniques. Intact FGF23 was measured by the Kainos assay (Tokyo, Japan). Menaquinone 7 levels were measured by liquid chromatography tandem mass spectroscopy (LCMSMS) consisting of an initial sample purification step prior to tandem MS detection (Magtivio BV, Nuth, the Netherlands). The assay performance was evaluated through participation in the international KEQAS scheme.

Quantification of vascular calcification by high resolution micro Computed Tomography

All groups of rats (5-9 per group) underwent *ex vivo* high resolution scanning of the thoracic and abdominal aorta including renal artery and cartilage of tibias using micro-computed tomography (*micro*-CT) at 55 kV and 200 µA (*micro*CT100, Scanco Medical, Bruettisellen, Switzerland). Thoracic and abdominal aorta were scanned and the resolution was set to 24.5 *µm* isotropic. Tissue with mineralization exceeding 50 milligrams of hydroxyapatite per cubic centimeter (mgHA/cm3) was segmented from the image using a single threshold analysis. The threshold was based on trabecular bone calibration values. Both calcified and soft tissue was reconstructed using 3D visualization tool to obtain pictures (Figure 2). Calcification areas and volume were then determined by calculating total bone volume (mm3). This method is sensitive to measure early signs of calcification, takes the whole tissue into account, and leaves the tissue intact for further histochemical analysis. The tibia cartilage calcification was added since MGP is synthesized by vascular smooth muscle cells (VSMCs) but also significantly by chondrocytes. Therefore, the impact of vitamin K metabolism on MGP also impacts cartilage. This was measured to validate the effects of vitamin K on MGP in the vessel wall.

Quantification of cartilage and bone calcification by high resolution micro Computed Tomography

Besides VSMCs, MGP is also synthesized by chondrocytes[24]. Knee joints were scanned in a closed holder at a resolution of 10 µm, with a source energy of 70 kVp, the intensity of 200 µA and an integration time of 300 ms. µCT image processing included Gauss filtering with σ = 0.8, a support of 1

voxel and a voxel size of 10 µm, as well as segmentation of the bone phase using a global threshold of 210 per mile of the maximum grey value, corresponding to 453 mg HA/ccm. Contours were drawn manually to determine the volume of interest (VOI) of the subchondral bone of the tibia plateau. For analysis of the knee joint itself, the contour was shifted in the proximal direction and only the region that was non-overlapping with the original contoured region or the femoral subchondral bone was then analyzed. From the segmented images, the volumetric bone mineral density (vBMD) was determined[25, 26].

Immunohistochemical analysis

Aortic arches, including carotid arteries and abdominal arteries, including renal arteries were collected upon sacrifice, fixed in formalin embedded paraffin. Paraffinized tissues were cut in tissue sections of 4 mm. The tissues were stained with Alizarin Red, Von Kossa, and for 8-OHdG and uncarboxylated MGP (ucMGP). Semi-quantitative analysis of Alizarin Red, von Kossa and ucMGP was performed by a blinded single reader. Scores for 2 tissue sections with 4 independent regions of the thoracic and abdominal aorta per animal group were determined. The extent of ucMGP signal was quantified on a 'absent' (0), 'light' (+), 'moderate' (++), or 'heavy' (+++). For antibody based immunohistochemical imaging, sections were stained with primary antibodies for ucMGP (1:25; IDS, Boldon, UK), 8-OHdG (1:150; Meridian Life Science, Memphis, US)). Secondary antibodies used were goat anti-mouse HRP conjugated IgG (1:1000, Dako) and anti-goat HRP conjugated IgG (1:400, Dako). Antibodies were visualized by red alkaline substrate kit I (Vector SK-5100; Vector Laboratories, Burlingame; CA); nuclei were counterstained with haematoxylin.

Statistical analysis

Statistical analysis was performed using Prism version 6 (GraphPad Software INC; San Diego, CA, USA). Data are presented as mean ±SD unless stated otherwise. All data were analyzed using Mann-Whitney for the difference between two groups and Kruskal-Wallis for the difference between three or more groups since the data were not normally distributed. Since there was no difference between the individual PB groups and the numbers in each group were small, we decided to pool the PB results.

Results

Blood chemistry

At the end of the study, 3/4Nx surgery resulted in increases of serum creatinine and urea concentrations from 37.1 µmol/L to 75.7 µmol/L and from 8.7 mmol/L to 16.1 mmol/L, respectively ($p < 0.01$ for both). Both calcium and phosphate levels did not change significantly (2.52 mmol/L to 2.39 mmol/L; $p= 0.131$ and 2.14 mmol/L to 2.09 mmol/L; $p= 0.586$, respectively). There were no significant differences between the groups in phosphate and calcium levels or kidney function at any time point (Table 1). The mean intact FGF23 serum level before sacrifice was elevated (2097 ± 2197) ng/L as compared to an average of 400 ng/L, in non-CKD rats, measured by the same assay[27]. There was no difference in intact FGF23 levels between the different groups ($p=0.074$) (Table 1) or pooled groups of animals with or without PBs (0.36), respectively (970 (650-2843) ng/L, median, interquartile range, for animals with PBs throughout; 1250 ng/L (805-4677) median, interquartile range for the animals without PBs. The FGF-23 level in the animals treated with calcium-containing PBs was 1289 (425-2759), median (interquartile range) versus the level in the calcium free BP's 908 (643-3120), median (interquartile range) this difference was not significant either ($p=0.99$). High vitamin K2 (MK-7) supplementation had a positive effect on increasing circulating plasma levels of MK-7 in comparison with low vitamin K2 intake ($p=0.0012$). There were no significant differences between high vitamin K2 (MK-7) and low vitamin K2 (MK-7) with and without PBs ($p=0.642$ and $p=0.826$, respectively, figure 5E). Moreover, we found no differences in circulating plasma levels of vitamin K1 (phylloquinone) between high vitamin K2 (MK-7) and low vitamin K2 diets ($p=0.321$, figure 5F)

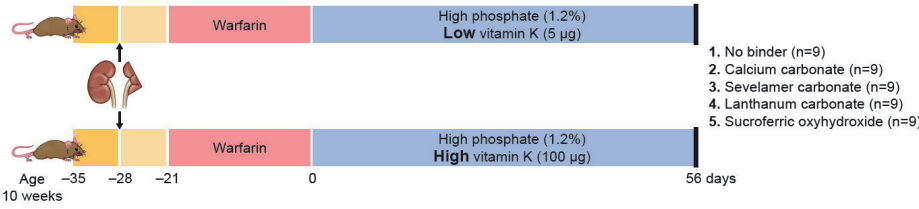

Figure 1. Study setup design. Animals entered the study and received after one week 3/4 nephrectomy. Next, animals were placed on 3 weeks warfarin/ vitamin K1 treatment to induce vitamin K deficiency. Subsequently, animals received a high phosphate diet with either a high or low vitamin K2 (MK-7) diet, combined with a phosphate binder. Both low and high vitamin K groups had a control group not receiving phosphate binder treatment.

	Vitamin K2 high group start		Vitamin K2 low group start		Vitamin K high end		Vitamin K low end	
	Control	PB group	Control	PB group	Control	PB group	Control	PB group
Creatinine (µmol/L) Median (IQ range)	23.9 (22.1-34.7)	27.2 (22.6-44.3)	28.1 (24.8-97.2)	28.45 (24.3-42.0)	54.0 (33.5-92.5)	53.0 (42.0-86.5)	47.0 (34.8-113.5)	70 (50.0-90.0)
Urea (SD) (mmol/L) Mean (SD)	6.9 (1.6)	11.4 (14.0)	12.1 (9.2)	8.5 (2.9)	12.9 (4.8)	16.1 (6.9)	16.0 (11.2)	17.0 (45.6)
Calcium (SD) (mmol/L) Mean (SD)	2.43 (0.65)	2.49 (0.45)	2.84 (0.24)	2.5 (0.43)	2.39 (0.22)	2.39 (0.23)	2.27 (0.37)	2.40 (0.20)
Phosphate (SD) (mmol/L) Mean (SD)	2.20 (0.44)	2.24 (0.94)	2.56 (0.85)	2.19 (0.49)	2.11 (0.42)	2.09 (0.58)	2.12 (0.78)	2.11 (0.80)
FGF-23 Median (ng/L) (IQ range)					5282 (663-7374)	731 (474-2433)	1241 (920-1370)	1130 (730-3520)

Table 1. Both baseline and end of study data for creatinine, urea, calcium, phosphate and FGF-23 of animals on high and low vitamin K2 diet, with and without PB use**.** There were no significant differences between any of the groups.

Vascular calcification detected by aortic micro-CT and tissue staining

In the low vitamin K2 group, all tested PBs failed to inhibit VC as measured by *micro*-CT in the thoracic (p=0.053) and abdominal aorta (p=0.81) compared to animals not taking PB (Figure 3A and 3B). In animals not treated with PBs, there was no statistically significant difference in VC between animals treated with a high or low vitamin K2 diet (p=0.95). However, rats in high vitamin K2 group on PBs developed significantly less VC as measured by *micro*-CT in both the thoracic (p=0.026) and abdominal aorta (p=0.023; Figure 3A and 3B), compared to control. There was no statistically significant difference among the different binders tested, nor compared to the no-binder control for individual binders (Supplemental figure 1). The presence of VC, as quantified by *micro*-CT, was confirmed by von Kossa and Alizarin Red staining after sectioning the same tissues. Both von Kossa and Alizarin Red staining revealed calcification in line with the *micro*-CT results (Figure 4A and 4B). Moreover, we detected the presence of 8-OHdG around calcified regions in the medial layer of the thoracic aorta. 8-OHdG was mainly present in animals on low vitamin K2 diet in combination with PBs (Supplemental figure 5). On the contrary, 8-OHdG levels were lower in animals treated with high vitamin K diet in combination with PBs groups (Supplemental figure 4).

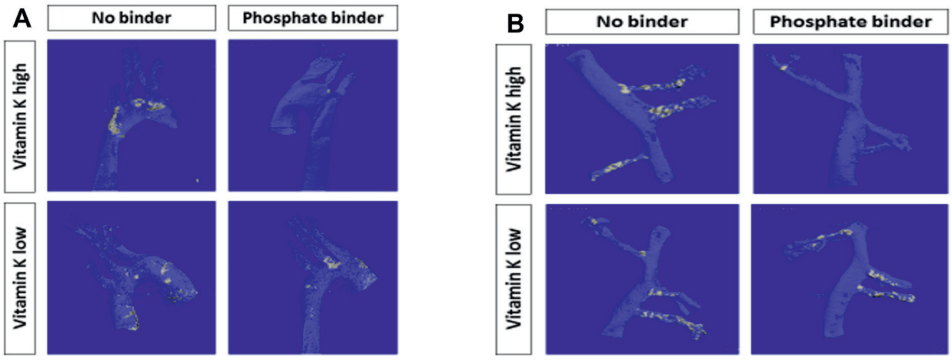

Figure 2. Representative images of vascular calcification measured by *micro*-CT scan. Figure 2A: aortic arch and thoracic aorta. Figure 2B: abdominal aorta with renal arteries

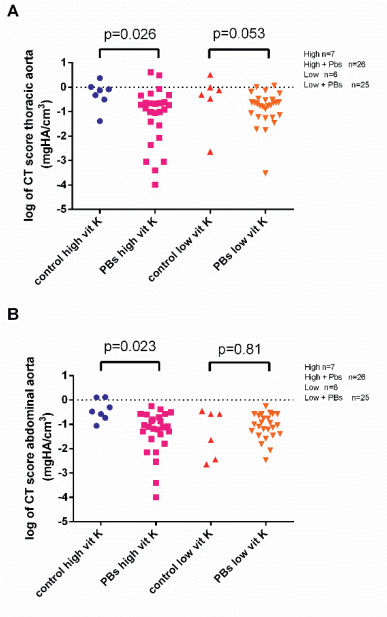

Figure 3. Log transformation of vascular calcification score measured by *micro*-CT scan. Figure 3A: aortic arch and thoracic aorta showing significant lower vascular calcification in high vitamin K2 with PB treatment. In the low vitamin K2 treatment, additional PB treatment reduced vascular calcification non-significantly. Figure 3B: abdominal aorta and renal arteries showing significantly lower vascular calcification in the high vitamin K2 with PB treatment groups. Vascular calcification score is depicted as hydroxyapatite per cubic centimeter (mgHA/cm^3) with a median and 95% confidence interval for control and pooled phosphate binder groups.

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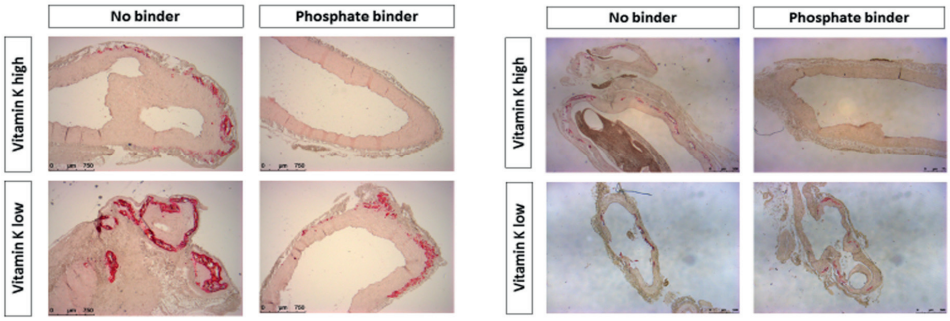


Figure 4 A. Alizarin red staining of the aortic arch and thoracic and abdominal aorta with renal arteries. At both anatomical sites, the high vitamin K2 with PB treatment showed less vascular calcification depicted by Alizarin Red

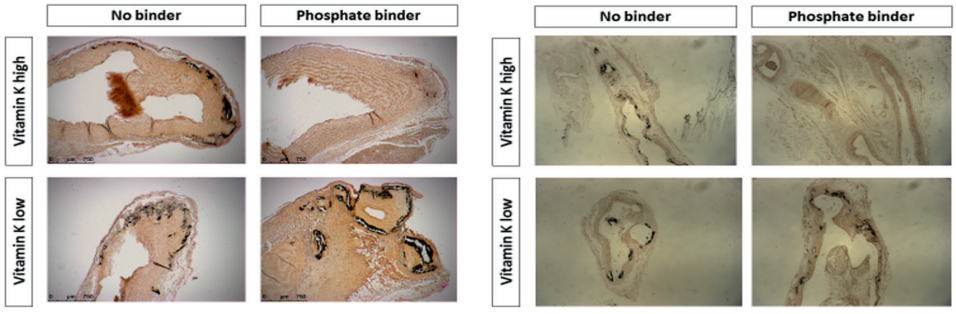


Figure 4 B. Von Kossa staining of the aortic arch and thoracic and abdominal aorta with renal arteries. At both anatomical sites, the high vitamin K2 with PB treatment showed less vascular calcification depicted by von Kossa.

ucMGP accumulation in the vascular wall

Immunohistochemical staining for ucMGP in the vessel wall revealed ucMGP present at sites of VC. Presence of ucMGP was mainly detectable in the low vitamin K2 treated groups. The low vitamin K2 groups had significantly higher expression of ucMGP in both the thoracic ($p<0.01$) as well as the abdominal aorta ($p<0.01$), compared to the high vitamin K2 groups (Figure 5C and 5D). There was no difference between the control and PBs in either the high ($p=0.95$) or low vitamin K2 treated groups ($p=0.58$). However, there was less ucMGP staining present in the group on combined PBs with high vitamin K2 diet compared to the group on PBs on low vitamin K2 diet, respectively for thoracic ($p=0.013$) and abdominal ($p=0.005$) aorta (Figures 5A and 5B, see Figure 6 for representative photos).

Micro-CT cartilage and bone

To further validate the protective effect on calcification of PB treatment in combination with high vitamin K2, we analyzed the amount of bone volume density in articular cartilage of the tibia and bone mineral density of the femur using *micro*-CT. In the PB groups on low vitamin K diet, cartilage calcification was evenly present and not different between groups ($p=0.90$). PB treatment in combination with high vitamin K2 resulted in significantly less tibial articular cartilage calcification compared to control (supplemental Figure 2). The measurement of the bone mineral density of the femur showed no significant differences between any of the treatment groups (Supplemental figure 3).

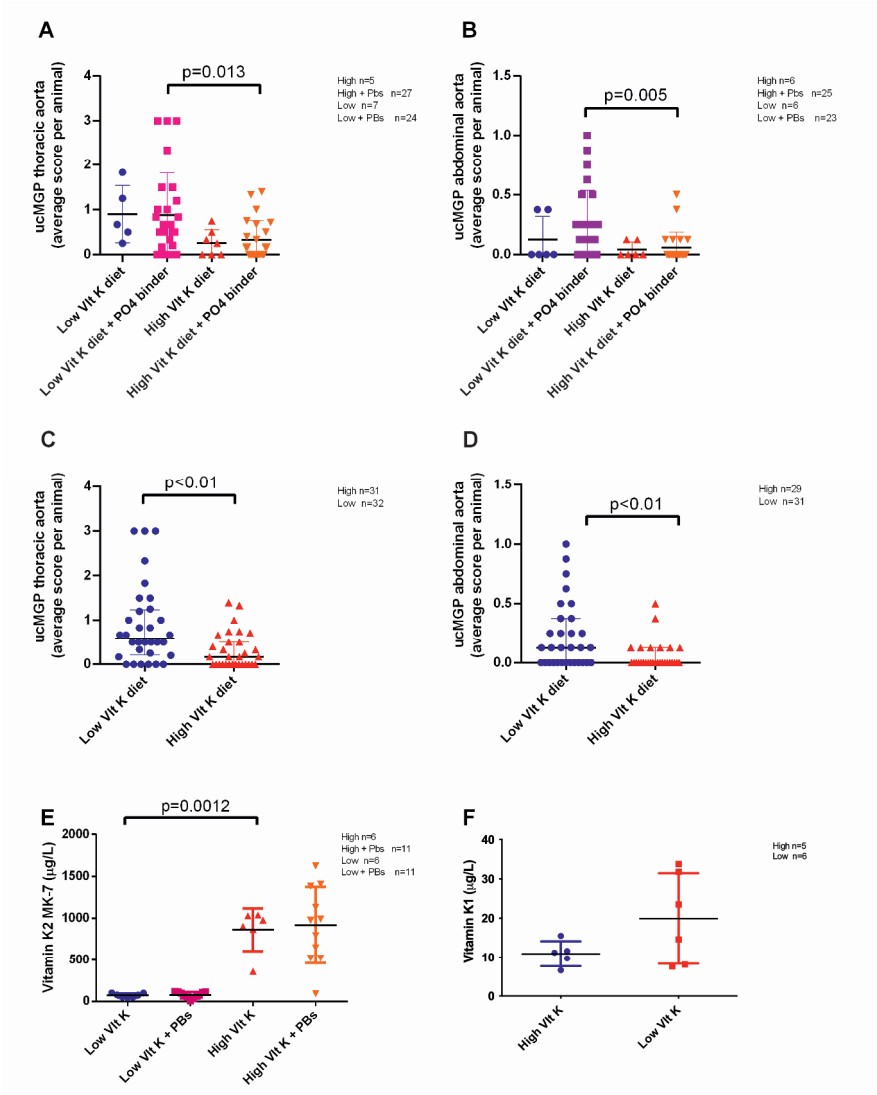


Figure 5. Vitamin K-status measured in vasculature and circulation. Figures 5A and B show ucMGP presence and is depicted as median and 95% confidence interval for A. aortic arch and thoracic aorta and B. the abdominal aorta and renal arteries. Significant lower ucMGP was present animals treated with high vitamin K2 and PB treatment. Figures 5C and D show the difference between ucMGP presence in animals treated with either high or low vitamin K2. The ucMGP score is depicted as median and 95% confidence interval for thoracic aorta. Figure 5E shows plasma levels of MK-7 in animals treated with high and low vitamin K2 (MK-7). Animals treated with high vitamin K2 has significant higher MK-7 plasma levels as compared to low vitamin K treatment, irrespective of PB treatment. MK-7 plasma levels are depicted as median and 95% confidence interval. **F** Comparison of plasma levels of phylloquinone in high and low vitamin K2 (MK-7) groups. The vitamin K1 plasma levels are depicted as median and 95% confidence interval.

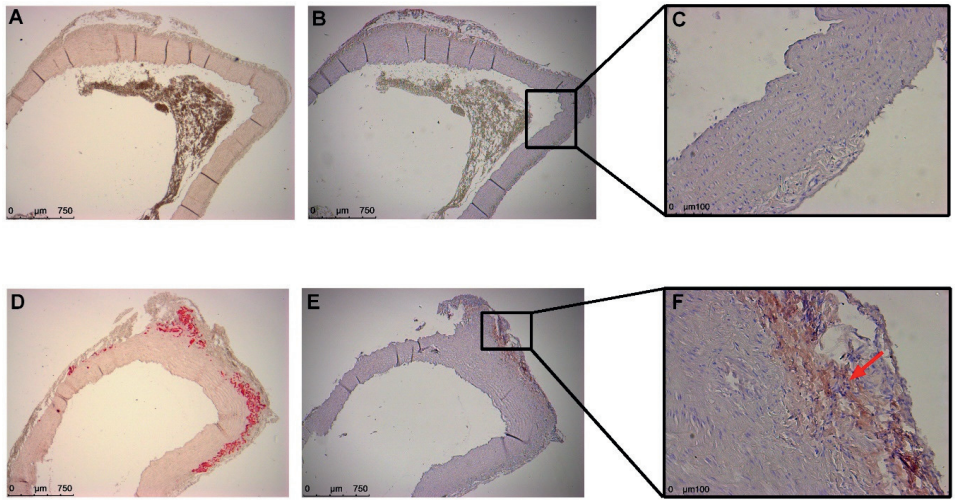


Figure 6. Alizarin red (Figures A, D) and ucMGP (Figures B, C and E, F) staining of the aortic arch and thoracic and abdominal aorta with renal arteries. At both anatomical sites, the high vitamin K2 (Figures A, B and C) with PB treatment showed less vascular calcification and also less ucMGP positive staining (arrows).

Discussion

In this study, we demonstrate that the use of PB treatment in a kidney failure (CKD) animal model with concomitant vitamin K deficiency, which mirrors dialysis patients, is not sufficient to prevent ectopic calcification. However, combining PBs with high vitamin K2 supplementation strongly attenuated VC. This protective effect on calcification is likely accomplished by the synergistic effect of combined PB treatment and vitamin K2 supplementation. To the best of our knowledge, this is the first *in vivo* preclinical study combining PB therapy with vitamin K2 supplementation. In animal models without CKD[22] and even in animals with CKD[28, 29] vitamin K supplementation has been shown to inhibit VC. Additionally, PB therapy in CKD animals demonstrated a reduction in VC to some extent[30–32]. However, none of these animal models combined CKD with vitamin K deficiency, as is the clinical situation for the majority of late-stage CKD patients[12]. Both vitamin K deficiency[33] and increased phosphate levels[34] are associated with increased morbidity and mortality in CKD patients. In our model, CKD animals were rendered vitamin K deficient by pretreatment with warfarin and provided a high phosphate diet. Additionally, animals were treated with different PBs in combination with a low intake of vitamin K2, thereby mimicking the clinical situation in late-stage CKD[33, 34]. Patients with late-stage CKD are recommended to lower both phosphate and potassium intake. These recommendations, however, also limit intake of vitamin K as leafy green vegetables (as a source of potassium) are rich in vitamin K1 and cheeses (rich in phosphate) are a major source of vitamin K2[13]. On top of these untoward effects of dietary intervention on vitamin K intake, different PBs have been shown, both *in vitro* and *in vivo,* to bind vitamin K, thereby limiting its bioavailability[15–17]. Sevelamer seems to bind vitamin K directly[16], however, there might also be an effect on microbiota by sevelamer and calcium carbonate treatment[35, 36]. Moreover, in patients on renal replacement therapy, an association of sevelamer carbonate use and increased dp-ucMGP levels was observed, suggesting that PB therapy aggravates vitamin K deficiency[18]. In our study, there was no difference in VC, vitamin K levels or ucMGP levels between the distinct PBs. This may be due to a type II error, given the small number of animals per phosphate binder. Nevertheless, the use of calcium-containing PBs in our study did not aggravate VC, as seen in clinical studies with calcium-containing PBs compared to calcium free PBs[4, 6, 7, 9, 10] either. The relatively short duration of our experiment, including a mild degree of kidney failure and the absence of hyperphosphatemia, compared with clinical late-stage CKD, may explain the absence of

difference in calcification with the use of calcium-containing and non-calcium-containing PBs. Also, circulating levels of MK-7 in rat plasma at the end of the study were measured. There was a significant increase in MK-7 plasma levels in animals fed a high MK-7 diet in comparison to animals fed a low MK-7 diet. There was no difference in MK-7 plasma levels between animals treated with or without PBs. To further verify whether local vascular vitamin K deficiency was present, we quantified ucMGP in thoracic and abdominal aorta tissue. In the vessel wall of CKD animals treated with PBs in combination with low vitamin K, ucMGP colocalized extensively with VC. However, when PBs were combined with high vitamin K2 intake, significantly less ucMGP and VC were present in the vessel wall. Our findings are in line with previous data in experimental animals and suggest that vitamin K deficiency is a risk factor for developing VC[22]. The inhibitory role of combined PB and vitamin K2 can be explained via a mechanism other than MGP activation. It has been shown that vitamin K protects VSMC differentiation and calcification into osteogenic phenotype under a high phosphate environment via downregulation of bone-specific genes[37, 38]. However, we could not find such an effect in our study (data not shown). Recent findings suggest a non-canonical role of vitamin K2 as an antioxidant[39]. Indeed, vitamin K2 ameliorated nicotine-induced VSMCs intracellular oxidative stress and subsequently calcification[40]. Additionally, VKORC1L1 (VKORC1-like 1), a paralogue enzyme of VKORC1, was found to regulate vitamin K-dependent intracellular antioxidant function in cell membranes[41]. Vitamin K has been shown to mediate a VKORC1L1-dependent increase in cell viability. Warfarin use or low vitamin K-status limits the function of VKORC1 as well as VKORC1L1, and thus might be an important contributor to oxidative stress. Here, we show that high vitamin K2 treatment, especially in combination with PB treatment, attenuates oxidative stress (Supplemental Figure 4). This effect was not observed in any of the low vitamin K2 treated groups (Supplemental Figure 5). We hypothesize that the combination of high vitamin K2 and PBs results in reduced VC, in part via decreased oxidative stress. Thus, both PB and vitamin K2 supplementation are required to reduce VC in our CKD model. VC in CKD patients is predominantly linked to medial calcification[34], a condition that is more present in the abdominal aorta and peripheral arteries[42]. VC in CKD is also located at the aortic arch and the thoracic aorta[43]. Therefore, we analyzed calcification in both the thoracic and abdominal aorta and found VC to be increased at both vascular sites in CKD animals treated with PBs and low vitamin K2 intake. UcMGP expression could be detected at both vascular anatomical sites, suggesting an involvement of vitamin K at both locations. Vitamin K antagonist treatment, inducing vitamin K deficiency, is known to induce vascular as well as cartilage calcification[44]. MGP is produced by both vascular smooth muscle cells and chondrocytes. Additionally, cartilage calcification is a feature that is present in dialysis patients[45, 46] suggesting a process similar to VC. Therefore, we analyzed the calcification of knee articular cartilage to confirm the impact of vitamin K2 treatment in combination with PB on MGP. Indeed, also in the articular cartilage, we found that the combination therapy resulted in significantly less calcification as compared to PB treatment in combination with low vitamin K2 intake, as such corroborating our findings in the arterial walls. The calcium paradox is a term relating to the loss of bone mineral content and subsequently the accumulation of calcium crystals in the arterial vessel wall. In this bone-vascular crosstalk, vitamin K has been put forward as a nutritional intervention to improve bone quality and simultaneously protect the vasculature[47, 48]. In our study, we show that the combination of vitamin K2 and PB treatment has beneficial effects on vascular and cartilage calcification. However, this treatment had no effect on bone mineral density (BMD) in our model. This is in line with previous research, where vitamin K2 did not affect the BMD of the femoral shaft in combination with teriparatide *in vivo*[49].

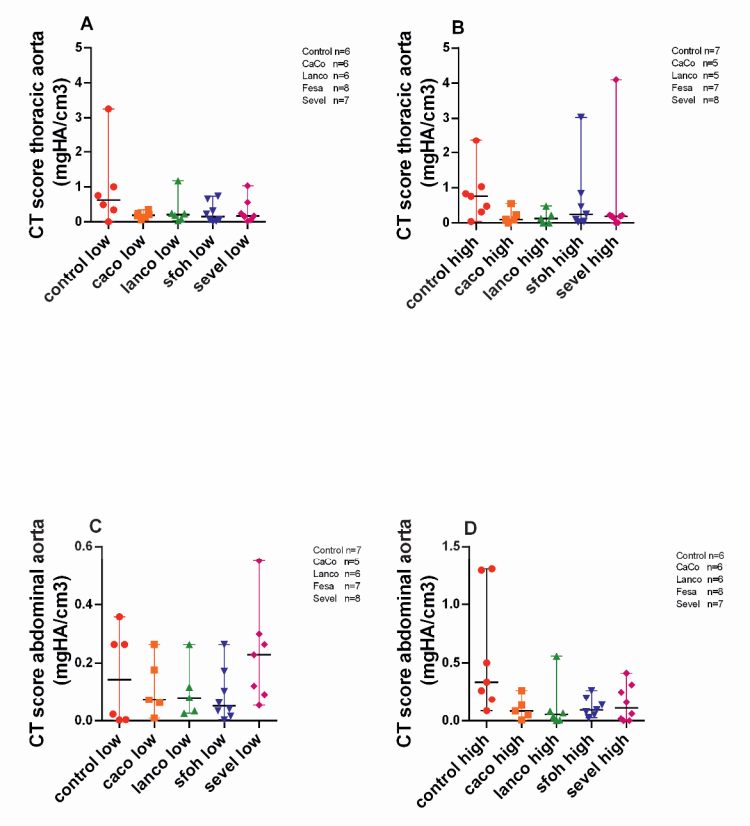
There are a few limitations of this study. In our rat model, the severity of CKD was less pronounced as compared to other CKD models[30]. This could be due to 3/4 nephrectomy procedure compared to animal models using adenine[31] or 5/6 nephrectomy[50] to induce CKD. In our model, there was no change in phosphate levels after the 3/4 nephrectomy as compared to baseline. This is, however, in line with what has been described previously[51, 52], and also in clinical CKD, hyperphosphatemia is a late stage feature[53]. There was no control group without CKD present, and thus we do not have data on normal phosphate levels in rats. We cannot exclude that PB treatment alone, in a setting of more

advanced CKD with hyperphosphatemia would attenuate VC. Also, we did not collect urine so we cannot exclude a difference in phosphate balance and there was an elevated intact FGF-23 as compared to non-CKD rats[27]. FGF-23 levels were elevated at the end of this study. In the absence of a control group without CKD, we compared FGF23 levels with previously published literature. This elevation was less pronounced as compared to CKD rat models, where FGF-23 has been measured by the Kainos assay[27]. In this model, a 25% adenine diet was given, and intact FGF-23 levels were some 10-fold higher compared to our model. This discrepancy might be due to the more pronounced CKD in the adenine model compared to our 3/4 nephrectomy. In addition, the adenine model is a tubulotoxic model which may also induce FGF23 resistance. Indeed, creatinine levels were also some 3-fold higher as in our study. We did not observe differences in FGF-23 levels between animals treated with PBs compared to control. This might be due to the relatively mild increase in FGF-23 levels (2097 ± 2197) ng/L as compared to 400 ng/L in non CKD rats[27]. Moreover, our CKD model has a high degree of complexity, including CKD, vitamin K-deficiency and high phosphate in combination with low or high vitamin K.

Our study also has several strengths. We included a broad variety of PBs in this model and measured a great number of parameters, which are known to have an effect on VC. We have used *micro*-CT as a highly sensitive and specific technique for the measurement of VC *ex vivo*. In this way, we analyzed total VC throughout the vasculature and kept the tissue available for (immuno)histochemical analysis. In this study, the use of PB monotherapy, effective in lowering serum phosphate levels, hardly inhibits the progression of VC[8, 54]. Also, vitamin K2 supplementation alone seems not to be sufficient to reduce the progression of VC in CKD[55]. Because vitamin K metabolism is likely to be affected by PBs, monitoring of vitamin K status should be considered to assess optimal treatment dosage. Although this is an experimental animal study, we put forward that – based on the safety of PBs and vitamin K - these results could be translated to clinical practice.

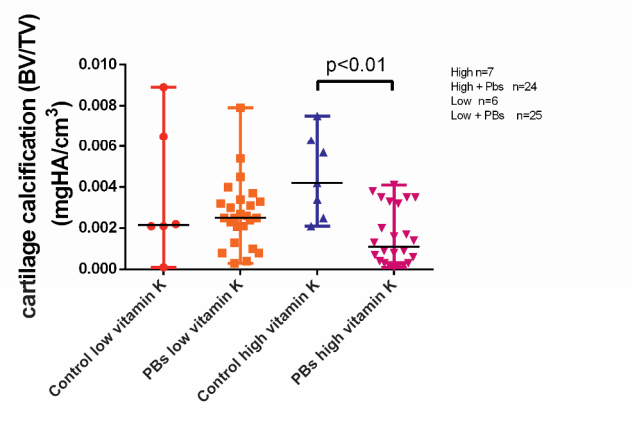
We conclude that high dose vitamin K2 may be needed in combination with PB use to significantly counteract VC. Future clinical studies should analyze the combination of vitamin K2 supplementation with PB therapy to hold the progression of VC, changes in arterial stiffness and cartilage calcification.

Supplemental data



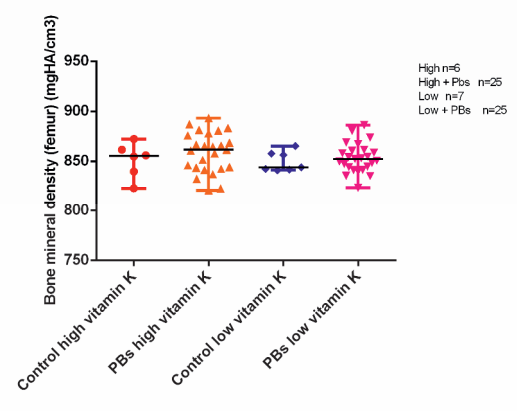
Supplemental Figure 1. Vascular calcification score measured by micro-CT scan in low and high vitamin K2 groups, with and without phosphate binders. Vascular calcification expressed as hydroxyapatite per cubic centimeter (mgHA/cm^3) with a median and 95% confidence interval at thoracic and abdominal aorta. No significant differences are observed, likely due to the low number of animals per group.

Abbreviations: caco - calcium carbonate, lanco - lanthanum carbonate, sevel - sevelamer carbonate and sfoh - sucroferric oxyhydroxide, control low: low vitamin K2 diet, high: high vitamin K2 diet.

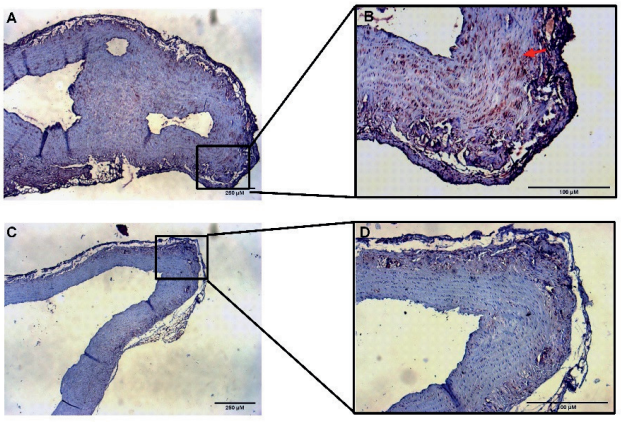


Supplemental Figure 2. Calcification of the articular cartilage (BV/TV) of the tibia plateau (knee joint) measured by micro-CT scan. Calcification expressed as hydroxyapatite per cubic centimeter (mgHA/cm^3) with a median and 95% confidence interval. In the high vitamin K2 group, use of PBs significantly reduced articular cartilage calcification.

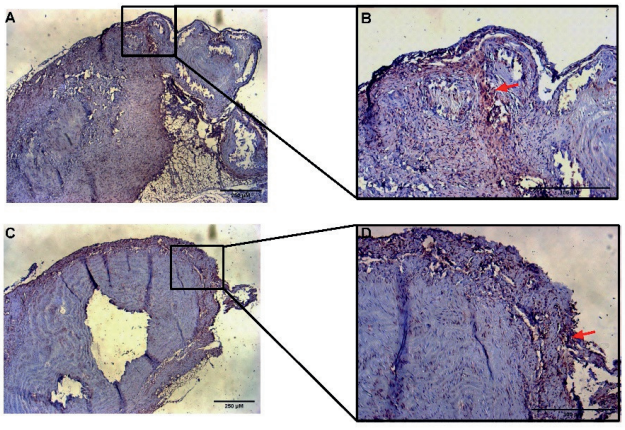
BV/TV = bone volume fraction



Supplemental Figure 3. Bone mineral density (BMD) of the femur (knee joint) in animals treated with low and high vitamin K2 with or without PB treatment. BMD measured using micro-CT scan and expressed as hydroxyapatite per cubic centimeter (mgHA/cm^3) with a median and 95% confidence interval. No significant differences were present between low and high vitamin K treatment, or with or without PB use.



Supplemental Figure 4. S4A: Representative image of 8-OHdG staining of the thoracic aorta in the high vitamin K2 treatment group without PB. Representative figure shown in 4x magnification. S4B: 8-OHdG staining in 10x magnification. Arrows indicate positive 8-OHdG presence. S4C: 8-OHdG staining of the thoracic aorta in the high vitamin K2 treatment group along with PB. Representative figure shown in 4x magnification. S4D: 8-OHdG staining in 10x magnification. Arrows indicate positive 8-OHdG staining which is less as compared figure S4B.



Supplemental Figure 5. S5A: Representative image of 8-OHdG staining of the thoracic aorta in the low vitamin K2 treatment group without PB. Representative figure shown in 4x magnification. S5B: 8-OHdG staining in 10x magnification. Arrows indicate positive 8-OHdG presence. S5C: 8-OHdG staining of the thoracic aorta in the low vitamin K2 treatment group along with PB. Representative figure shown in 4x magnification. S5D: 8-OHdG staining in 10x magnification. Arrows indicate positive 8-OHdG staining which is similar in both figures.

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CHAPTER

Menaquinone-7 Supplementation Improves Osteogenesis in Pluripotent Stem Cell Derived Mesenchymal Stem Cells

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Abstract:

Development of clinical stem cell interventions are hampered by immature cell progeny under current protocols. Human mesenchymal stem cells (hMSCs) are characterized by their ability to self-renew and differentiate into multiple lineages. Generating hMSCs from pluripotent stem cells (iPSCs) is an attractive avenue for cost-efficient and scalable production of cellular material. In this study we generate mature osteoblasts from iPSCs using a stable expandable MSC intermediate, refining established protocols. We investigated the timeframe and phenotype of cells under osteogenic conditions as well as the effect of menaquinone-7 (MK-7) on differentiation. From day 2 we noted a significant increase in RUNX2 expression under osteogenic conditions with MK-7, as well as decreases in ROS species production, increased cellular migration and changes to dynamics of collagen deposition when compared to differentiated cells that were not treated with MK-7. At day 21 OsteoMK-7 increased alkaline phosphatase activity and collagen deposition, as well as downregulated RUNX2 expression, suggesting to a mature cellular phenotype. Throughout we note no changes to expression of osteocalcin suggesting a non-canonical function of MK-7 in osteoblast differentiation. Together our data provide further mechanistic insight between basic and clinical studies on extrahepatic activity of MK-7. Our findings show that MK-7 promotes osteoblast maturation thereby increasing osteogenic differentiation. Keywords: vitamin K, menaquinone-7, osteogenesis, pluripotent stem cells, mesenchymal stem cells.

INTRODUCTION

Mesenchymal stem cells (MSCs) are the predominant source of cells for endogenous repair after bone fracture[1]. Application of MSCs in regenerative medicine has been widespread and explored as a therapeutic source following damage to bone, cartilage, cardiac, tendon, and immune-related disorders[2]. The ability to more specifically program and direct cells beforehand could improve regenerative outcome[3–5]. Induced pluripotent stem cells (iPSCs) allow for an unlimited supply of patient specific cells. Furthermore, the establishment of clinically validated lines could streamline the process of applying regenerative cell-based therapies to the clinic[6]. Current protocols are scarce in variety of chemicals that are used for differentiating bone, mainly consisting of Dexamethasone, Beta-Glycerol Phosphate and L-Ascorbic Acid (Vitamin C)[7]. It is clear, that such limited number of stimuli cannot replace physiological bone formation and are prone to achieve suboptimal osteogenesis conditions, thereby contributing to variability in these processes[8]. Additional supplementation to mineralisation media might aid the *in vitro* development of bone and could be beneficial to enhancing the expression profile of bone- related markers. Vitamin K was first discovered in the 1930s for its role in coagulation, and the extrahepatic activity of vitamin K has been widely unexplored[9]. Vitamin K is a hypernym for multiple analogs each with their own distinct properties and structure. The past decades have begun to elucidate a potent role for vitamin K2 in health and disease beyond coagulation[10]. Vitamin K2 analog menaquinone-7 (MK-7) has been implicated in a range of studies, from basic biochemistry to long term clinical studies[11–14]. The majority of these studies have implicated an important role for MK-7 in bone health and metabolism, yet studies on the mechanistic role of MK-7 on bone healing are lacking[15,16]. Using iPSCs to generate MSCs is the next step in combining the best from stem cell technology and tissue engineering[17]. Further, differentiating cells from pluripotency toward mesenchymal and osteogenic lineage enables application of a novel tool for interrogation of developmental pathways and phenotypes in bone formation[18]. This can potentially answer age-old questions with regards to mechanisms of endochondral transdifferentiation, and the role of macrophages in bone formation[19–23]. Present methods of determination of osteogenic processes include detection of osteoblastic markers such as RUNX2, Col1A1, and Osterix along with ALP activity and calcium deposition[24,25]. However, oxidative stress status or cell migration are rarely explored and should be delineated to enrich the knowledge of differentiation and repair processes in osteogenesis[26]. Due to the aforementioned obstacles to clinical application of stem cell therapies, we investigated the role of vitamin K analog menaquinone-7 [MK-7 (VK2)], in the differentiation of iPSCs to osteoblasts using a stable iMSC intermediate. The intermediate phase allows for reduced costs and technical demands, developing an application toward widescale of tissue engineering based solutions. In this study we find that supplementation of MK-7 to osteogenic medium promotes differentiation of iMSCs toward osteoblasts significant beyond that of osteogenic medium alone.

MATERIALS AND METHODS

Generation and Maintenance of Pluripotent Stem Cell Line

The iPSC line iPSC-UkB-Ctrl-XXwas generated from PBMCs via expansion to a cellular intermediate of EPCs, using Stem Span SFEMII (StemCell Technologies) and addition of Erythropoietin (R&D Systems), Stem Cell Factor (PeproTech), Interleukin- 3 (PeproTech), Insulin Growth Factor-1 (PeproTech), and dexamethasone (Sigma-Aldrich). Following expansion, EPCs were electroporated with Epi5 Reprogramming (Thermo Fisher Scientific) Lonza 4D-Nucleofector X-Unit (Lonza). Cells were plated on Corning ESC-grade Matrigel (Corning) in ReproTeSR (Stem Cell Technologies) before changing to mTeSR 1 (Stem Cell Technologies) when colonies first appeared. Following iPSC clones were picked

and expanded before characterization. iPSC- UkB-Ctrl-XX line is cultured on Corning ESC-grade Matrigel and in mTeSR 1. Cells are passaged when colonies are too large using 0.5mM EDTA in PBS and cultured in a humidified incubator at 37◦C and 5% CO2.

Generation and Maintenance of iMSCs

iMSCs were generated as previously described[27]. Briefly, cell line iPSC-UkB-Ctrl-XX was passaged as normal the day before starting differentiation. Differentiation medium was added and refreshed every 2 days for 2 weeks. iMSC differentiation medium contains Dulbecco's Modified Eagle Medium (DMEM) low glucose (1 g/L D-Glucose), 10% Fetal Bovine Serum (FBS), 1% Penicillin/Streptomycin (Pen/Strep), 2mM glutamine and 0.05mM L-ascorbic acid. Following the initial 2 weeks, iMSCs were passaged with a split ratio of 1:2 on to 0.1% gelatine-coated 6-well plates for two further passages before adhering to untreated plastic. At this stage, cells assume morphology similar to that of primary MSCs and were maintained in differentiation medium with a split ratio of either 1:2 or 1:3. Cells were cultured in a humidified incubator at 37◦C and 5% CO2.

Osteogenic Differentiation of iMSCs

Osteogenic differentiated iMSC were used between 8 and 20 passages. iMSCs were seeded on appropriate size plates dependent on analysis with two varying densities depending on time in culture. For earlier timepoints (2, 7, and 10 days), cells were seeded at 10^5 cells per cm^2 whereas for the later time point (21 days), cells were seeded at 2.6×10^4 per cm^2. The following day, medium was changed to osteogenic medium composed of DMEM, FBS (10%), Pen/Strep (1%), 100 nM Dexamethasone, 10mM B-Glycerol—phosphate, and 0.05mM L-ascorbic acid. For MK-7 (synthetic menaquinone-7, kind gift, NattoPharma, Oslo, Norway) supplementation a final concentration of 10μM was added or equal volume of isopropanol vehicle control.

Chondrogenic Differentiation of iMSCs

Differentiation medium was as follows; high glucose DMEM, TGF-B1 10 ng ml−1 (PeproTech), 100 nM dexamethasone (Sigma-Aldrich), insulin (Sigma-Aldrich). Cells were seeded at a density of 2.6×10^4 per cm^2. Medium was refreshed three times per week and cells lysed after 2 weeks of differentiation induction. Control cells were cultured in standard medium with appropriate vehicle controls.

Adipogenic Differentiation of iMSCs

Adipogenic differentiation was induced by seeding cells at 2×10^4 cell per cm^2. Differentiation medium was composed of high glucose DMEM, FBS (10%), Pen-Strep (1%), 0.5mM isobutylmethylxanthine and 1mM dexamethasone. Medium was refreshed every 2 or 3 days and cells were lysed for analysis after 2 weeks of differentiation. The control cells were cultured in standard medium with appropriate vehicle controls.

Vasculogenic Differentiation of iMSCs

Differentiating iMSCs to vascular smooth muscle-like cells was done using vasculogenic differentiation medium composed of high glucose DMEM, FBS (10%), Pen-Strep (1%), PDGF-BB 10 ng ml−1 (PeproTech) and TGF-B1 5 ng ml−1 (PeproTech). Cells were seeded at a density of 2×10^4 cells per cm2.

Medium was refreshed every 2–3 days for 14 days before analysis. Control cells were cultured in standard medium with appropriate vehicle controls.

Real-Time Reverse-Transcription Polymerase Chain Reaction

Total RNA was isolated from differentiated cells using TRIzol reagent (Invitrogen) and quantified using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific). Five hundred nanogram of total RNA was transcribed using iScriptTM Reverse Transcription Supermix (BioRad) for RT-qPCR in a 20 µl reaction. The resulting cDNA was diluted one and a half times, and 4mL was amplified using Power SYBR Green PCR System following manufacturers recommendations. Real-time qPCR was performed using the Quantitect SYBR green PCR kit (Qiagen) in a LightCycler 480 II (Roche) with 50 ng of cDNA and 0.5µM of each primer. The sequences of primers used are provided in Supplementary Table 1. Relative expression for each gene was normalized against GAPDH and expressed as fold change over control. Fluorescence curves were analyzed with LightCycler 480 Software (Version 1.5) and relative quantification was performed with the 2^Ct method. Data from at least 3 different differentiations of our line were combined and reported as mean ± SD.

Flow Cytometric Analysis

iMSCs were dissociated, washed and adjusted to a cell suspension of concentration 1×10^6 in ice-cold PBS, 10% FBS and 1% sodium azide at 4°C. Next, the conjugated primary antibody was added in 3% FBS in PBS for 30min in dark at room temperature. After the incubation, cells were washed 3x by centrifugation at 400 g for 5min and resuspended in 1ml ice-cold PBS, 10% FCS, 1% sodium azide. Cells were kept at ice until time of analysis.

O-Cresolphthalein Assay

Cells in 48 well plates were washed twice with PBS. The mineralized matrix was dissolved in 1M HCl and put on a shaker overnight. To quantify the amount of calcium per sample, Randox O-cresolphthalein kit was used to assess the amount of calcium embedded in extracellular matrix. Values were converted to µg calcium and adjusted to protein levels. All samples were assayed in triplicate.

DC Protein Assay

For normalization of the calcium content of the cells, DC protein assay was performed. 1M HCl cell suspension was neutralized and lysed with 1M NaOH 0.2% SDS and incubated on a shaker overnight. Plates were read at 750 nm using Cytation3 (BioTek). Standard curve was created and sample absorbances were calculated, giving the protein content µg/µl protein. All samples were assayed in triplicate in three independent experiments.

Immunofluorescence

Cells were fixed with 4% PFA, permeabilized with 0.1% Triton X-100, blocked in 1% BSA/PBS and incubated with primary antibodies (COL1a1, Applied Logistics). The following secondary antibodies were used: anti-rabbit-FITC (Dako, F0205) and anti-mouse-FITC (Dako, F0232). Nuclei were stained with DAPI (Sigma–Aldrich). Cells were analyzed using Cytation3.

Western Blotting

VSMCs were lysed in 0.1M Tris pH 8.1, 0.15M NaCl, 1% triton x-100 0.2mM NaVO3 and 1:50 protease inhibitor cocktail (Sigma). Protein concentration was determined using DC protein assay (Bio-Rad) and lysates were separated on Any kD Mini- PROTEAN TGX Precast Protein Gels (BioRad). Samples were transferred to nitrocellulose membrane (BioRad) and incubated overnight with anti-Runx2 (MBL, D130-3) and anti-Col1A1 (BD, 610153). Protein was detected using HRP-conjugated secondary antibodies (anti-mouse: p0447, Dako; anti-rabbit: 7074S, Cell Signaling, anti-goat: P0449, Dako) and

visualized by enhanced chemiluminescence (Pierce ECL Western Blotting Substrate, ThermoFisher Scientific). All samples were blotted in triplicate.

Alkaline Phosphatase Activity

Cells were lysed in 1% Triton X-100 in PBS, subjected to 2 freeze-thaw cycles and centrifuged at 13,000 g for 5min. ALP activity in the supernatants was measured at 405 nm using 4-Nitrophenyl phosphate disodium salt hexahydrate (Sigma) as substrate. Enzyme activity (U) was normalized to protein concentrations. All samples were assayed in triplicate.

Alizarin Red Assay

On the final day of differentiation, medium was aspirated, and wells were washed twice with PBS. Next, cells were fixed in 4% formaldehyde in RT for 40min. Then cells were washed twice with PBS and stained with 2% Alizarin Red for 1h, followed by washing cells twice with PBS. Mineral deposits were visualized using light microscopy at 20X magnification

Reactive Oxygen Species

To measure oxidative stress, we measured reactive oxygen species (ROS) using 2, 7-dichlorofluoroscein diacetate (DCFDA, Merck) which is oxidized to 2, 7-dichlorofluoroscein in the presence of the oxidants. Mesenchymal stem cells supplemented with osteogenic media with or without MK-7 (10μM final concentration) and left for 48 h, or 7 days with medium refreshed twice. After 2 or 7 days, media was replaced with Krebs-Ringer Phosphate Glucose Buffer (KRPG) in the presence of 20μM DCFDA in the dark at 37°C and 5% CO_2. Next, the fluorescence was measured (Excitation 485, Emission 529) with Cytation Cell Imaging Multi-Mode Reader (Bio-Tek Instruments) for a total of 45min. Fluorescence intensity was normalized to the protein content.

Migration Assay

Mesenchymal stem cells were seeded in cell culture plate until confluence was reached. Migration assay started at 0 h followed by scratching a monolayer of cells with a pipette tip. Cells were washed twice with PBS and supplemented with control and osteogenic media with or without MK-7 (10μM final concentration) and left for 24 h. Gap closure was calculated in reference to timepoint = 0 using Image J [ImageJ 1.52q (64-bit)].

xCELLigence Proliferation Assay

To monitor continuous real-time proliferation XCELLigence system was used Cells were seeded in microelectrode plate control and osteogenic media with or without MK-7 (10μM final concentration) to measure impedance. Cell impedance was measured at 15min intervals up to 168 h. Electrical impedance was a measurement of cell number and recorded as Cell Index (CI). Cells were kept in 37°C and 5% CO_2 until the end of the experiment.

Statistical Analysis

Results are presented as replicates in three or more independent experiments ± standard deviation. Statistical analysis was performed using GraphPad Prism (v8.4.3, Prism 8 for macOS, GraphPad Software, USA). Two-tailed unpaired Student's t-test was used for comparisons between two groups, or a one-way analysis of variance with a post-hoc test of Tukey's analysis when more than two groups were compared. Statistical significance between two groups that did not display normal distribution was performed using Mann-Whitney U-test. Statistical significance denoted by $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$.

RESULTS

Characterization of iPSC-UkB-Ctrl-XX and Generation of iMSC-Ctrl-1

Following generation of iPSC-UkB-Ctrl-XX, cell line was characterized by immunofluorescence for pluripotency markers (Figure 1A), trilineage differentiation (Ecto-/endo-/mesoderm) and karyotyped as normal (data not shown). Following differentiation to mesenchymal lineage, cells were FACS sorted to check for markers associated to mesenchymal and hematopoietic stem cells (Figure 1B). CD73 was used as marker for mesenchymal stem cell and cells highly positive[28], whereas CD34 used as a marker for hematopoietic stem cells showed negative[29]. This gives us the confidence moving forward into osteogenic differentiations that what we are observing is a model for mesenchyme for osteoblast to osteocyte differentiation without possible mechanisms that involve osteoclasts.

Differentiation Potential of iMSCs

Using previously established protocols for primary cells we tested whether differentiation of iMSCs was possible into the canonical triad of expected mesenchymal differentiations as well as vasculogenic potential (Figure 2A). We found that upon differentiation iMSCs responded successfully to stimuli as verified by RT-qPCR for markers synonymous with the various progeny (Figure 2B). This proof of efficacy provides us with a definitive cell source capable for performing further studies into mesenchymal stem cell differentiations.

Variant Time Course of Osteogenic Differentiation

Next, we interrogated the mesenchymal osteogenic trajectory at a series of timepoints of osteoblast differentiation (Figure 2C). We investigated expression of a variety of genes associated with bone formation under osteogenic treatment (Osteo), osteogenic treatment with MK-7 supplementation (OsteoMK-7) and the respective controls (control and MK-7). These conditions were used to interrogate osteoblast differentiation at various stages considered early, mid and late differentiation, days 2, 10, and 21, respectively (Figure 2C). Runt-related transcription factor 2 (RUNX2) is a master transcription factor for early osteogenic differentiation. We measured expression at earliest time point and found RUNX2 to be significantly upregulated in OsteoMK-7 treatment compared to Osteo and both controls ($p < 0.005$, Figure 3A). BMP- 2 is suggested to play an important role in early bone formation and OCN (osteocalcin), is a vitamin K dependent protein (VKDP) present in mature osteoblasts. Therefore, we investigated whether our treatments change the expression of these markers. Expression of BMP-2 and OCN at day 2 did not vary between any treatments (Figures 3B,C). At mid-osteoblast differentiation (day 10), RUNX2 was further upregulated in osteogenic differentiation, although this was no longer in favour of OsteoMK-7 condition (Figure 3D). Further, at this timepoint we noticed an upregulation of COL1A1 expression under Osteo ($p < 0.0001$) and OsteoMK-7 ($p < 0.0001$) conditions, without favourability to OsteoMK-7 (Figure 3E). OCN expression did not differ in expression between any treatments at day 10 (Figure 3F). At late-osteoblast differentiation (day 21), both RUNX2 and COL1A1 have an even higher fold upregulation (~10-fold for both) compared to the controls then that of day 10 (Figures 3G,H). Also, at the late-osteoblast differentiation there is no difference in expression of either of the genes measured for OsteoMK-7 treatment. Furthermore, we did not observe any difference in OCN expression under any treatments compared to control (Figure 3I).

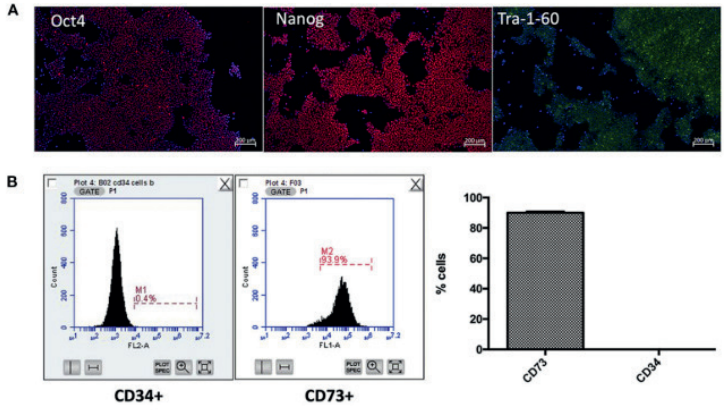

Figure 1. Characterization of iPSC-UkB-Ctrl-XX and generation of iMSC. Immunocytochemistry, (A) confirms pluripotency of cell line by expression of pluripotent markers Oct4, Nanog and Tra-1-60. (B) FACS sorting for CD73 and CD34 confirms that no cells are expressing hematopoietic stem cell marker CD34 and are positive for mesenchymal stem cell marker CD73

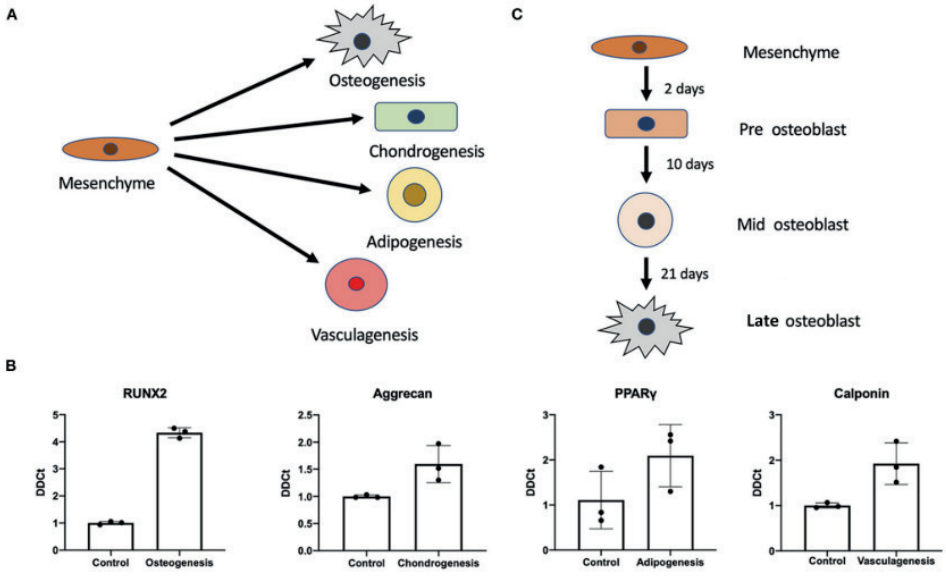

Figure 2. Mesenchymal stem cell differentiation potential. Differentiation potential of mesenchymal stem cells. (A) Visual representation of the different cellular iMSCs have potential to generate (osteogenic, chondrogenic, adipogenic, and vasculagenic lineages). RT-qPCR confirms differentiation of these four lineages by expression of commonly used markers RUNX2, aggrecan, PPARgamma, calponin (respectively) (B). Variant time course of osteoblastic differentiation that was interrogated to check time points for pre-osteoblast, mid-osteoblast and mature osteoblast phenotype (C).

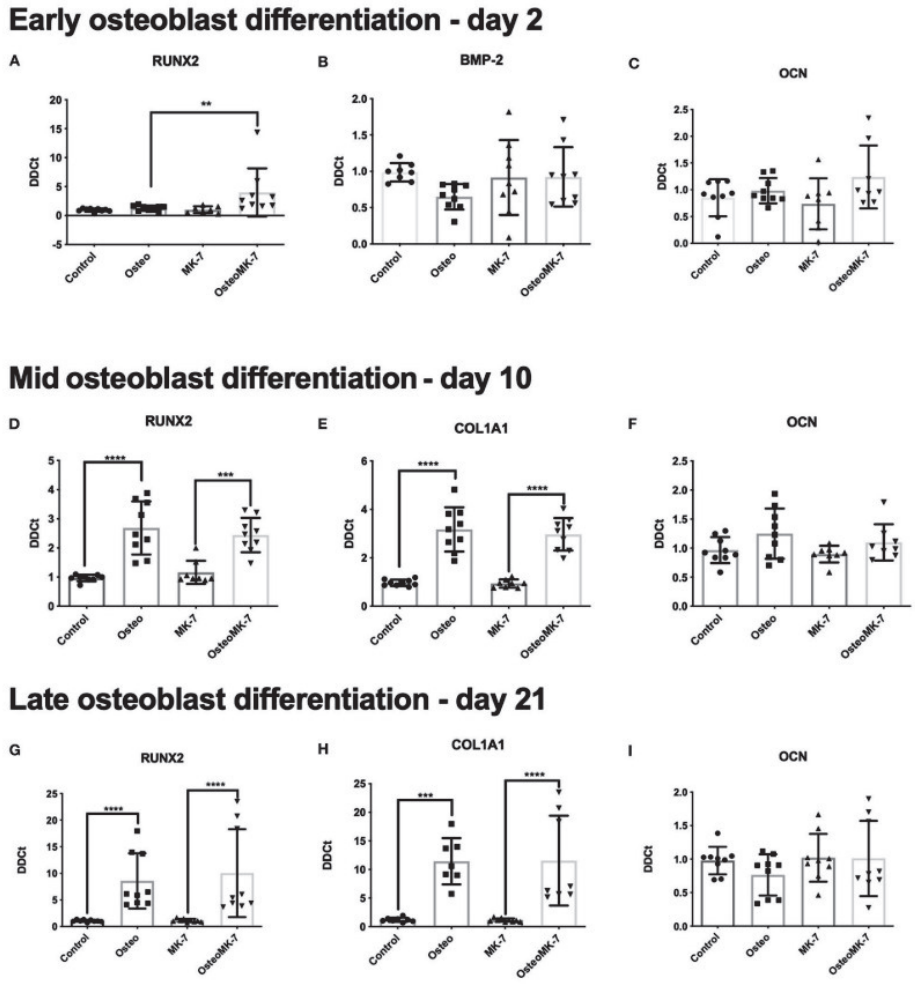

Figure 3. RT-qPCR expression of markers at various time points in differentiation. RT-qPCR of RUNX 2 (A,D,G) at various time points reveals its upregulation from controls as early as timepoint day 2 wherein RUNX2 is significantly upregulated under OsteoMK7 compared to controls and Osteo ($p = 0.0040$). At earliest timepoint BMP2 expression was not modulated between any treatments (B). COL1A1 (E,H) is significantly upregulated in osteogenic differentiation from day 10 ($p < 0.0001$). However, osteocalcin (C,F,I) has no differences in expression via any of the treatment over any time point in the differentiation. **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

Phenotype of Late Osteogenic Differentiation

RUNX2, COL1A1 and OCN are considered important players in the development and maturation of osteogenic processes. RUNX2 expression rises in early osteoblast phenotype, whereas high expression of COL1A1 and OCN are considered hallmarks of late-stage osteoblast differentiation. We investigated the cellular phenotype following 21 days of osteogenic differentiation. At the late osteogenic time point, western blot analysis reveals RUNX2 is significantly downregulated under OsteoMK-7 compared to Osteo and both controls ($p < 0.0001$, Figure 4A). Protein expression of COL1A1 reveals a significant upregulation under both Osteo and OsteoMK-7 conditions compared to their respective controls ($p < 0.001$, Figure 4B) but no difference was found between Osteo and OsteoMK-7. Expression of OCN confirm our transcriptional findings, as there is little to no expression of OCN in our cells under any condition (Figure 4C). Immunocytochemistry at day 21 points toward a different mode of COL1A1

expression between osteogenic and control treatments. Although the control samples show that nodules of iMSCs can resemble the osteogenic mode of COL1A1 (Figure 4D), image analysis between Osteo and OsteoMK-7 of fluorescence per cell reveals an upregulation of COL1A1 in OsteoMK-7 compared to Osteo. However, we found this not to be statistically significant ($p = 0.067$, data not shown). Next we determined the osteogenic phenotype of iMSCs.

Alkaline Phosphatase (ALP) activity was significantly upregulated in OsteoMK-7 compared to control and MK-7 ($p < 0.05$, Figure 4F), whereas no statistical difference was found between Osteo and controls. Additionally, both increased AR staining and calcium deposition (o-cresophthalein assay) was observed under osteogenic treatments compared to controls ($p < 0.0001$) for both Osteo and OsteoMK-7 (Figures 4E,G). There was no statistical difference between Osteo and OsteoMK-7 in ALP activity and calcium deposition (Figures 4F,G).

Effect of MK-7 on Migration, ROS and Cell Cycle Regulator P21

Since it is known in bone repair that following fracture and initial hematoma formation, osteoblastic or chondrocytic cells migrate to repopulate the fracture site before mineral deposition occurs[30]. Therefore, we determined whether cellular migration and proliferation was modulated following Osteo or OsteoMK-7 treatment. For cellular migration we used in vitro scratch wound assay. Scratch wound assay demonstrated that MK-7 increases gap closure compared to other conditions (Average closure—control 68.95%, osteo 51.97%, MK7 72.8%, OsteoMK7 58.34%, Figures 5A,B). Further, there was a statistical significance between control and Osteo on gap closure, however between MK-7 and OsteoMK-7 no such observation was present (Figures 5A,B). Next, we investigated the proliferative capacity of cells using the xCELLigence proliferation assay. Following the trend of the findings from scratch wound, both control and MK-7 have the greatest initial proliferation (Figure 5C). However, as the 7-day time frame progresses OsteoMK-7 proliferative capacity increases significantly beyond that of the other conditions, and significantly beyond that of Osteo ($p < 0.0196$). Given that ROS production has been implicated as a driving factor in cellular adhesion and migration, reduced wound healing and osteoporosis[31–33], we investigated ROS species production. Measuring intracellular oxidative stress using DCFDA probe revealed that OsteoMK-7 cells had a significantly reduced level of ROS production compared to Osteo at day 2 ($p<0.005$, Figures 5D,E).

Following 1 week of culture, OsteoMK-7 maintains reduced levels of ROS species, although this was found to be no longer statistically significant ($p < 0.091$, Figure 5C). Lastly, given that osteogenic phenotype is a terminal differentiation, cell cycle arrest of osteocytes will provide feedback between ROS production and cell cycle regulator P21. We found P21 expression to be significantly upregulated in osteo and OsteoMK-7 treatments compared to respective controls ($p < 0.05$, Figure 5F).

Changes to Dynamics of COL1A1 Expression From

From day 2, immunocytochemistry reveals changes to the mode by which COL1A1 is expressed. Image analysis reveals that not all iMSCs are expressing COL1A1 in any condition at day 2. There is a significantly greater total COL1A1 coverage in control samples at day 2 as quantified by percentage surface area coverage per cell and the product of area per cell IntDen per cell (Figures 6A,C). Similar to that of day 21, we note a differential mode by which COL1A1 is expressed. In control treatments, COL1A1 positive expression appears to be purely cytoplasmic, whereas in both osteogenic treatments, strands or fibers of COL1A1 are detected (Figure 6F). Additionally, RT-qPCR confirms a significant ($p < 0.05$) upregulation in COL1A1 expression under osteogenic conditions although no difference between Osteo and OsteoMK-7 was observed (Figure 6E). Using ImageJ analysis, we normalized thresholds equally amongst all images before reducing background, then converting to 8-bit black and white

photos which were used for particle analysis and area measurements. This reveals that there were a significantly greater number of particles per cell in osteogenic treatments compared to respective controls ($p > 0.0001$, Figure 6B). Although there was a greater number of particles in osteogenic treatments, we noted no difference in number of particles between Osteo and OsteoMK-7 (Figure 6B). However, when examining the area coverage of these particles (IntDen per cell), we found there to be a significantly greater coverage under OsteoMK-7 compared to Osteo ($p < 0.05$, Figure 6D).

DISCUSSION

In this study, we derived iMSCs using a simple low-glucose DMEM medium with l-ascorbic acid as previously described[27]. As this basic medium has no addition of cytokines, growth factors, or compounds we consider it to be cost-efficient. This was sufficient for generation of a population of CD73+/CD34− iMSCs. We demonstrated that these cells not only possess the plasticity as expected by primary MSCs, but can both proliferate and maintain this plasticity up to passage 20. We further showed that supplementation of osteogenic differentiation medium with MK-7 increased expression of osteogenic markers at both early and late timepoints of osteoblast differentiation. Additionally, we noted an improved osteoblast phenotype assessed by a variety of assays at early and late time points. The present study shows for the first time that MK-7 can influence the in vitro osteogenic differentiation of pluripotent stem cell derived MSCs.

Our first aim was to determine whether reproducibility of the most cost-efficient method for deriving iMSCs from iPSCs was robust. Following 2 weeks of over confluency, two passages on gelatine coated plates (0.1%w/v), and plastic adherence, confirmation of mesenchymal lineage by FACS analysis. We demonstrate iMSCs possess the plasticity as expected via the canonical differentiation triad, with an added vasculogenic phenotype.

We next aimed to decipher various stages of osteogenic differentiation. As our iMSC cell line was negative for hematopoietic markers, we are confident that all observations at various timepoints are via mesenchymal to osteoblast differentiation[34]. We hypothesize that any observations and understanding derived would be independent of osteoclast involvement in bone formation. Moreover, we interrogated the differentiation trajectory, given basic and clinical science reports on pro-osteogenic properties of MK-7, such as promoting bone formation, bone density and strength, as well as inhibiting bone loss[35]. Furthermore, vitamin K deficiency is associated with increased fracture rate and decreased bone mass density in a variety of patient cohorts[36]. Therefore, we determined whether MK-7 supplementation may further propagate an osteogenic phenotype of iMSCs. We selected three timepoints based on previous reports to determine insight to early-, mid- and late osteoblast formation[7]. At early timepoint, master regulator of osteogenesis, RUNX2 was significantly upregulated in osteogenic medium with MK-7, an observation that hasn't been reported before, and one suggesting that MK-7 might drive early osteogenic differentiation. Additionally, we did not note a difference in expression of BMP2 or OCN at this time point, confirming what has been reported by others[37]. This suggests to a potentially unknown mechanism by which MK-7 might drive the early stages of osteogenic differentiation from the iMSC intermediate. At both the mid- and late-osteoblast differentiation stages we observed an upregulation of RUNX2 and COL1A1 as expected, although there is no further benefit of MK-7 activity at this stage.

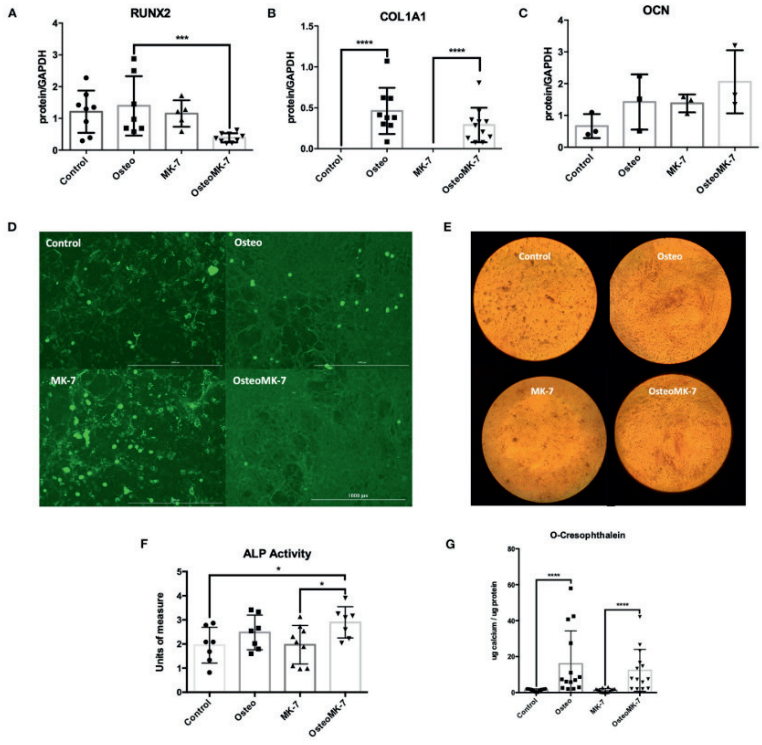

Figure 4. Protein expression at late osteoblast differentiation. Western blotting following 21 days differentiation for RUNX2 shows that following 21 days osteogenic treatment with MK-7 that RUNX2 is significantly downregulated compared to other samples (A) ($p = 0.0003$). Collagen 1A1 is upregulated in osteogenic conditions without any difference compared to MK-7 supplementation and OCN has no difference in expression (B,C) ($p \leq 0.0001$). Immunocytochemistry staining for COL1A1 shows that a completely different mode of collagen deposition has occurred following osteogenic treatment, although there are nodules present in the control and control MK-7 treated cells that resemble COL1A1 osteogenic deposition (D). Representative photos of the cells stained with Alizarin Red at day 21 (magnification 10x) (E). At day 21 control, osteogenic and MK-7 treated groups show no difference in ALP levels. Only osteogenic media supplemented with MK-7 was able to induce significant upregulation in ALP activity (F) ($p = 0.0418$). Quantification of calcium deposition by o-cresophthalein assay at day 21 (G). *p < 0.05, ***p < 0.001, ****p

Expression of OCN maintains itself as unaffected by either osteogenic or MK-7 stimulation at these stages. This is contrary to reports that used MC3T3-E1 cell line and primary BM-MSCs wherein an upregulation in expression of OCN has been reported[38]. It is plausible that OCN expression is different in iPSC derived cells than in cell lines or primary MSCs, and that changes to the carboxylation status of OCN might in part explain why we do not note differences in OCN expression. It has been reported that RUNX2 is downregulated and possibly even not expressed in mature osteoblasts[39]. Our finding corroborates as to a more mature phenotype as protein expression of RUNX2 was significantly downregulated in MK-7 supplemented osteogenic medium. We note little to no protein expression of OCN in any conditions, suggesting that OCN expression is not modulated by MK-7 in osteoblast differentiation.

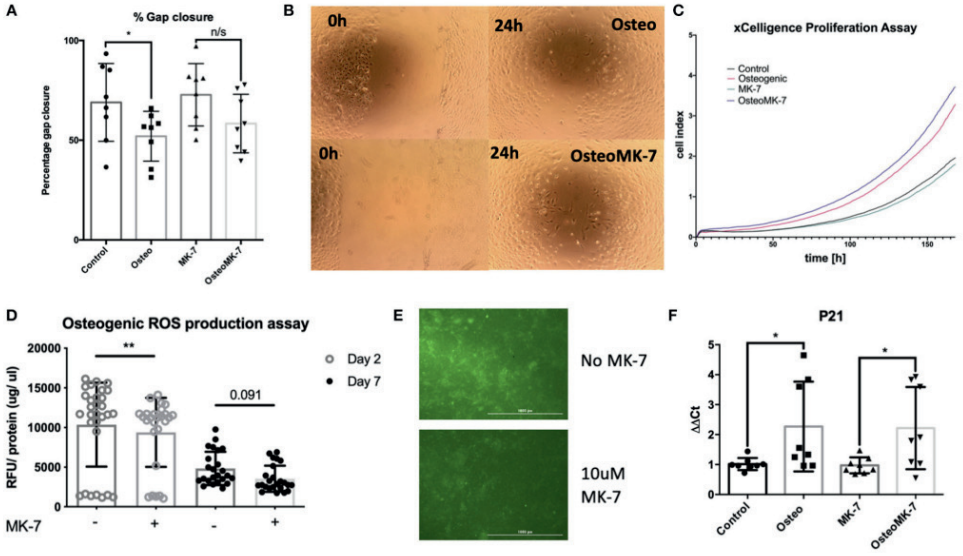

Figure 5. Activity of MK-7 on Migration, ROS and cell cycle regulator P21. Scratch wound assay reveals that MK-7 has the greatest gap closure followed by control, then OsteoMK7 and Osteo last (A). While the difference between osteogenic medium and control alone is statistically significant ($p = 0.0499$), there is less of a difference in gap closure between OsteoMK-7 and MK-7 (B). xCELLigence proliferation assay reveals that OsteoMK-7 has the greatest increases in proliferation over 1 week compared to the other conditions (C). OsteoMK-7 had significantly higher proliferation ($p < 0.0196$) compared to Osteo alone. Further MK-7 supplementation significantly reduces the production of ROS species under osteogenic conditions compared to the osteogenic control (D,E) ($p = 0.0047$). Wherein by day 7 of treatment the differences are no statistically significant but remain (D) also shown by visual representation of the DCFDA staining between the two different conditions (E). Further at day 2 we note by RT-qPCR analysis, a significant upregulation of cell cycle regulator P21 under both osteogenic conditions compared to controls (F) ($p = 0.0379$, $p = 0.0499$). *$p < 0.05$, **$p < 0.01$.

Furthermore, significant upregulation of ALP activity and calcium deposition suggests a more mature osteoblast phenotype. No difference for MK-7 supplementation compared to osteogenic medium was found in calcification propensity, which might be due to the excess calcium ions in cell culture medium present for in vitro hydroxyapatite formation. Collagen is the major collagen constituent of bone. Here, we note a different mode of COL1A1 expression, with strands or fibers of collagen present in totality over the osteogenic differentiation. In controls, COL1A1 appears to be present in nodules wherein a cluster of iMSCs have possibly spontaneously osteogenically differentiated[40]. We show a greater surface area of osteogenic differentiation with MK-7 although not statistically significant. Despite there being no increased in vitro calcification on adding MK-7, our findings suggest a more mature osteogenic cellular phenotype by downregulation of RUNX2, increased collagen deposition, and increased ALP activity. Since upregulation of RUNX2 is indicative of an early pro-osteogenic event by MK-7 activity, we investigated the potential of wound healing. MK-7 supplementation increased gap closure and increased proliferation compared to osteogenic medium alone. It is widely accepted that oxidative stress accelerates the rate of bone loss and is one of the risk and pathogenic factors in osteoporosis[41–43]. Our group has previously noted increases in production of ROS species to be associated with ectopic vascular calcification in vitro[44]. Intriguingly, ROS in osteogenic iMSCs were significantly downregulated by MK-7 treatment, which is in concordance with literature wherein antioxidants decreased oxidative stress in osteoblasts[45]. This suggest that MK-7 counteracts oxidative stress in developing bone *in vitro* and should be further explored for its antioxidative properties. As

we note increases in cell number with MK-7 supplementation, nodularity of conditions inducing osteogenic events, and given that ossification is considered a terminal differentiation process, we analyzed expression of cell cycle regulator P21. Increases in P21 expression are typically associated with transcriptional regulation of cell cycle arrest[46]. In both osteogenic conditions P21 is significantly upregulated, however increases in proliferation under OsteoMK-7 has been noted. This suggests there might be an overriding regulatory mechanism induced by MK-7. Our data suggests that MK-7 decreases ROS production while increasing cellular migration, proliferation, and potentially overriding cell cycle regulation by P21 during osteogenesis, providing novel insights into the activity of MK-7 on osteogenesis. Differences in collagen expression amongst samples were apparent and detectable from as early as day 2. Under osteogenic conditions many of the cells appear to have COL1A1 positive fibers, an observation that is completely absent in controls. Furthermore, at this time point the dynamics by which extracellular COL1A1 is expressed is different in osteogenic treatment and MK-7 increases COL1A1. Combined, our data reveal novel insights into the mesenchymal osteogenic differentiation as well evidence for use and efficacy of MK-7 in promoting an osteogenic cellular phenotype. We did not find OCN expression modulated at any time point. Additionally, in the early timepoint we note no change to BMP-2 activity, which contradicts with literature[37]. This suggests to additional non-canonical roles of MK-7 in pluripotent mesenchymal differentiation, that remains elusive from our findings. Furthermore, we purposefully chose to use iMSCs between passages 10-20 to determine whether previously noted increased passaging capabilities were also reproducible in our model. Given that primary mesenchymal stem cells typically senesce by passage 8, this further advocates for use of iMSCs as a more practical and scalable cellular resource[47].

We note that there are particular limitations to this study. Firstly, our findings have only been validated in vitro. It is entirely plausible that the benefit to adding MK-7 is a cell culture phenomenon wherein decreasing cellular stress under high phosphate conditions, for increased cellular proliferation and differentiation. Additionally, we note no differences in calcium deposition, despite increased ALP activity. As we did not vary calcium concentrations this could justify why we do not find differences in calcification with MK-7 supplementation. This study provides a framework for future studies, both *in vitro* and *in vivo* for the role of MK-7 in bone formation and fracture healing. This corroborates observations, from longitudinal and interventional trials on increased bone mineral density following either vitamin K2 supplementation[35] or an increased amount of vitamin K2 in the diet[36]. Furthermore, the role of VKDPs in iPSC derived products has not been widely explored. Additionally, our data suggest non-canonical function of VKDPs in early differentiation events, that can be modeled using iPSCs. Lastly, the increasing osteogenic cellular phenotype might prove beneficial toward development of tissue engineering solutions such as ex vivo bone grafts. In turn, development of biological products over ceramic or metallic based solutions could be more advantageous over artificial products. There is much we do not know about differentiation processes and extrahepatic activity of vitamin K2 analogous. This paper provides a first account into several mechanisms of osteogenesis from iPSCs and the influence of MK-7 on osteogenic processes.

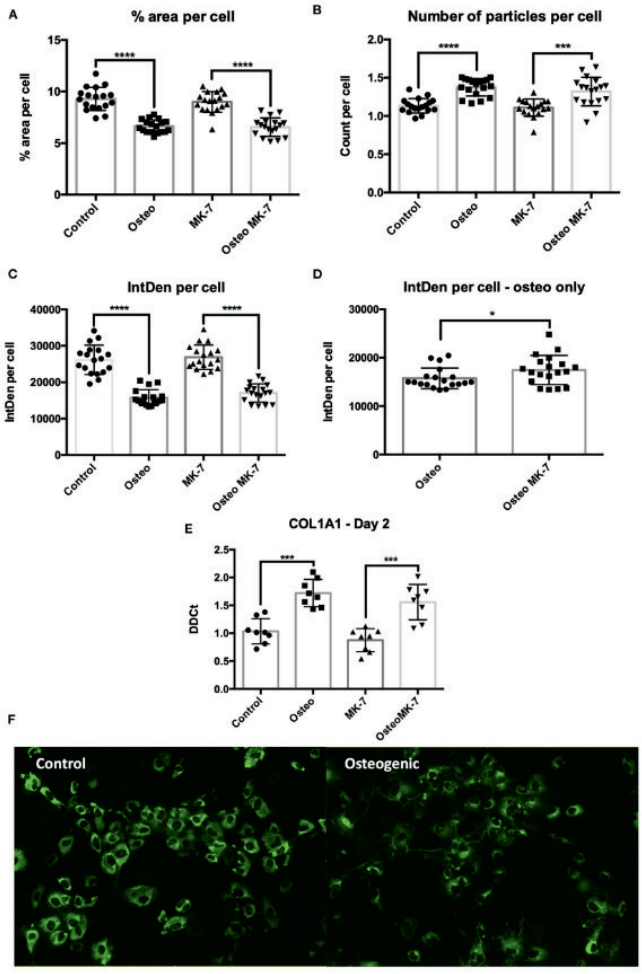

Figure 6. Changes to dynamics of COL1A1 expression. Immunocytochemistry followed by ImageJ analysis reveals that there is a greater surface of COL1A1 expression in control mesenchymal stem cells compared to their respective controls (A,C). The dynamics by which COL1A1 is expressed can be quantified with regards to number of particles expressing COL1A1, wherein there is a significantly greater number of particles, averaging of a smaller size in both osteogenic treatments compared to their respective controls (B,E). When osteogenic treatment is compared to osteogenic treatment with MK-7 supplementation. MK-7 supplementation upregulates the total IntDen (product of area and mean gray values) compared to just osteogenic treatment (D). RT-qPCR confirms significant upregulation of COL1A1 expression at day 2 compared to controls ($p < 0.05$) (E). Representative photos of collagen deposition under control and osteogenic conditions at day 2 (F). *$p < 0.05$, ***$p < 0.001$, ****$p < 0.0001$.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at:
https://www.frontiersin.org/articles/10.3389/fcell.2020. 618760/full#supplementary

Gene	
RUNX2	QIAGEN, QT00020517
BMP-2	QIAGEN, QT00012544
OCN	F: GGCAGCGAGGTAGTGAAGAG R: CGATAGGCCTCCTGAAAGC
COL1A1	F: TGTGGCCCAGAAGAACTGGTACAT R: ACTGGAATCCATCGGTCATGCTCT
GAPDH	F: AACGGATTTGGTCGTATTGGGC R: CTTGACGGTGCCATGGAATTTG
P21	F: GCAGACCAGCATGACAGATTTC R: GCGGATTAGGGCTTCCTCTT

Supplementary table 1. qPCR Primers

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CHAPTER

5

Calcification of vascular smooth muscle cells and chondrocytes – the inhibitory role of menaquinone-7 and calcium channel blockers

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Abstract

Background: Vascular calcification (VC) is now recognised as one of the main pathological hallmarks of cardiovascular disease. Phenotypic switching of vascular smooth muscle cells (VSMCs) plays a crucial role in initiating VC and involves transdifferentiation towards synthetic and chondrocyte-like VSMCs. VC resembles physiological bone mineralisation in which extracellular vesicle (EV) release plays a key role. Our aim was to investigate how different VSMCs phenotypes respond to calcifying media and if this calcification process could be blocked by calcium channel blockers and menaquinone-7 (MK-7).

Methods: VSMCs were differentiated into contractile, synthetic and chondrocyte-like cells and the calcification potential was compared to articular chondrocytes.

Results: After 48h of exposure to calcium and calcium-phosphate-rich media, we noted significantly more calcification and EV release in synthetic and chondrocyte-like VSMCs, as compared to contractile VSMCs. Contractile VSMCs calcified only in calcium-phosphate rich media without the notice of EV release. We observed no calcification development in articular chondrocytes under any of the calcifying conditions. Calcifying conditions increased calcium influx and reactive oxygen species production, these phenomena were less present in articular chondrocytes. Calcification of synthetic VSMCs could be ameliorated by supplementation of MK-7. Further, calcification of both synthetic and chondrocyte-like VSMCs could be blocked by the L-type voltage-dependent calcium channels (VDCCs) inhibitors Gabapentin and Amlodipine.

Conclusions: Calcification driven by synthetic and chondrocytic-like VSMCs depends on oxidative stress and L-type VDCCs. Our findings suggest new strategies to treat VC pharmacologically.

Introduction

Pathological calcification is the process of undesired calcium deposition in soft tissue[1]. Hydroxyapatite (HA) is the most stable calcium-mineral mediating physiological calcification and can be found in bones and teeth[2]. One of the most detrimental variants of ectopic calcification is vascular calcification (VC), which progresses with age. It has been now recognized as an orchestrated sequence of events contributing to vascular pathology and an independent predictor of cardiovascular mortality and morbidity[3]. Vascular smooth muscle cells (VSMCs) are found abundantly in the tunica media of the vasculature, maintaining vascular tone and integrity. Upon injury, VSMCs are the main drivers of VC.

VC can be found in two respective forms. Firstly, intimal calcification, defined as the pathological build-up of calcification in atherosclerotic plaques, associated with atherosclerotic burden and increased risk of rupture. Intimal calcification is associated with inflammation and impaired lipid metabolism, together creating the perfect storm to develop cardiovascular disease (CVD)[4]. Secondly, VC can be found as medial calcification, also known as Mönckeberg's sclerosis. Medial calcification is often found in patients suffering from chronic kidney disease (CKD) and diabetes mellitus type II patients, but also in the ageing population[5-7]. CKD patients are characterized by dysregulated mineral metabolism and display increased levels of circulating phosphate, which contributes to the progression of VC[8-10]. In CKD patients, VC correlates with premature mortality, resulting in a reduced life expectancy[11]. In such an unfavourable environment, VSMCs lose their functional contractile phenotype[12]. In response to injury, VSMCs undergo phenotypic switching to synthetic and chondrocyte-like VSMCs which correlates with the development of VC[13] and atherosclerosis[14]. *In vitro*, synthetic VSMCs are characterized by increased generation of oxidative stress and extracellular vesicle (EV) release resulting in the initiation and progression of calcification[15]. Prolonged exposure of VSMCs to elevated levels of phosphate alters phenotype from contractile to chondrocyte-like VSMCs[16]. Chondrocyte-like VSMCs express cartilage and bone-specific genes, including sRY-Box transcription factor 9 (SOX9), runt-related transcriptional factor 2 (RUNX2), bone-morphogenic-protein-2 (BMP-2), proteoglycan 4 (PRG4) and osteocalcin (OC)[17-19]. Additionally, histological analysis of calcified aortas of end-stage CKD patients reveals a positive presence of several cartilage and bone-associated proteins including RUNX2, Collagen1A1 and BMP-2[20]. VSMC phenotypic alterations occur concurrently with EV release, widely recognized as VC mediators[3]. Non-calcifying EVs release by articular chondrocytes has been identified, suggesting their role is not exclusively limited to matrix mineralization[21]. This suggests that articular chondrocytes and chondrocytes-like VSMCs do not possess equal mineralization capabilities.

VC treatment is impeded due to its multifactorial origin, hence the current therapies are unable to prevent or hold the progression of VC. Targeting VC is further impaired because of its late diagnosis, due to the lack of sensitivity of imaging modalities. Several strategies aiming to stop the progression of VC are being explored. One promising target are voltage-dependent calcium channels (VDCCs), which facilitate calcium entry into VSMCs and chondrocytes contributing to ectopic calcification[15,22]. Amlodipine and Verapamil, two VDCCs L-type inhibitors are currently utilized in clinical trials for the treatment of hypertension, whereas Gabapentin is largely unexplored for its effect in the vasculature[23,24]. Verapamil has been shown to inhibit VC *in vitro* and reduce the calcifying potential of EVs[25]. Amlodipine is known to reduce medial elastocalcinosis *in vivo*[26]. Another intervention that is currently explored to treat VC is vitamin K supplementation. Low vitamin K status is associated with coronary artery calcification and cardiovascular events in both cardiovascular patients and the elderly population[27,28]. Furthermore, vitamin K supplementation has been used to hold the progression of atherosclerosis[29] and aortic valve calcification[30]. Vitamin K functions as a cofactor for the vitamin K-dependent matrix gla-protein (MGP), a potent inhibitor of VC[31]. The non-canonical role of vitamin K as an antioxidative agent has also been described, expanding the function of vitamin K in the

vasculature[32,33]. Recently, *in vitro* treatment of VSMCs with the vitamin K-derivative MK-7 (menaquinone-7) showed reduced VC, by targeting EV release and oxidative stress in a nicotine-induced model of calcification[34].

In this paper, we investigated the molecular pathways regulating phenotypic switching of VSMCs and their subsequent calcification potential. Also, we compared the calcification potential of chondrocyte-like VSMCs with articular chondrocytes. Finally, we tested different VDCC inhibitors and MK-7 in vitro to inhibit VSMC calcification, with the aim to present potential future clinical agents to alleviate VC.

Materials and Methods

Human Articular Chondrocytes and VSMCs isolation

Human articular chondrocytes were isolated from the osteoarthritic patients undergoing arthroplasty as described before[35]. Successful isolation of articular chondrocytes was confirmed by immunocytochemical characterization for positive expression of Collagen type-2A1 and -10A1. Culture medium consisted of DMEM/F12 (Invitrogen, #11320033), 10% FBS (Gibco, #12340030), penicillin/streptomycin (Gibco #15140148, 100 U/ml and 100 mg/ml respectively) and 1% non-essential amino acids (NEAA; Invitrogen, #11140050). Cells between passages 1-4 were used for experiments. VSMCs were isolated from aneurysm patients undergoing surgery. Briefly, VSMCs were cultured in M199 (Gibco, #12340030) supplemented with 20% FBS (Gibco #10270106, penicillin/streptomycin (Gibco #15140148, 100 U/ml and 100 mg/ml respectively) and Amphotericin B (Gibco, #15290018, 25ug/ml) on a 6 well plate (Costar #CLS3516) and were left until cells migrated from the tissue. All cells were cultured in a humidified atmosphere at 37°C, 5% CO2. Successful isolation of VSMCs explants was confirmed by immunocytochemical characterization for positive expression of alpha-smooth muscle actin (α-SMA), calponin (CNN), smooth muscle protein 22-alpha (SM22α), phosphorylated myosin light chain 2 (p-MLC) and absence of S100 C Calcium Binding Protein 4 (S100A4) (data not shown).

VSMC differentiation

Differentiation medium was as follows; M199 media (Gibco, #12340030) supplemented with 10% FBS (Gibco #10270106), penicillin/streptomycin (Gibco #15140148, 100 U/ml and 100 mg/ml. VSMCs were seeded at density of 10.000 cells/cm^2 on the same plate and treated with either, 200 IU/ml Heparin (LeoPharma #01004003), 20 ng/ml Platelet Derived Growth Factor-BB (PDGF-BB, PeproTech #10014B) or 2.6 mM inorganic phosphate (NaH_2PO_4/Na_2HPO_4, Sigma #S0751 and #1065861000) to obtain contractile, synthetic and chondrocyte-like VSMCs, respectively[15,16,36]. The medium was refreshed every other day.

Calcification assays

Cells were seeded at a density of 10.000 for VSMCs and 30.000/cm^2 for articular chondrocytes. Two different methods of calcification induction were used: high calcium (+3.6 mM Ca) or high calcium and phosphate (+0.8/1.0 mM Ca/P) in the presence of 0.5% FBS (Gibco #10270106) including control media (control media concentrations are: 1.8 mM Ca/1.0 mM P) for 48h. For calcification inhibition studies, Gabapentin (30 µM) (Sigma, #G154), Verapamil (30µM) (Sigma, #V4629), Amlodipine (30µM) (Sigma, #A5606) or MK-7 (50µM) (kind gift, Nattopharma, Oslo, Norway) were added to the calcifying media. Quantification of deposited calcium was performed using the o-Cresolphthalein method (Randox, #CA8309) according to the manufacturer's instructions. Briefly, cells were washed twice with

PBS and calcium deposits were solubilized in 0.1 M HCl and normalized to protein concentration using micro-BCA Protein Assay (Thermo Scientific, #5000111). Calcification was additionally visualized using Alizarin Red S staining. In the MK-7 inhibitory assay, fluorescent-labeled Fetuin-A Alexa Fluor-546 was added at the start of the experiment to visualize mineral deposits as described before[37].

Nano-particle tracking analysis

EVs analysis was performed using a nanoparticle tracker (ZetaView, Particle Matrix, USA) in scatter mode. Optimal scanning conditions were established using previously described protocols[38]. Briefly, media was aspirated from cells and spun at 500 g for 5 minutes. Further, calibration beads and cell supernatants were diluted in PBS to a final volume of 1 ml. Technical duplicates were measured for each sample including a washing step with distilled water in between measurements.

Alizarin Red staining

Cells were washed twice with PBS and fixed in 4% Paraformaldehyde (PFA Merck #1.04005.1000) for 15 minutes at room temperature. Next, cells were washed twice with PBS and incubated in 40 mM Alizarin Red solution pH 4.2 (Sigma, #A5533) for 60 minutes then washed twice with PBS and visualised using light microscopy.

Immunofluorescence

Briefly, cells were washed twice with PBS, fixed in 4% PFA (Merck #1.04005.1000) and permeabilised with (2% BSA and 0.1% Triton X-100 (Sigma #A3059 and Biorad #1610407 respectively)) for 1h at room temperature. Next, cells were incubated with the following primary antibodies COL2A1 (Southern Biotech #1320-01), alpha-smooth muscle actin (α-SMA, Sigma #122437), smooth muscle protein 22-alpha (SM22α, Abcam #14106), phosphorylated myosin light chain 2 (pMLC, Dako #124PA2), COL10A1 (Calbiochem, #234196), COL2a1 (Southern Biotech, 1320-01) and calponin (CNN, Sigma #23777) overnight at 4°C. The next day, cells were incubated with secondary antibodies: anti-rabbit-FITC (Dako, F0205), anti-goat-FITC (Dako, F23334) and anti-mouse-FITC (Dako, F0232). Nuclei were stained with 0.5 µg/ml DAPI (Sigma, #D9542). Fluorescent signal was analysed using Cytation 3 (BioTek).

RNA extraction and RT-qPCR

Total RNA from cells was isolated using TRIzol reagent (Invitrogen, #15596026) and quantified using a spectrophotometer (NanoDrop 2000, Thermo Scientific). 1 µg of total RNA was transcribed using iScript Reverse Transcription Supermix (Biorad, #1708840) to cDNA in a 20 ul reaction. Real-time qPCR was performed using the Quantitect SYBR green PCR kit (Qiagen, #204143) in a LightCycler 480 II (Roche) with 5 ng of cDNA and 0.5 µM of each primer. Fluorescence curves were analysed with LightCycler 480 Sofware (Version 1.5) and relative quantification was performed with the $2^{-\Delta\Delta Ct}$ method. Relative expression for each gene was normalised against GAPDH and expressed as fold change over control data from at least 3 different differentiations and reported as mean ± SD.

Intracellular reactive oxygen species (ROS) detection

To measure oxidative stress, we measured ROS using 2,7–dichlorofluorscein diacetate (DCFDA, Merck, #287810) which is oxidised to non-permeant fluorescent 2,7–dichlorofluoroscein (DCF) in the presence of oxidants. In brief, cell media were incubated with 20 µM DCFDA in Krebs-Ringer

Phosphate Glucose Buffer (KRPG) kept in the dark at 37° C and 5% CO_2. Next, fluorescence was measured (Excitation 485, Emission 529) in the Cytation 3 (Biotek) for a total of 40 minutes. Fluorescence intensity was normalised to cell count.

Micro BCA protein assay

Micro BCA protein assay was performed to normalise for protein amount. In brief, 0.1 M HCl cell suspension (used in calcification assay) was neutralized with 0.1 M NaOH, 0.2% SDS (Sigma #06203 and Biorad #1610416 respectively) and incubated on a shaker for 2h. Protein concentration was measured at 750nm using Cytation 3 (Biotek). All samples were assayed in duplicate in 3-4 independent experiments.

Statistical analysis

Data are shown as mean ± SD and were obtained in three or more independent experiments. Statistical significance between the groups was calculated using Mann-Whitney, Kruskall-Wallis with Dunn's and Tukey's multiple comparisons tests were used. Statistical analysis was carried out using GraphPad Prism 8.2.0. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Results

Phenotypic characterization of VSMCs and articular chondrocytes

VSMCs were characterized by immunofluorescence for contractility (Figure 1A) and articular chondrocytes for collagen-specific markers (Figure 1B). We found that both αSMA and CNN showed significantly lower fluorescence intensity in synthetic and chondrocyte-like VSMCs (Figure 1C, 1D; $p < 0.001$). Moreover, S100A4 was found to be significantly upregulated in both synthetic and chondrocyte-like VSMCs (Figure 1E; $p < 0.01$). Additionally, we detected both collagen type-2A1 and type-10A1 in articular chondrocytes (Figure 1B).

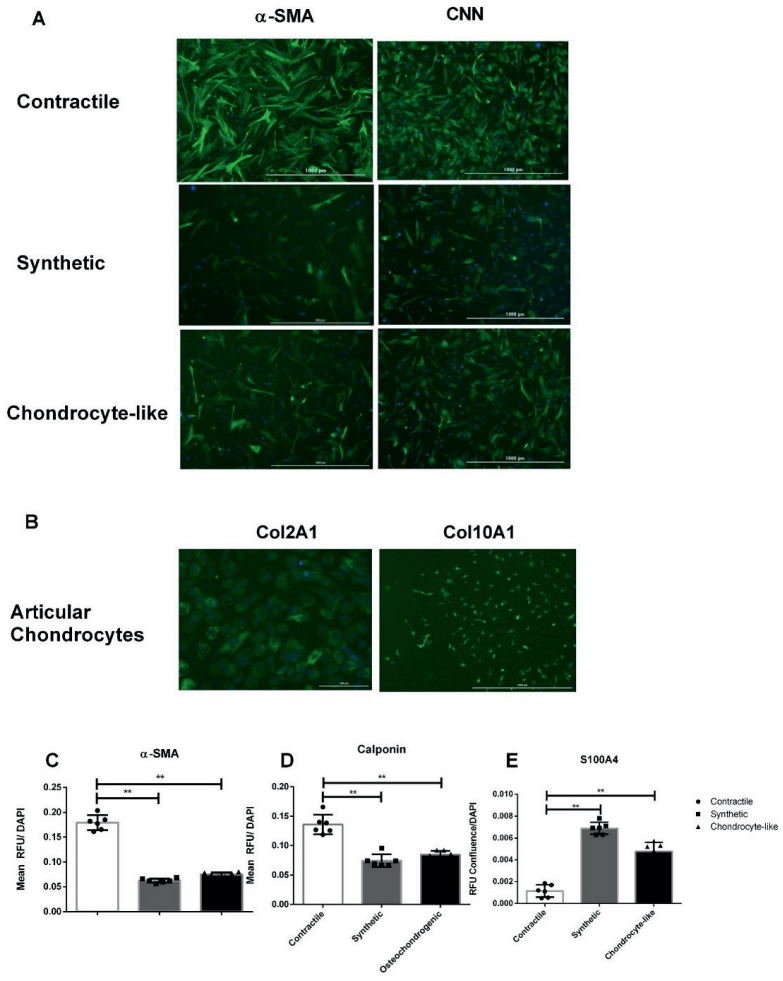

Figure 1. Phenotypic changes of vascular smooth muscle cells (VSMCs) is associated with alterations in contractile markers expression. Expression of collagen markers in articular chondrocytes. A, Immunocytochemical staining of contractile proteins in contractile, synthetic and chondrocyte-like VSMCs showing the differences in alpha-smooth muscle actin (α-sma) and calponin (CNN) expression. Scale bars: 100μM. Representative image taken from 3 independent experiments. **B**, Immunocytochemical stainings of collagen proteins (collagen2A1 and 10A1) confirming its presence in articular chondrocytes. **C, D**, Quantitative analysis of α-sma and CNN markers followed by VSMC phenotypic switching in synthetic and chondrocyte-like cells. **E,** Quantitative analysis of S100A4 marker followed by VSMC phenotypic switching in synthetic and chondrocyte-like cells.

In vitro calcification of vascular smooth muscle cells and articular chondrocytes

Next, we investigated the rate of calcification of different VSMCs phenotypes and articular chondrocytes. Contractile VSMCs and articular chondrocytes showed no apparent calcification when exposed to elevated extracellular calcium concentrations, whereas synthetic and chondrocyte-like VSMCs showed increased calcification (Figure 2A; $p < 0.05$ and $p < 0.01$, respectively). Chondrocyte-like VSMCs calcified more compared to articular chondrocytes (Figure 2A; $p < 0.001$). Calcifying medium containing both increased calcium and phosphate resulted in calcification of contractile, synthetic and chondrocyte-like VSMCs, but not of articular chondrocytes (Figure 2C; $p < 0.01$).

Similarly, chondrocyte-like VSMCs calcified significantly more than articular chondrocytes under increased calcium and phosphate conditions (Figure 2C; $p < 0.05$).

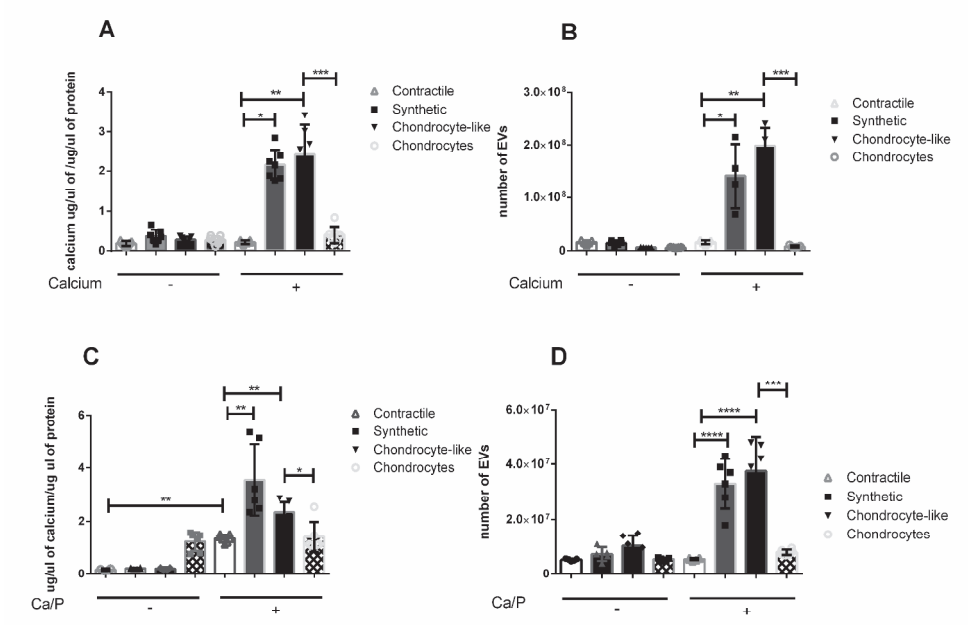

Figure 2. Calcification and extracellular vesicles (EVs) release of VSMCs and articular chondrocytes *in vitro. A,* overall levels of calcium deposition in contractile, synthetic, chondrocyte-like and articular chondrocytes. VSMCs were treated with +3.6mM Ca for 48 h. Calcification was observed in synthetic and chondrocyte-like cells but not in contractile phenotype and articular chondrocytes . **B**, overall levels of EVs release in contractile, synthetic, chondrocyte-like and articular chondrocytes. VSMCs were treated with +3.6mM Ca Ca for 48 h. EVs release was observed in synthetic and chondrocyte-like cells but not in contractile VSMCs neither articular chondrocytes. **C,** overall levels of calcium deposition in contractile, synthetic, chondrocyte-like VSMCs and articular chondrocytes. VSMCs were treated with (+0.8mM Ca, +1mM P) for 48 h. Calcification was observed in contractile, synthetic and chondrocyte-like VSMCs but not in articular chondrocytes. **D**, overall levels of EVs release in synthetic, chondrocyte-like and articular chondrocytes. VSMCs were treated with (+0.8mM Ca, +1mM P) for 48 h. EVs release was observed in synthetic and chondrocyte-like cells but not in contractile VSMCs, neither articular chondrocytes.

Increased calcium and phosphate triggers VSMC extracellular vesicle release

To investigate the mechanisms of increased calcification, we measured EV release of VSMCs and articular chondrocytes. NTA analysis demonstrated increased EVs release from synthetic and chondrocyte-like VSMCs under elevated calcium concentrations (Figure 2B; $p < 0.05$, $p < 0.01$ respectively). Chondrocyte-like cells also released more EVs than articular chondrocytes (Figure 2B; $p < 0.001$). Calcifying media containing both calcium and phosphate resulted in an increase in EVs in both synthetic and chondrocyte-like VSMCs (Figure 2D; $p < 0.01$). EV release was not altered by increased calcium and phosphate levels in contractile VSMCs and articular chondrocytes.

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Oxidative stress is increased in chondrocyte-like vascular smooth muscle cells and decreased in articular chondrocytes

Contractile, synthetic and chondrocyte-like VSMCs showed higher baseline oxidative stress compared to articular chondrocytes (Figure 3A; $p < 0.001$). Culturing cells for three days with increased phosphate levels, chondrocyte-like VSMCs showed a significant increase in ROS production compared to contractile VSMCs (Figure 3B; $p < 0.05$).

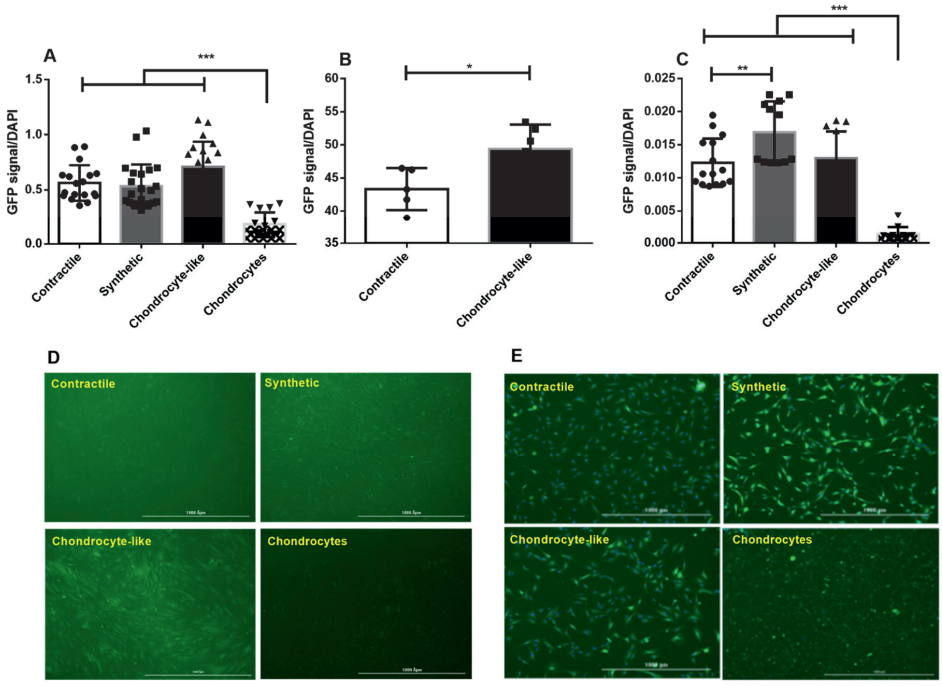

Figure 3. Reactive oxygen species (ROS) generation and calcium influx is increased during vascular smooth muscle cells (VSMCs) phenotypic switching. A, The level of oxidative stress contractile, synthetic, chondrocyte-like VSMCs and chondrocyte cells. No differences were found between VSMCs phenotypes however articular chondrocytes exhibited significantly less reactive oxygen species (ROS) than any of the VSMCs phenotypes. **B**, Chondrocyte-like VSMCs show increased ROS production in comparison to contractile phenotype at day 3 of the differentiation. **C**, Intracellular calcium influx is greater in synthetic VSMCs following phenotypic switching. A much lower calcium influx was detected in articular chondrocytes. **D,** Immunocytochemical images taken at the end of the differentiation of contractile, synthetic, chondrocyte-like VSMCs and articular chondrocytes. Cells were loaded with 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA) and fluorescence was measured as indicative of ROS. **E,** Fluorescent images of contractile, synthetic, chondrocyte-like VSMCs and articular chondrocytes followed by phenotypic switching. VSMCs were incubated with Fura-2-AM and excited at 340 and 380 nm followed by emission measurement. Cells were treated with normal extracellular Ca^{2+} after 1 min of live monitoring.

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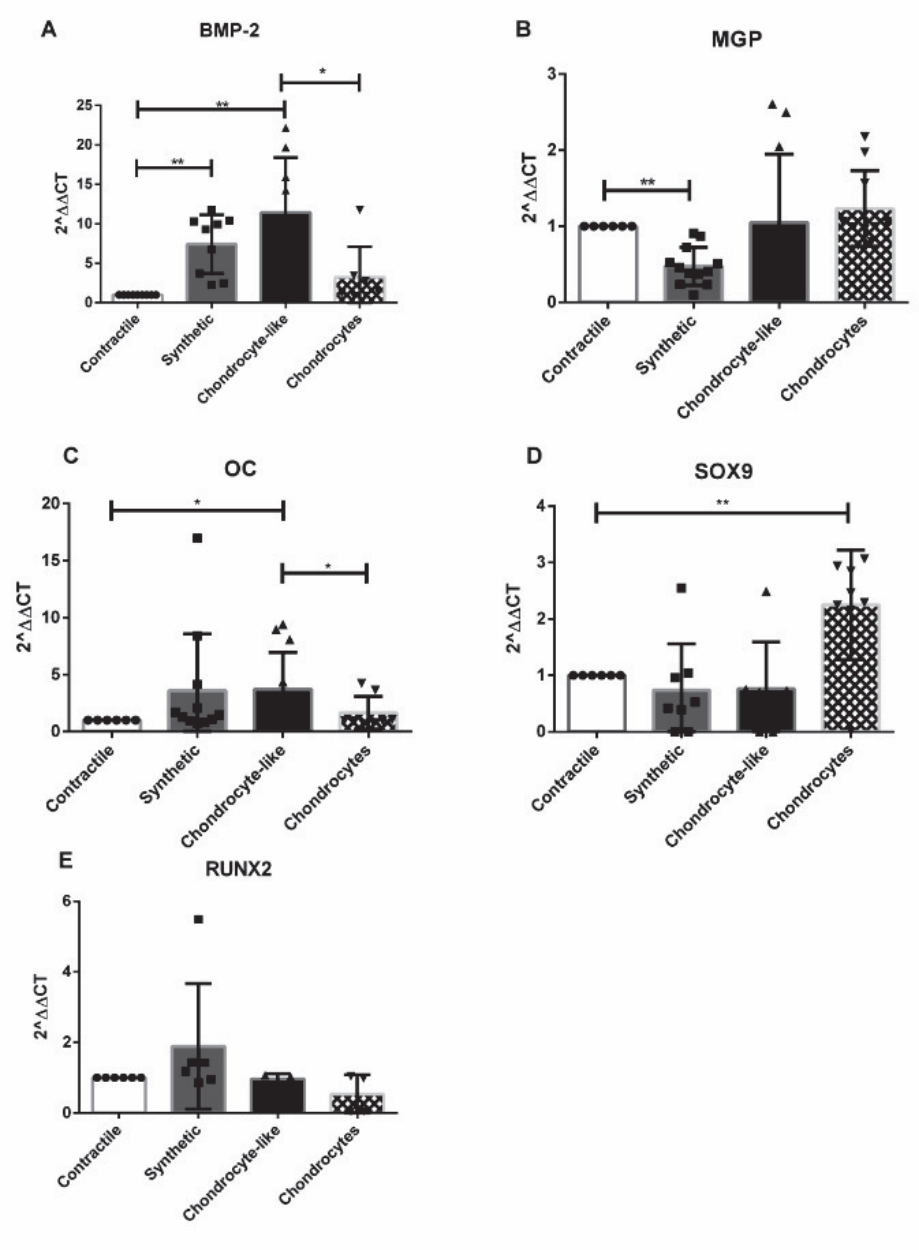

Figure 4. Gene expression analysis of contractile, synthetic, chondrocyte-like vascular smooth muscle cells (VSMCs) and articular chondrocytes. A, Bone morphogenic protein-2 (BMP-2) mRNA is upregulated in synthetic and chondrocyte-like VSMCs in comparison to contractile cells. Chondrocyte-like VSMCs express significantly more BMP-2 mRNA than articular chondrocytes. **B,** Matrix Gla protein (MGP) is downregulated in synthetic cells. **C,** Osteocalcin (OC) is upregulated in chondrocyte-like cells in comparison to contractile VSMCs. Chondrocyte-like VSMCs express significantly more OC mRNA than articular chondrocytes. **D,** SOX9 is upregulated in articular chondrocytes in comparison to contractile VSMCs. **E,** no differences were found in RUNX2 expression across VSMCs, neither in chondrocytes.

Intracellular Ca^{2+} levels are increased in synthetic vascular smooth muscle cells and decreased in articular chondrocytes.

Next, we measured calcium uptake in VSMCs and articular chondrocytes. We observed increased intracellular calcium in synthetic compared to contractile VSMCs (Figure 3C; $p < 0.01$). Articular chondrocytes revealed significantly lower baseline calcium uptake (Figure 3C; $p < 0.001$). No differences were found between contractile and chondrocyte-like VSMCs.

Differential comparison of bone and cartilage gene expression between vascular smooth muscle cell phenotypes and chondrocytes

BMP-2 mRNA expression levels were higher in synthetic and chondrocyte-like VSMCs compared to contractile VSMCs. Compared to chondrocyte-like VSMCs, articular chondrocytes showed decreased BMP-2 levels (Figure 4A; $p < 0.01$ and $p < 0.05$, respectively). Levels of MGP mRNA were found to be significantly decreased in synthetic compared to contractile VSMCs (Figure 4B; $p < 0.01$). OC was upregulated in chondrocyte-like VSMCs, yet significantly lower in articular chondrocytes (Figure 4C; $p < 0.05$). Moreover, we detected upregulation of SOX9 in articular chondrocytes compared to contractile VSMCs (Figure 4D; $p < 0.01$). We did not find any differences in Runx2 expression between the VSMC phenotypes and articular chondrocytes (Figure 4E).

Vitamin K2 inhibits calcification of synthetic but not chondrocyte-like VSMCs in a high calcium environment

Next, we investigated the role of MK-7 in the inhibition of VC *in vitro.* The concurrent addition of MK-7 (50 μM) under high calcium conditions (+3.6mM Ca) significantly reduced calcium deposition in synthetic VSMCs after 48h with no significant effect seen in chondrocyte-like VSMCs (Figure 5A; $p < 0.01$). We also observed a reduction of mineral deposits in treated VSMCs using fluorescent Fetuin-A Alexa Fluor-546 (Figure 5B).

Gabapentin and Amlodipine inhibit calcification of synthetic and chondrocyte-like vascular smooth muscle cells

Gabapentin and Amlodipine supplementation (30μM) in calcifying media containing both calcium and phosphate decreased calcification of synthetic and chondrocyte-like VSMCs after 48h (Figure 6A; $p < 0.001$), whereas Verapamil did not elicit any significant effect. Gabapentin and Amlodipine did not affect EV release (Figure 6B). We observed a large reduction of calcium deposits in cells treated with gabapentin (30μM) using Alizarin Red staining (Figure 6C).

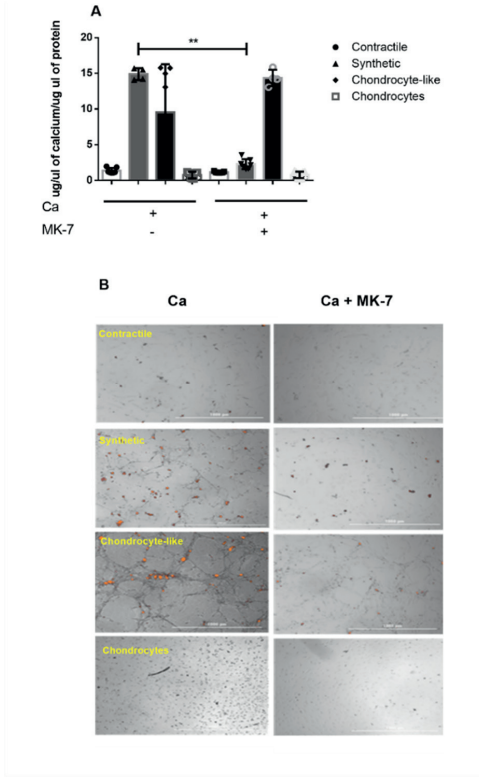

Figure 5. Vitamin K2 (MK-7) inhibits vascular calcification *in vitro*. A, vitamin K2 (MK-7) (50μM) inhibits calcification of synthetic vascular smooth muscle cells (VSMCs) in calcium rich media (+3.6mM Ca) after 48h. Vitamin K2 (MK-7) was added concurrently to calcifying media and calcium content was determined after 48h. **B**, Detection of calcified nodules in contractile, synthetic, chondrocyte like VSMCs and articular chondrocytes with and without MK-7. Mineral deposits were visualised using Fetuin-A labelled with fluorescent Alexa-546.

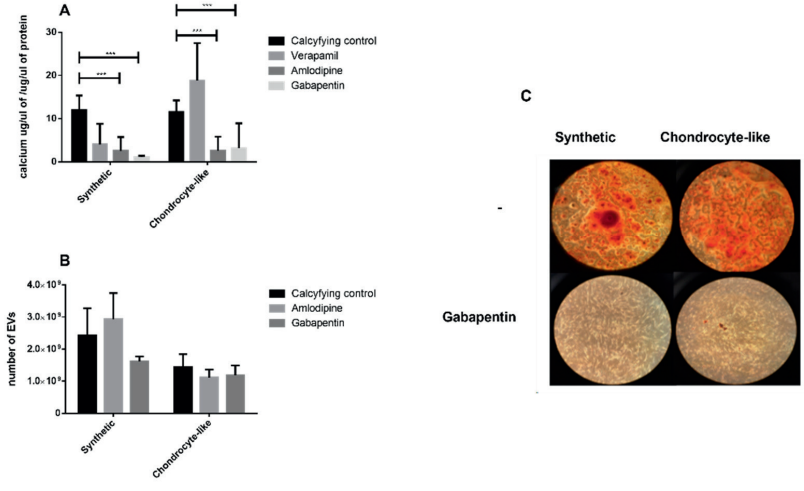

Figure 6. Gabapentin and Amlodipine inhibit vascular calcification *in vitro.* A, Gabapentin (30µM) and Amlodipine (30µM) significantly reduces the calcification of synthetic and chondrocyte-like vascular smooth muscle cells (VSMCs) in calcium and phosphate-rich media (+0.8mM Ca, +1mM P), whereas Verapamil has no effect after 48h of incubation. **B**, both Gabapentin and Amlodipine have no effect on reducing the extracellular vesicle (EVs) release under these calcifying conditions. **C**, visualization of mineral deposits with and without Gabapentin treatment (30µM) using Alizarin Red staining method.

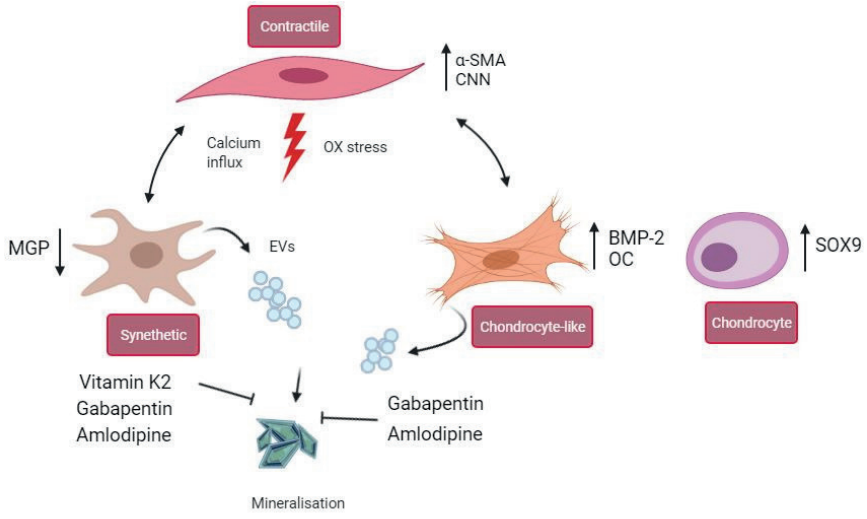

Figure 7. Overview of signaling pathways triggered by vascular smooth muscle cells (VSMCs) phenotypic switching including articular chondrocytes (AC) comparison. Healthy VSMCs express contractile markers including alpha-smooth muscle actin (α-sma) and calponin (CNN). This, followed by platelet-derived growth factor (PDGF-BB) stimulation, synthetic VSMCs, loose contractility markers and increase intracellular calcium influx. During chondrocyte-like differentiation in the presence of elevated phosphate, VSMCs generate reactive oxygen species (ROS) and upregulate chondrocyte-like and bone markers including bone-morphogenic protein-2 (BMP-2) and osteocalcin (OC). Articular chondrocytes express sRY-Box transcription factor 9 (SOX9) more abundantly than healthy VSMCs. Intracellular calcium entry and ROS generation are much lower in articular chondrocytes. Synthetic and chondrocyte-like cells calcify significantly more than contractile VSMCs followed by extracellular vesicle (EVs) release. Articular chondrocytes calcify and release much fewer EVs than chondrocyte-like VSMCs. Vitamin K2 (MK-7) reduces the level of vascular calcification (VC) in synthetic VSMCs. Amlodipine and Gabapentin reduce the level of VC in synthetic and chondrocyte-like VSCMs.

Discussion

In the present study, we show that VSMCs are more prone to calcification upon phenotypic switching, whereas contractile VSMCs and articular chondrocytes seem to be resistant. Also, increased calcium and phosphate, a condition seen in CKD patients, further increased the calcification potential of VSMCs. In this CKD setting, dysregulated calcium and phosphate balance has an impact on VSMCs, contributing to the development of medial calcification and atherosclerosis[39,40]. It has been shown that increased calcium levels induce EV release in synthetic VSMCs, resulting in increased calcification[41]. We show that intracellular calcium influx is increased leading to more calcification in synthetic VSMCs, confirming our previous results[15]. We noticed increased intracellular calcium influx in synthetic VSMCs, a phenomenon also observed in human glioblastoma cells, where the opening of

calcium channels in the plasma membrane has been shown to be directly proportional to the dose of PDGF-BB stimulation[42]. Orchestrating calcium influx and its corresponding intracellular signaling cascade is a highly adaptable mechanism, which varies among cells. Calcium entry in both chondrocytes and VSMCs is partly mediated via the calcium-sensing receptor (CaSR)[43,44]. In addition, VSMCs derived from aortic aneurysm showed a reduced expression of CaSR receptor compared to VSMCs of apparently healthy aorta[45]. VSMCs used in our experiments were also isolated from aneurysm tissue, likely explaining a greater calcium influx compared to articular chondrocytes, who are known to not express functional CaSR[46]. Chondrocytes can be considered less excitable cells than VSMCs, manifested by low levels of calcium influx[47], highlighting the significance of our data. Furthermore, external stimuli, including mechanic and compressive loading as well as hydrostatic pressure, promote intracellular calcium signaling in chondrocytes[48–50]. Thus, our cell culture conditions suggest that articular chondrocytes received insufficient force to stimulate calcium-sensing and subsequent uptake.

Physiological levels of ROS are required for maintenance of the contractile VSMC phenotype[51]. High levels of ROS are perilous and result in an increased inflammatory response linked to cardiovascular pathologies including CKD and atherosclerosis[52–54]. Chondrocyte-like VSMCs had increased ROS suggesting that oxidative stress might be an early event in phosphate-driven calcification progression. It has been shown that transient exposure to phosphate decreased the mitochondrial membrane potential, contributing to increased VC[55]. Similarly, phosphate stimulated VSMCs display increased ROS and inflammation[56]. Moreover, ROS are known inducers of calcification *in vitro* via mitochondrial free radicals[36,57]. ROS control the signalling and expression of BMP, particularly BMP-2[58]. BMP-2 is known to induce bone formation as well as VSMC calcification *in vitro*[59]. Furthermore, BMP-2 is known to exacerbate oxidative stress via activation of pro-inflammatory NADPH oxidase enzymes, subsequently inducing calcification[59,60]. The detailed mechanism underlying the differences in ROS generation by articular chondrocytes and contractile and synthetic VSMCs warrant further investigation. To the best of our knowledge, we present differences in ROS-driven calcification between VSMCs and articular chondrocytes.

To further elucidate the observed differences between VSMC and articular chondrocyte calcification, we examined EV release. EVs have been shown to be the nidus for VC *in vivo,* as well as disease mediators in atherosclerosis and hypertension[3,61,62]. EVs are the consequence of VSMC oxidative stress and are released into the extracellular matrix[63]. Indeed, oxidative stress is associated with EV release in VSMCs[36]. If not phagocytosed, these EVs are the starting point for mineralization[15]. Our data show that synthetic and chondrocyte-like VSMCs shed more EVs compared to contractile VSMCs, which is supported by literature[15,64]. Previously, contractile VSMCs have been shown to be inert to calcification[15], and that increased calcium exposure did not induce calcification. However, we did observe induced calcification of contractile VSMCs under high calcium-phosphate conditions, yet without increased EV release. This suggests that the calcification of contractile VSMCs might be mediated by different mechanisms. Additionally, neither increased calcium nor calcium-phosphate conditions could induce EVs release and calcification of articular chondrocytes. Since articular chondrocytes exhibited lower ROS levels, we believe this might prevent EV release and subsequent calcification. Articular chondrocytes are also known to produce extensive networks of glycosaminoglycans (GAGs), which are long polysaccharide chains that can bind calcium via their sulphate and carboxyl group[65]. In our calcifying environment, these negatively charged GAGs might bind positively charged calcium ions, preventing further calcium internalisation into the cells thereby reducing EV generation[66].

Endogenous BMP-2 levels drive calcification development *in vitro*[59], therefore we measured BMP-2 gene expression in VSMC phenotypes and articular chondrocytes. We found increased levels of BMP-2 expression in synthetic and chondrocyte-like VSMCs, which implies that BMP-2 might drive calcification in synthetic and chondrocyte-like VSMCs. In contrast, articular chondrocytes expressed much lower levels of BMP-2 than chondrocyte-like cells. This could explain the difference in calcification potential between chondrocyte-like VSMCs and articular chondrocytes. Further, MGP is a potent calcification inhibitor that is produced by chondrocytes and VSMCs[67]. In our study, we observed reduced levels of MGP in synthetic VSMCs but MGP was unchanged in chondrocytes supporting the calcification profiles we found[68].

Phosphate is known to increase MGP gene expression in chondrocytes[69]. The levels of phosphate that were used were supraphysiological compared to ours. This might explain why we did not observe an upregulation of MGP in chondrocyte-like cells. On the other hand, chondrocyte-like cells are prone to mineralization, and thus they might change towards bone-like cells. Indeed, we detected significant upregulation of OC expression in chondrocyte-like VSMCs. OC is an osteoblast-secreted protein linked to calcified tissues[70,71]. We did not observe SOX9 upregulation of VSMCs. This contradicts the literature, where it is reported that SOX9 is upregulated in VSMCs treated with β-glycerol phosphate[72]. To differentiate VSMCs we used inorganic buffer composed of sodium dihydrogen phosphate and disodium hydrogen phosphate solutions in comparison to β-glycerol phosphate which is an organic phosphate donor, likely differently utilised by VSMCs. This suggests that SOX9 expression is dependent on the type of phosphate donor. Additionally, we detected SOX9 upregulation in articular chondrocytes, which is in line with previous data showing that SOX9 is an essential transcriptional factor in chondrocytes[73].

Recently, MK-7 has been shown to alleviate VC via reduction of ROS and subsequent EVs release36. In our study, we show that MK-7 attenuates the calcification of synthetic VSMCs. VSMCs are known to express vitamin K-dependent proteins (VKDPs), including MGP and gla-rich-protein (GRP), known to be potent inhibitors of calcification. MGP and GRP are located within EVs to prevent calcification[74]. VKDPs require activation by the gamma-glutamyl carboxylase enzyme (GGCX) to fulfil their function[75,76]. Vitamin K is an unequivocal cofactor for GGCX and could thus be a treatment to prevent vascular calcification. The absence of an effect of MK-7 in reducing calcification of chondrocyte-like VSMCs could be explained by the reduced levels of MGP and increased levels of OC.

Increased calcium levels induce calcification of VSMCs, which might be caused by increased uptake of calcium via L-type VDCCs that enable the influx of calcium ions into the cell[15]. It has been shown that Verapamil, an L-type VDCC inhibits VC of VSMCs *in vitro*[25]. In our study, Verapamil had no notable effect on EV release nor calcification. Two other L-type VDCC, Gabapentin and Amlodipine did effectively inhibit VSMC calcification and decreased calcification in synthetic and chondrocyte-like VSMCs. The inhibitory action of Gabapentin on calcium signalling is not fully understood. Gabapentin is known to inhibit the $\alpha_2\delta$-1 subunit of L-type VDCC, which is also expressed by VSMCs[22,77]. Thus, Gabapentin might inhibit the $\alpha_2\delta$-1 subunit of L-type VDCC, preventing calcium internalization, thereby reducing calcification. Additionally, Gabapentin can activate adenosine-1 receptors, which are known to inhibit calcification[78,79]. Whether the inhibitory role of Gabapentin in our VSMCs *in vitro* model is mediated via these receptors is subject to further investigation. Amlodipine is known to bind to L-type VDCC in the aorta, reducing calcium influx and aortic calcium oscillations[80]. Amlodipine has been shown to have no effect on calcification *in vitro,* yet the concentrations that were used in these studies were lower than those in our experiments[81]. Gabapentin and Amlodipine had no significant effect on EV release, suggesting that their inhibitory actions are mediated via different mechanisms.

Despite providing insights into articular chondrocyte and VSMC calcification, our study bears some limitations. Our *in vitro* model of phenotypic switching does not account for the full spectrum of signals that mediate VSMCs differentiation. Moreover, articular chondrocytes used in our study are derived from osteoarthritis patients due to the limited supply of healthy cartilage in the laboratory.

Conclusion

Our results show that VSMC calcification is dependent on phenotypic switching. Chondrocyte-like cells possess higher calcification ability than articular chondrocytes, possibly because of the gain of calcification activators. Differentiation of VSMCs induces oxidative stress and intracellular calcium uptake that can partially explain the increased calcification. We provide evidence that oxidative stress and calcium uptake are potential therapeutic targets to inhibit VSMC induced calcification. We present data that MK-7 inhibit calcification of synthetic VSMCs possibly via reduction of oxidative stress. L-type VDCC blockers, Amlodipine and Gabapentin, successfully ameliorated calcification of both synthetic and chondrocyte-like VSMCs. The effect is likely mediated by calcium uptake inhibition, however the precise mechanism warrants further investigation.

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CHAPTER

6

General discussion

General discussion

Cardiovascular disease (CVD) has received great research attention due to its global prevalence and adverse societal impact. Despite progressive exploration of the field, CVD is still one of the leading causes of death. One of the main contributors and predictors of CVD is vascular calcification (VC). VC manifests in either tunica intima or the medial layer of arteries and consists of hydroxyapatite (HA) deposits[1]. Medial calcification is also known as Mönckeberg's sclerosis and involves vascular smooth muscle cells (VSMCs) and calcification takes place in the absence of lipid accumulation and inflammation. For a long time, VC has been looked upon as a passive, non-regulated process, happening passively due to old age or end-stage of disease. More recently, however, the pathology of VC is known to be actively orchestrated by a variety of stimuli including minerals such as calcium and phosphate, phenotypic switching of VSMCs and loss of calcification inhibitors[2]. Both calcium and phosphate subserve the development of HA in both skeletal mineralisation and pathological calcification of arteries[2–4]. Accelerated bone loss and subsequent increased vascular calcification, commonly known as the calcium paradox, is often seen in women suffering from osteoporosis and in patients with chronic kidney disease (CKD). Bone and vascular mineralisation share many similarities, including expression of bone-related proteins in the vasculature as well as the presence of extracellular vesicles (EVs), both features involved in calcification. The work described in this thesis contributes to the role of vitamin K and vitamin K-dependent proteins (VKDP) in relation to VSMC mediated VC. In **chapter 2** we provide an up-to-date review of current literature on the interaction between bone and vascular tissue with a key role for vitamin K and VKDPs. In **chapter 3** we describe a novel *in vivo* pre-clinical model of vitamin K deficiency, high phosphate and CKD allowing us to study the combination of phosphate binders (PBs) and vitamin K to combat VC. **Chapter 4** presents evidence that vitamin K acts as an inducer and enhancer of osteogenesis in induced pluripotent stem cells (IPSCs) *in vitro.* In **Chapter 5** we elaborate on different mechanisms that induce mineralisation of VSMCs and chondrocytes and demonstrate that both vitamin K2, amlodipine and gabapentin can reduce VC *in vitro*.

The key findings of my thesis are:

1. Nephrectomy, vitamin K-antagonist treatment and high phosphate diet induces VC in a CKD animal model
2. In a CKD animal model of vitamin K deficiency and high phosphate, neither phosphate binder therapy nor vitamin K2 supplementation alone was sufficient to prevent VC. However, the combination of vitamin K2 with phosphate binder treatment significantly attenuated VC.
3. Vitamin K2 (MK-7) improves osteogenesis in iPSC-induced iMSCs in a novel non-canonical way by reducing oxidative stress and improving collagen networks.
4. Phenotypic switching of VSMCs controls the magnitude of VC *in vitro* which can be further ameliorated by vitamin K2, amlodipine and gabapentin.

Novel rodent nephrectomy induced CKD model

In my thesis, I investigated the initiation and progression of VC. Clinically, VC is a common pathology among patients suffering from CKD. A common complication of kidney function decline is hyperphosphatemia, described as toxic levels of circulating phosphate contributing to the formation of HA crystals. As a result, advanced CKD patients suffer predominantly from medial calcification in the arterial wall resulting in increased arterial stiffness and subsequent cardiovascular events. Additionally, end-stage CKD patients experience bone abnormalities[5,6]. Accelerated bone loss and simultaneous VC, commonly known as calcium paradox is often a consequence of the uremic milieu

present in end-stage CKD patients. In addition, CKD patients often are vitamin K deficient due to dietary restrictions, the use of PBs that are known to bind vitamin K in the gastrointestinal tract, or increased utilization of vitamin K[7,8] (Figure 1). Partly nephrectomy of rats, combined with a high phosphate diet, mimics renal insufficiency and is widely used to study the development and progression of CKD as well as possible therapeutic interventions. The surgical kidney ligation is accompanied by medial calcification development and increased circulating levels of phosphate, creatinine and urea. To improve our current understanding of CKD progression, we developed a novel *in vivo* model that mimics end-stage CKD including also vitamin K deficiency. Following ¾ nephrectomy in Sprague-Dawley rats, we administered vitamin K antagonist warfarin, resulting in the depletion of vitamin K. Warfarin has been shown to aggravate VC[9]. At the end of the study, animals suffered from increased concentrations of serum creatinine and urea when compared to sham animals. Moreover, medial HA deposits were found in the aortic arch and renal arteries. That is in line with other studies where nephrectomy combined with warfarin and high phosphate diet, resulted in medial calcification and deposits of HA in the vasculature[10,11]. So far, many CKD models are used, yet do not precisely mimic the pathological progression and aetiology of CKD. In a 5/6 nephrectomy and high phosphate diet CKD rat model sevelamer and calcium carbonate were able to reduce kidney calcification score, yet vascular calcification was not measured[12]. In another nephrectomy model, lanthanum carbonate reduced urinary phosphate excretion which suggests their beneficial role in reducing phosphate absorption, yet without screening for VC[13]. Sucrofferic oxyhydroxide was shown to attenuate renal inflammation and attenuation of glomerulosclerosis in an early CKD model, however before any development of VC and in the presence of a low phosphate diet[14]. In a similar study, lanthanum hydroxide suppressed the development of VC and lowered circulating phosphate[14]. Other research of CKD nephrectomy models coupled with PBs in the diet show no survival benefit, kidney function improvements, simultaneously inducing hypercalcemia and acidosis[15]. Other studies show that sevelamer hydrochloride aggravates the progression of aortic VC in a nephrectomy model of CKD[16]. It is worth noticing that all these CKD models were in the presence of sufficient vitamin K. Our model extends by adequately mimicking CKD status by introducing vitamin K deficiency and thus to study the pathogenesis and treatment options for VC.

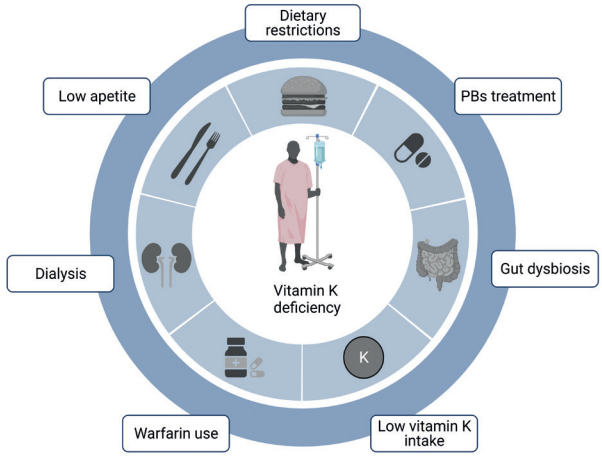

Figure 1. Factors contributing to vitamin K deficiency in chronic kidney disease (CKD) patients

Vascular calcification in a CKD rodent model of the disease – role of Vitamin K and phosphate binders

VC is a late-stage consequence of CKD, being a great risk factor for cardiovascular events and mortality[17]. Mineral deposits manifest as HA in which phosphate is one of the main components. To reduce the burden of elevated phosphate in CKD patients PBs are used routinely to bind phosphate in the gastrointestinal tract notably reducing its circulating levels. In dialysis patients, circulating phosphate levels above 5.5 mg/dL have been noted as an independent risk factor for cardiovascular morbidity and mortality[18]. Although PBs have been documented to effectively reduce phosphate concentrations in blood, there is no clear evidence advocating phosphate binder use for reducing VC and mortality[19].

In our animal model, the use of both calcium-based and non-calcium based PBs in combination with low vitamin K diet was not sufficient to reduce vascular calcification. Nevertheless, the use of calcium-containing PBs in our study did not aggravate VC, as seen in clinical studies with calcium-containing PBs compared to calcium-free PBs[20–22]. However, combining PBs with high vitamin K2 (MK-7) supplementation strongly attenuated VC. This protective effect on calcification is likely accomplished by the synergistic effect of combined PB treatment and vitamin K2 supplementation. This is likely due to the reduction of circulating phosphate levels by PBs throughout the diet and aiding vitamin K dependent calcification inhibitors by improving vitamin K status. Indeed, it has been shown that vitamin K supplementation inhibits VC in a CKD animal model[23]. Additionally, in several clinical intervention trials, vitamin K supplementation reduced arterial stiffness, slowed the progression of VC and improved the levels of dp-ucMGP in end-stage CKD patients[24,25]. Vitamin K is known to activate calcification inhibitors, amongst them MGP. MGP is a potent inhibitory protein synthesised by VSMCs. Thus, MGP is a promising target to reduce the burden of VC in CKD. PBs have been shown to impair vitamin K metabolism by binding fat-soluble vitamins in the GI tract, including vitamin K [7,19]. It is now well accepted that CKD patients are sub-clinically vitamin K deficient as deduced by the measurement of inactive dp-ucMGP[26]. High levels of dp-ucMGP correlate with increased VC, risk of fractures and low bone mineral density[27]. In our study, ucMGP colocalized extensively with VC in the vessel wall of CKD animals treated with PBs in combination with low vitamin K diet, suggesting local vascular vitamin K deficiency. Our findings are in line with previous data in experimental animals and suggest that vitamin K deficiency is a risk factor for developing VC[28]. However, when PBs were combined with high vitamin K2 intake, significantly less ucMGP and VC were present in the vessel wall. Recent studies showed the superiority of PBs sevelamer hydrochloride and sucroferric oxyhydroxide which both did not show vitamin K binding *in vitro*[29]. On the other hand, some studies showed an effect of sevelamer hydrochloride on increasing dp-ucMGP, suggesting impairing vitamin K status[30,31]. In our in vivo experiment, PBs combination therapy did not affect vitamin K status as measured by ucMGP staining in both thoracic and abdominal aorta. Additionally, we examined articular cartilage in the tibia to assess the efficacy of PBs and vitamin K supplementation on cartilage calcification. It has been shown that some 65-97% of CKD patients experience OA, where cartilage calcification is a major aspect of the common pathology[32,33]. Cartilage is known to express VKDP such as MGP and GRP[34], acting as calcification inhibitors[35,36]. Because of the presence of these VKDP in cartilage, vitamin K supplementation might rescue cartilage calcification. We found that also in articular cartilage the combination therapy resulted in significantly less calcification, corroborating with our findings in the arterial wall.

The role of vitamin K in osteogenesis – a novel induced pluripotent stem cell model

Induced pluripotent stem cells (IPSCs) have been extensively mobilized to study disease pathology. IPSCs appear advantageous to primary cell model predecessors. The use of iPSCs for osteogenic bone differentiation has been reported, yet conventional protocols require optimization and do not adequately resemble physiological conditions[37]. To take this further, we developed a novel osteogenesis protocol that enabled us to study the effect of vitamin K on the differentiation of iPSCs into osteoblasts. Vitamin K has been shown to stimulate osteoblast differentiation via upregulation of bone-specific markers, reduction of oxidative stress and increased mineralization[38–40]. Vitamin K serves as cofactor for gamma-glutamylcarboxylase (GGCX) which converts glutamic acid residues (Glu) into Gla residues, essential for the activation of VKDPs. One of the VKDP involved in HA deposition in bone is osteocalcin (OC). Uncarboxylated, inactive OC possesses weak binding to HA crystals. The property of vitamin K is thought to be the key mechanism by which OC controls the development and growth of HA crystals on the bone surface. Vitamin K also plays a role in non-canonical pathways where it is involved in activating the steroid and xenobiotic (SXR) receptor. The SXR receptor is involved in extracellular matrix remodeling and bone collagen assembly[41]. These pathways warrant further investigation, thus we explored the non-canonical pathways of vitamin K in our iPSCs derived model of osteogenesis. We show that *in vitro* supplementation of vitamin K2 (MK-7) improves collagen deposition in comparison to osteogenic media alone. This is in line with another study, where vitamin K2 improved bone strength via improving collagen crosslinking[42]. Oxidative stress is a known inducer of bone deterioration and inhibition of osteoblast function[43]. We explored the role of vitamin K in the management of reactive oxygen species (ROS) and found it to reduce ROS production during osteogenic differentiation. The basis for this hypothesis follows previously published studies where vitamin K prevented oxidative injury in developing neurons and in cultured osteoblasts[44,45]. Very recently, vitamin K has been postulated as a potent free radical scavenger superior to other commonly known agents such as α-tocopherol[46]. Our development of a novel osteogenesis protocol *in vitro* using iPSCs provides a potentially translational model for the clinical practice of bone regeneration. The beneficial implementation of vitamin K in our model will help to develop the area of bone growth to a more favourable microenvironment for osteoblast growth and maturation. Unlike bone marrow-derived stem cells, iPSCs cells allow researchers to establish a personalised bone model with the promise of successful regenerative therapy. Moreover, vitamin K might earn a more prominent role as a potent-vitamin antioxidant.

Phenotypic switching of VSMCs is a key feature in vascular calcification – effects of vitamin K2, Amlodipine and Gabapentin

Phenotypic switching of VSMCs has been linked to vascular remodelling resulting in atherosclerosis, arteriosclerosis (Mönckeberg's sclerosis) and aneurysm formation. Synthetic VSMCs can give rise to HA deposits in the vasculature [47,48]. With the discovery of VC as an orchestrated and active process, high phosphate concentrations have also been found to induce phenotypic switching of VSMCs to cells that closely resemble osteo/chondrocytic cells. Contractile VSMCs are found to be inert for inducing vascular calcification[49]. To glean more information, we examined different VSMC phenotypes and their calcification potential and compared them to articular chondrocytes. Additionally, these cells were used to examine therapeutic agents to combat calcification.

In chapter 5 we show that contractile VSMCs exhibit a different phenotypic pattern compared to synthetic and chondrocyte-like VSMCs. When losing their contractility, VSMCs are more prone to calcification. This is in accordance with published research where synthetic and chondrocyte-like

VSMCs were prone to calcifying conditions[49,50]. We also report that articular chondrocytes calcify less than chondrocyte-like VSMCs. It has been shown that synthetic and chondrocyte-like VSMCs express pro-calcifying genetic and protein patterns that could account for the pro-calcifying state. We also showed that calcification of synthetic and chondrocyte-like VSMCs is accompanied by an increased EV release and ROS generation which both are involved in the process of VC. EVs are mediators of native bone mineralization in both endochondral and intramembranous ossification. This indicates that the release of calcifying EVs plays an important role in both physiological and pathological calcification[61,62]. In opposition, we show that articular chondrocytes release fewer EVs than both synthetic and chondrocyte-like VSMCs. Moreover, we show that phenotypic switching of VSMCs is necessary to induce EV mediated mineralisation[49,50]. This is in line with EVs from atherosclerotic aortas showing greater calcification potential than EVs isolated from non-atherosclerotic aortas[51]. In the vessel wall that is subject to calcification, presence of chondrocytes and osteoblasts has also been recorded[52]. Our data provide evidence that calcium influx might be responsible for the difference in VSMC calcification. Intracellular calcium influx has been shown to mediate VC *in vitro* and in CKD *in vivo* models[49,53]. We noted a greatly reduced influx of calcium into articular chondrocytes which could account for the scarce HA deposits upon calcifying conditions. That is in concordance with literature as chondrocytes do not actively transport calcium inside the cell unless mechanically stimulated[65]. Furthermore, we showed that vitamin K2 (MK-7) reduced mineralisation of synthetic VSMCs despite significant upregulation of BMP-2. MGP is present in both VSMCs and articular chondrocytes, acting as local inhibitor of calcification. Carboxylated MGP, which is extra negatively charged, binds HA crystals which in turn halts crystal growth. Another protective mechanism of MGP is facilitated by direct binding to BMP-2 thereby preventing it from binding its receptor[54]. This suggests that *in vitro* calcification of synthetic VSMCs can be prevented by the interaction of cMGP-BMP-2.
Calcium channel blockers are used as angina and hypertension treatment and have atheroprotective properties[55]. Calcium channel blockers were found to reduce VC *in vitro* and *in vivo* and reduced progression of CAC in hypertensive patients[56,57]. Amlodipine is a calcium channel blocker used to treat high blood pressure by inhibiting the influx of extracellular calcium ions into myocardial and peripheral VSMCs. Gabapentin is widely used for neuropathic pain, epilepsy, and hypertension. It binds with high affinity to alpha-2 delta voltage-gated calcium channels through which it elicits its action. So far there is no data on Gabapentin in CVD progression. We show that Gabapentin and Amlodipine significantly reduced the level of calcification in contractile, synthetic and chondrocyte-like VSMCs, yet without affecting EV levels. Further studies are needed to elucidate the precise molecular mechanism of gabapentin and amlodipine in VSMCs. Our work elucidates the molecular mechanisms that affect the phenotypic transition of VSMCs thereby facilitating mineralisation and may provide important pathways towards a novel remedial methodology of VC.

Future perspectives and concluding remarks

In my thesis, I aimed to investigate the role and function of vitamin K in VC and bone mineralisation in translational and basic research. In Chapter 2 we review the current literature on calcium supplementation among post-menopausal women and calcium-based phosphate binder treatment in dialysis patients and discuss underlying molecular mechanisms of calcium-induced VC. The mechanism via which calcium supplements negatively impact the vasculature is likely mediated via increased EV [62]. Recent meta-analyses showed that calcium supplementation in osteoporotic women is associated with increased cardiovascular and all-cause mortality, especially myocardial infarction. This is contradictory with other meta-analyses showing that supplemental calcium intake does not increase CVD[58,59]. In our review, we propose therapeutic strategies combining calcium supplements with

vitamin K2 treatment to prevent or hold the progression of VC. We suggest vitamin K as an appealing target to combat the calcium paradox.

In Chapter 3 we report the generation of a novel animal model of vitamin K deficiency in CKD. In this model, we tested a therapeutic regimen of PBs in combination with vitamin K2. Vitamin K deficiency, a feature that is commonly present in CKD patients, was induced by feeding the animals the vitamin K-antagonist (VKA) warfarin. The use of high vitamin K in the diet along with PBs successfully reduced the magnitude of VC. In our model, the increased presence of ucMGP in vascular tissue was noticed, strongly correlating with increased VC. Currently, phosphate management in dialysis resorts to dialysis or PB treatment. Often, current therapies are not efficient enough in lowering circulating phosphate, and novel drug candidates are being tested. Current clinical trials focus on surrogate measures of CVD and describe vitamin K deficiency as a marker for all-cause mortality[60]. One clinical trial is currently testing the efficacy of the novel compound KHK7791 on hyperphosphatemia in HD patients. Primary and secondary measures including measurement of calcium-phosphate product levels over time will determine the effectiveness of the compound in the treatment of hyperphospahetamia[61]. Our data provide clinically relevant insight for combining high vitamin K2 with KHK7791 to counteract HA deposition. This is in line with clinical trials that are currently undertaken. The iPACK trial assesses the effect of vitamin K1 in end-stage CKD patients. The primary endpoint is coronary artery calcification (CAC) while secondary endpoints are femoral and lumbar fracture incidence and cardiovascular events[62]. The second interesting clinical trial VIKIPEDIA will investigate low vs high dose vitamin K2 (MK-7) on arterial stiffness in haemodialysis patients as measured by pulse wave velocity analysis[63].

In my thesis, I present a new IPSCs derived model of bone differentiation. Our successful implementation of vitamin K2 in osteogenic assays creates a bridge in the development of bone regeneration techniques and basic research. We present vitamin K2 (MK-7) as an agent with antioxidant properties. Our study expands on the role of vitamin K in CVD and bone health. Our data provide a connection between oxidative stress and CVD as an agent of important biological activity. The currently ongoing clinical trial “TAKEOVER” assesses the antioxidant activity of vitamin K as a measurement of oxidative stress and inflammatory markers aside from its canonical role in VKDP carboxylation[64]. Furthermore, in chapter 5 we explored the role of VSMCs phenotypic switching in the development of VC *in vitro*. Our data provide evidence of the inhibitory effect on calcification of vitamin K2 (MK-7), amlodipine and gabapentin and creates a platform to translate the use in clinical practice. Our data could provide a roadmap in establishing new treatments using calcium channel inhibitors in the treatment of VC. Finally, my thesis provides evidence that VSMCs drive the mechanism of VC and that vitamin K possesses a beneficial action in CVD and bone development. We advocate its further research into clinically studies.

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CHAPTER

Summary

In this thesis, I investigated the role of vitamin K in both the skeletal and vascular system. I focussed on processes involved in vascular calcification (VC) and bone mineralisation aiming to glean the molecular mechanisms behind it. This thesis entails a literature review followed by *in vivo* and *in vitro* studies trying to elucidate these aspects.

Chapter 2 elaborates on the role of vitamin K on calcium supplementation in the bone-vascular axis. Calcium supplements are generically prescribed to combat bone-loss and increase bone strength. We discuss the impact of vitamin K and vitamin K dependent proteins (VKDPs) in bone and vascular. Data in postmenopausal women suggest that calcium supplementation significantly correlates with cardiovascular morbidity and morbidity yet provides little benefit on bone. This phenomenon is described as the "calcium paradox" and describes the simultaneous decline in bone calcium mineral content with the deposition of calcium at extra-osseous sites. Dialysis patients are also often prescribed calcium supplements in the form of phosphate binders (PBs) and their use is associated with increased VC. Initiation and progression of VC in dialysis patients correlates with impaired mineral metabolism represented by elevated serum levels of phosphate. Any increase in calcium results in pathological calcium-phosphate precipitation. In chapter 1, we impose the supplementation of vitamin K in the dietary regimen. We provide a literature overview that shows that vitamin K supplementation can benefit both vasculature and bone, preventing calcium residing in soft tissues including arteries and improving skeletal parameters.

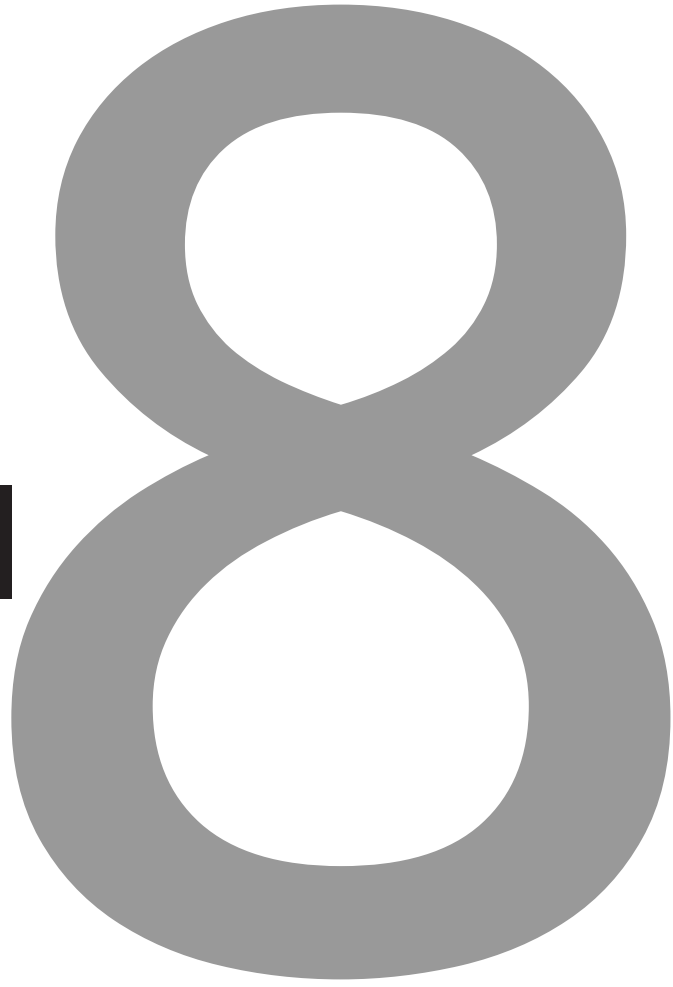
In **Chapter 3** we describe a novel preclinical *in vivo* model of vitamin K deficiency in CKD, representing the clinical situation of end-stage CKD patients. Using this model, we showed that the use of PB treatment is not sufficient to prevent ectopic calcification. However, combining PBs with high vitamin K2 supplementation, counteracting the vitamin K deficiency, strongly attenuates VC. This protective calcification effect is likely accomplished by the synergistic effect of combined PB treatment and vitamin K2 supplementation. Incorporation of vitamin K2 into PBs treatment also improved the levels of vascular matrix Gla-protein, a vitamin K dependent protein involved in the inhibition of vascular calcification. This study aids the understanding of the role of vitamin K in CKD and suggests the supplementation of vitamin K in the clinical setting.

In **Chapter 4** we investigated the role of vitamin K2 in bone formation. Vitamin K2 is known to exert a plethora of effects on the skeletal system resulting in increased bone mineral density. We report the successful differentiation of osteoblast using an iPSCs model of osteogenesis. Our data suggest a non-canonical function of VKDPs in early differentiation events, that can be modelled using iPSCs. We show that vitamin K2 induces an osteogenic cellular phenotype that might prove beneficial towards the development of tissue engineering solutions. This is accompanied by reducing oxidative stress diminishing the inflammatory response during bone formation. Our data provide the first account of several mechanisms of osteogenesis from iPSCs and the influence of vitamin K2 on the osteogenic processes.

Chapter 5 provides insights into the inhibitory role of vitamin K2 and the calcium-channel blockers, amlodipine and gabapentin in VSMC mineralisation. We characterised the contractile, synthetic and chondrocyte-like VSMC phenotype and compared these to articular chondrocytes. Phenotypic modulation of VSMCs into synthetic and chondrocyte-like VSMCs is accompanied by loss of contractile markers, increased oxidative stress and greater calcium influx. Differentiated cells are more prone to mineralisation which is reflected by greater extracellular vesicle (EV) release. Chondrocytes release fewer EVs and calcify to a lesser extent than chondrocyte-like VSMCs. We identified several genes that might drive the calcification of VSMCs and account for the difference in the amount of mineralisation. Additionally, we provide evidence that vitamin K2 can inhibit VC *in vitro.* Finally, using our *in vitro*

model we demonstrate the inhibitory role of amlodipine and gabapentin in reducing VC of contractile, synthetic and chondrocyte-like VSMCs via a EV independent mechanism.

CHAPTER



Societal impact

Societal Impact

Raising global awareness about the severity and impact of age-related diseases such as cardiovascular and bone disease has greatly increased in recent years[1]. Yet the deleterious effects on the elderly society are progressing and current therapies aiming to reduce bone loss and prevent soft tissue calcification are limited. The increasing ageing population brings with it the burden of lifestyle disease and thus huge socio-economic and public health care concerns[2]. Current therapies are costly, and their development is labour intensive and arrives with an adverse panel of side effects. For that reason, nutraceutical modulators are tested to elucidate their efficacy on disease progression. Dietary vitamin K attracts great attention as the "new kid on the block" in preventing debilitating symptoms of vascular calcification (VC) and reducing bone loss due to its multifactorial, yet specific roles. Therefore, supplemental vitamin K to modulate cardiovascular disease is a promising treatment.

Although calcium supplements are broadly used, their contribution as to whether they benefit bone health by reducing fracture risk and bone fragility is still a matter of debate[3]. Our review provides the latest take on the effects of calcium supplementation in post-menopausal women and the effect of calcium-based phosphate binders in patients suffering from chronic kidney disease (CKD). Here we discuss underlying molecular mechanisms of calcium intake in relation to ectopic calcification and bone metabolism. We imply that combining calcium supplements with vitamin K could reduce the risk of post-menopausal bone loss and simultaneously prevent VC by activating vitamin K-dependent proteins in both bone and vasculature. In the end, preventing the negatively associated vascular effects of calcium supplementation would be an easy, safe and cost-effective add-on for healthy living.

The increasing awareness of the presence of VC, and the notion that VC is an independent risk factor and predictor of CVD, has led to research models to investigate diagnosis and treatment of VC[4]. In chapter 3, we addressed the impact of VC using a basic research approach aiming to unravel molecular mechanisms in detail. Our pre-clinical approach would allow translation into clinical practice, to generate an impact on improving patient care. We developed a novel *in vivo* rodent model closely mimicking the clinical situation of CKD patients, known to be prone for VC. Our rodent model underwent ¾ nephrectomy receiving a high phosphate diet and was subjected to vitamin K deficiency by warfarin treatment. High phosphate levels and vitamin K deficiency are common risk factors in CKD patients[5,6]. Using our *in vitro* model we demonstrated that vitamin K2 (MK-7) co-supplementation with phosphate binders (PBs) could effectively reduce the magnitude of VC as compared to vitamin K2 or PB use alone. The use of calcium-based PBs was a standard treatment for many years, but because of the prejudicious effects via increasing VC are replaced by non-calcium-based PBs[7]. However, non-calcium based PBs lower phosphate levels yet do not improve cardiovascular disease in CKD patients[8]. This might be due to the ability of non-calcium-based PBs to bind, next to phosphate, also fat-soluble vitamins such as vitamin K. Although our in vivo study could not prove that PBs complex vitamin K thereby inducing vitamin K deficiency our data show that supplementation of vitamin K2 in combination with PBs results in less VC and cartilage calcification. This is supported by the fact that PBs increase dp-ucMGP levels, a vitamin K-dependent protein produced in vascular and cartilage tissue as a result of vitamin K deficiency[9]. To our knowledge, our rodent model is the first to address more precisely the clinical situation of CKD patients which could also be used by other researchers. Our data may serve as a scaffold for improving medical guidelines for the nephrology community and might aid standardisation of routine testing of vitamin K levels in the CKD population.

Rodent studies are an inevitable step in pharmaceutical, toxicological and nutritional research. With the advent of transgenesis, animal experimentation in these fields has even expanded, despite major efforts to reduce or substitute animal use. However, a patient is not simply a 75kg rodent, and translation of research from animals to humans is often not possible. Therefore, we embarked on using human induced pluripotent stem cells (iPSCs) cells to elucidate molecular mechanisms of bone formation *in vitro*. We implemented novel protocols for iPSCs differentiation towards induced mesenchymal stem cells (iMSC) to test the effects of vitamin K2 (MK-7) under osteogenic conditions.

A multitude of strategies trying to mitigate biological bone properties using primary cells exhibited unsatisfactory results mostly due to limited cell acquisition and availability. Generation and use of iPSCs prevent costly and time-consuming animal work and provide a limitless supply of patient-specific cells. It also circumvents the ethical implications associated with traditional primary stem cells surpassing the use of embryos or oocytes. To our knowledge, our model is the first to address a novel protocol of iPSCs differentiation using vitamin K2 (MK-7). Our iPSCs can be further translated into more advanced models, e.g. organoid cultures to glean and expand the database of molecular pathways underlying disease of interest. Of note, iPSC mediated bone formation is a noteworthy tool in the development of personalized medicine entangled to patients' specific needs as it is not subject to immune-reaction rejection.

In chapter 5, we used primary VSMCs and articular chondrocytes to assess *in vitro* molecular mechanisms of vascular and cartilage calcification. In the vasculature, VSMCs can under stress differentiate to osteo/chondrocyte-like VSMCs[10]. To unravel molecular pathological mechanisms leading to calcification could benefit clinical practice. Using our *in vitro* setup, we demonstrated the beneficial effects of vitamin K2 (MK-7) in VSMCs cultures, by preventing calcification. These data support the paradigm where vitamin K2 could be advocated as a dietary alternative treatment reducing or regressing cartilage degeneration and holding progression of vascular disease. Moreover, using this *in vitro* approach we could show that Amlodipine and Gabapentin, used in neuroscience research to inhibit calcium channel activity, reduce VSMC calcification thereby providing a novel therapy for VC.

In conclusion, my thesis puts forward a role for vitamin K in preventing VC and cartilage calcification in CKD. Moreover, I provide novel treatment options for reducing ectopic calcification by inhibiting calcium channels. Our data lay the foundation for further elucidating the context of bone loss and subsequent vascular disease progression.

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CHAPTER

9

Curriculum Vitae

Curriculum Vitae

Grzegorz Boguslaw Wasilewski was born on the October the 11th , 1992 in Lublin, Poland. As a child he moved to the United Kingdom and started education there. He attended and graduated from St. Pauls Catholic College, Sunbury where he undertook, Biology, Chemistry, Maths and Physics classes. After finishing college, he started Medical Biochemistry at Royal Holloway University of London for which he obtained the bachelor's degree in 2015. Upon completion of the bachelor's program, Grzegorz began his Master in biomedical sciences specialising in neuroscience in the context of Alzheimer's disease under the supervision of Dr. Pavlos Alifragis. In 2017, he started a PhD program at Maastricht University, department of Biochemistry in collaboration with Nattopharma and the Norwegian Research Council under the supervision of Prof. Leon Schurgers. The aim of his PhD project was to investigate the calcium paradox and the role of vitamin K in chronic kidney disease patients and osteoporotic women.

CHAPTER

10

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