The renal-bone axis: the effect of renal impairment and vitamin D supplementation on bone turnover, Wnt-signalling and inflammation markers in older people

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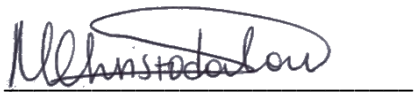
This dissertation is submitted for the degree of Doctor of Philosophy. This work is the result of my own work except were indicated in the text.

Signature



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Abstract

This PhD thesis is focused on the renal-bone axis which involves an endocrine feed-back loop and cross-talk between these two organs. Several hormones and regulators play key roles in this extensive cross-talk, including: parathyroid hormone (PTH), fibroblast growth factor-23 (FGF23), 1,25-dihydroxy vitamin D, 25-hydroxy vitamin D and Sclerostin. Ageing is associated with a decrease in both renal function (most commonly measured as the estimated glomerular filtration rate (eGFR)), bone loss and reduced tissue sensitivity to regulating hormones. The mechanisms involved in this cross-talk are poorly understood. Although changes in plasma concentrations of these factors are well-characterized in advance renal impairment, less is known in the early stages of renal impairment and how this may affect the response to vitamin D supplementation. The main aim of the research presented in my thesis was to develop a better understanding on the renal-bone cross-talk. Therefore, I investigated the consequences and changes caused by renal impairment on renal-bone axis and the effect of vitamin D supplementation on the renal-bone axis.

More specifically, the focus of my research was on the effect of vitamin D supplementation on FGF23 and markers of Wnt-signalling. FGF23 plays a key role in the renal-bone axis, together with parathyroid hormone and vitamin D metabolites. FGF23 is a phosphaturic hormone which is produced and secreted by osteocytes and acts in the kidney to induce phosphate excretion in order to maintain homeostasis. It also affects bone metabolism through its regulatory function on the Wnt-signalling pathway. In addition, recent evidence showed associations of FGF23 with iron and inflammation markers.

This study utilized data and samples from the dose-ranging vitamin D interventional randomized controlled trial 'the VDOP study'. This study was conducted in collaboration between Medical Research Council, Human Nutrition Research, The University of Newcastle and University of East Anglia. Further, a systematic review and meta-analyses of RCTs with vitamin D and its analogues in chronic kidney disease (CKD) patients and reviewed current guidelines.

Most chapters (2-5) are based on my published papers or are currently under review for publication (references and details are provided in each chapter).

The systematic review of RCTs showed that Vitamin D treatment of CKD patients has an inconsistent effect on PTH, although meta-analysis showed a significant overall effect. Calcifediol and analogues consistently suppress PTH, but the reported increase in FGF23 with $1,25(OH)_2D$ analogues warrants caution. Current guidelines for the first CKD stages (G1-G3a) follow general population recommendations for the prevention of vitamin D deficiency. Use of calcitriol or analogues is restricted to stages G3b-G5 and depends on patient characteristics.

The VDOP study showed that vitamin D supplementation in older people leads to a decrease in intact-PTH (iPTH) and increase in procollagen type I N-propeptide: beta-C-terminal telopeptide (PINP:CTX) ratio. This suggests a protective effect of supplementation on bone metabolism although no significant effect on bone mineral density (BMD) or pronounced changes in regulators of the Wnt-signalling pathway were found. There was an increase in FGF23. This warrants caution due to its negative associations with bone and cardiovascular health.

Further analyses with data categorised by eGFR (G1-2 >60 and G3a/b <60 ml/min/1.73m^2) showed that even a moderate decline in eGFR has a negative impact on vitamin D metabolism, Wnt-signalling and bone turnover markers. Vitamin D supplementation had beneficial effects on markers of the renal-bone axis in older people of both groups. The response to vitamin D supplementation was however dependent on renal function. Supplementation improved vitamin D status and Klotho in the group with moderate renal impairment (G3a/b) to concentrations comparable to those found in the group with the higher renal function (G1-2). Plasma intact-FGF23 (iFGF23), $1,25(OH)_2D$, PINP increased only in the group with the higher renal function (G1-2).

Moreover, in subgroup analysis comparing people with early renal impairment (CKDG3a/b) and normal renal function (CKDG1 eGFR >90 ml/min/1.73m^2), alterations in regulators of the renal-bone axis, inflammation and iron status were observed in early CKD. Early renal impairment was associated with changes in regulators of calcium, phosphate, vitamin D and bone metabolism and in iron status and inflammation. After vitamin D supplementation, differences between the two groups were no longer significant for iPTH, Klotho, iron and tumor necrosis factor-α (TNFα). The response of iron and inflammation markers, to vitamin D

supplementation differed between the groups. Plasma iron, Interleukin 10 (IL10) increased and TNFα decreased in the group with renal impairment. In the group with normal renal function, no changes were observed in markers of iron status and inflammation, except for an increase in IL10. Regression analyses showed that plasma c-terminal FGF23 and iFGF23 were predominantly predicted by eGFR and regulators of calcium/phosphate metabolism.

In conclusion, this study identified changes in the renal bone-axis with early renal impairment and differences in the response between groups with normal and early CKD. Vitamin D supplementation may partly abate the effects of renal impairment. Diagnosis of renal impairment at an early stage may provide opportunities for the prevention of the progression of renal disease and CKD-Mineral Bone Disease.

List of Abbreviations

1,25-dihydroxy vitamin D; calcitriol	$1,25(OH)_2D$
1a hydroxylase	1a(OH)ase
24 hydroxylase	24(OH)ase
24,25-dihydroxy vitamin D	$24,25(OH)_2D$
25-hydroxy vitamin D	25(OH)D
Adequate intake	AI
Aldosterone receptor antagonists	ARAs
Alkaline phosphatase	ALP
Angiotensin inhibitors	ACE
Autosomal dominant hypophosphatemic rickets	ADHR
Analysis of variance	ANOVA
Analysis of covariance	ANCOVA
B2-microglobulin	B2-M
Blood urea nitrogen	BUN
Bone mineral content	BMC
Bone mineral density	BMD
Body mass index	BMI
Bone sialoprotein	BSP
Bone specific alkaline phosphatase	BAP
Calcium	Ca
Calcium-sensing receptor	CaSR
Cardiovascular disease	CVD
Chlorine	Cl
Chronic kidney disease	CKD
Chronic Kidney Disease Epidemiology Collaboration	CKD-EPI
Chronic kidney disease- mineral bone disease	CKD-MBD
Cloning-stimulating factor	CSF
Cockroft Gault	CG
Computerized tomography	CT
Coronary artery calcification	CAC
C-parathyroid receptor	C-PTHR
Creatinine	Cr
C-reactive protein	CRP
c-terminal fibroblast growth factor 23	cFGF23
C-terminal telopeptide of type-I collagen	CTX
Cystatin C	CC
Deoxypyridinoline	DPD
Dickkopf-related protein 1	DKK1
Dietary reference intake	DRI
Dietary reference values	DRV
Dual-energy X-ray absorptiometry	DXA
Estimated Glomerular Filtration Rate	eGFR
European Food Safety Authority	EFSA
Extended release	ER
Extracellular signal regulated kinase-1	ERK1/2
FGF receptors	FGFRs

Fibroblast growth factor 23	FGF23
Filtration fraction	FF
Follicle-stimulating hormone	FSH
Glomerular filtration rate	GFR
Hemodialysis	HD
Immediate release	IR
Individual participant data	IPD
Interleukin 6	IL6
Interleukin 10	IL10
Interquartile range	IQR
Institute of Medicine	IOM
Intact fibroblast growth factor 23	iFGF23
International units	IU
Intact parathyroid hormone	iPTH
Janus kinase-3	JAK3
Kidney disease improving global outcome	KDIGO
Kidney Disease Outcomes Quality Initiative	KDOQI
Kidney injury molecule-1	KIM-1
Kinase with-no-lysine kinase 1	WKN1 kinase
Kinase with-no-lysine kinase 3	WKN3 kinase
Kinase with-no-lysine kinase 4	WKN4 kinase
Low-density lipoprotein receptor-related protein	LRP
Luteinizing hormone	LH
Mesenchymal stem cells	MSC
Modification of Diet in Renal Disease	MDRD
N- terminal telopeptide of type-I collagen	NTX
Na+/H+ exchange regulatory factor	NHERF
Na+-Cl− cotransporter	NCC
Osteocalcin	OC
Osteoprotegerin	OPG
Parathyroid hormone	PTH
parathyroid receptor-1	PTHR
Peroxisome proliferator-activated receptor-γ	PPARγ
Plasma membrane Ca2+ ATPase 1b	PMCA1b
Potassium	K
Peripheral quantitative computed tomography	pQCT
Phosphate transporter	PiT-2
Procollagen type-I N peptide	PINP
Pyridinoline	PYD
Randomized Controlled Trials	RCT
Receptor activator of nuclear factor kappa-B	RANK
Receptor activator of nuclear factor kappa-B ligand	RANKL
Recommended Dietary Allowance	RDA
Reference Nutrient Intake	RNI
Renal plasma flow	RPF
Renin-angiotensin-aldosterone system	RAAS
Sclerostin	SOST

Serum glucocorticoid regulated kinase-1	SGK1
Sodium	Na
Sodium-calcium exchanger	NCX1
Sodium-dependent phosphate transport protein 2a	NaPi2a
Sodium-dependent phosphate transport protein 2b	NaPi-2b
Sodium-phosphate cotransporter 2a	Npt2a
Sodium-phosphate cotransporter 2b	Npt2c
Standard deviation	SD
Tartrate-resistant acid phosphatase	TRAP
Tissue nonspecific alkaline phosphatase	Tnap
Transforming growth factor- β	TGF-β
Transforming growth factor-a	TNF-a
Transient receptor potential cation channel subfamily V member 5	TPRV5
Transient receptor potential vanilloid type 6	TRPV6
Tumor-induced osteomalacia	TIO
Type-1 procollagen C-terminal propeptide	P1CP
Ultraviolet B	UVB
Urinary retinol binding protein	uRBP4
Vitamin D binding protein	DBP
Vitamin D receptor	VDR
Vitamin D receptor activator	VDRA
X-linked hypophosphatemic rickets	XLH
αKlotho	Klotho

Academic outputs from this PhD research

Conference oral presentations

Christodoulou M., Aspray J. T., Piec I., Washbourne C., Tang C. Y. J., Fraser D. W. and Schoenmakers I. (2021) Total and free 25-hydroxyvitamin D associations with markers of bone turnover and Wnt signalling in older people supplemented with vitamin D, ***Vitamin D Workshop* 13-14 Oct**.

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Conference poster presentations

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Publications

Christodoulou M, Aspray TJ, Schoenmakers I. Vitamin D Supplementation for Patients with Chronic Kidney Disease: A Systematic Review and Meta-analyses of Trials Investigating the Response to Supplementation and an Overview of Guidelines. Calcif Tissue Int. 2021 Aug;109(2):157-178. doi: 10.1007/s00223-021-00844-1. Epub 2021 Apr 25. PMID: 33895867; PMCID: PMC8273061.

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CHAPTER 1a: Introduction

1. Vitamin D

Vitamin D can be obtained from diet either from animal sources (cholecalciferol D_3) or plant sources (ergocalciferol D_2). It can be also produced endogenously after Ultraviolet B (UVB) exposure of the skin, form 7-dehydrocholesterol[1]. The main dietary sources of vitamin D are food of animal origin which includes egg yolk, oily fish, meat, fat and liver[2]. Nowadays fortified foods are also available and commonly used. Foods are fortified either with vitamin D_3 or D_2 such as margarines, milk, breakfast cereals[2]. The only plant food with significant amount of vitamin D_2 are wild mushrooms[2]. The endogenous production of vitamin D varies with season, latitude and skin exposure to the sun[1]. Sunscreen and clothing have been reported to reduce the production of 7-dehydrocholesterol[3,4]. Also, the rate of cutaneous vitamin D synthesis is lower in people with darker skin thus the exposure time is longer for any equivalent amount of vitamin D produced[2]. The pigment melanin which gives the skin the brown colour, absorbs UVB[2]. Therefore, less UVB reaches the layer of the skin where vitamin D is synthesized from 7-dehydrocholesterol[2].

Vitamin D supplements contain either D_3 or D_2 and can be used orally or by intramuscular injection[2]. According to a meta-analysis, supplementation with vitamin D_3 is more efficient to maintain vitamin D status than D_2, due to its longer half-life[5]. Vitamin D status is assessed on the basis of the plasma concentration of 25-hydroxyvitamin D (25(OH)D) (*see section 1.1*). In the UK, 25(OH)D serum levels <25nmol/L is defined as the threshold of deficiency[2]. Vitamin D deficiency is associated with an increased risk of skeletal disorders. During growth, vitamin D deficiency can lead to development of rickets[2]. Early diagnosis and vitamin D supplementation treatment can reverse those defects[2]. Vitamin D deficiency can also result to the development of osteomalacia[2]. The symptoms are severe aching in bone and muscles, making walking and standing painful because of impaired bone mineralisation[2]. Vitamin D deficiency is one of the factors contributing to the development of osteoporosis during ageing[2]. Osteoporosis is a progressive skeletal disorder[2]. Its main characteristics are loss of bone mass, increased bone fragility and fracture risk[2].

Recommendations for vitamin D intake for the general population and for specific patient groups differ because of altered metabolism and requirements associated with disease

process such as with chronic kidney disease. There are also differences between countries in population guidelines. The UK Dietary Reference Nutrient Intake (RNI) is 10 µg/day (400 international units (IU)/day) for all adults[6], in North America the Recommended Dietary Allowance (RDA) is 15 µg/day (600 IU/day) for adults and 20 µg/day (800 IU/day) for people over 70 years old, which is double the amount of UK recommended intake[7]. Both the UK and US recommendations are mainly based on the relationship with bone health, although also other health outcomes were considered[7].

Excessive vitamin D intake can lead to vitamin D toxicity which is characterized by increased serum calcium and 1,25-dihydroxy vitamin D ($1,25(OH)_2D$; calcitriol)[2]. This generally is associated with a plasma 25(OH)D >220ml/L, but some individuals may be more sensitive. Cutaneous synthesis of vitamin D does not lead to vitamin D toxicity[2]. The Scientific Advisory Committee on Nutrition (2016) reported a safer upper limit up to 3000 IU/d[8]. The US Institute of Medicine (IOM) (2011) established the Tolerable Upper Intake Level for vitamin D intake up to 100 µg/d (4000 IU/d) for adults ≥19y of age[9]. Also in Europe, the European Food Safety Authority (EFSA) (2012) established the Tolerable Upper Intake Level at 100 µg/d (4000 IU/d) including pregnant and lactating women[10].

1.1. Vitamin D metabolism

Vitamin D itself is not biologically active. It is converted to the active hormone in the body through a chain of enzymatic reactions[11,12]. Vitamin D from the diet or the skin is transported to the liver through the lymph or blood bound to the vitamin D binding protein (DBP)[11]. In the liver vitamin D is hydroxylated predominantly at the position C-25 by 25-hydroxylase resulting in the formation of 25(OH)D[13]. It is suggested that the molecule can be hydroxylated in other positions as well[13]. 25-hydroxyvitamin D is the major circulating form of vitamin D in the human body[14]. It is used as a marker to assess vitamin D status due to its long half-life (2-3 weeks)[14]. After hydroxylation in the liver 25(OH)D is transported bound to DBP to the kidney. In the kidney, megalin (low-density lipoprotein receptor) allows 25(OH)D to enter the cells through an endocytic process[15]. In the kidney, another hydroxylation reaction occurs at the position of C-1 by 1a hydroxylase (1a(OH)ase), resulting in the formation of $1,25(OH)_2D$ which is the hormonally active form of vitamin D. The half-life of calcitriol is very short (~4 hours)[16]. This reaction primarily takes place in the proximal renal tubular epithelial cells of the kidney.

The 1a(OH)ase enzyme, (encoded by the gene *CYP27B1*) is mostly expressed in the kidney but it is also found in the placenta, macrocytes, macrophages and other organs. These organs/cells hydroxylate 25(OH)D for their own use or for cells close to them (auto- and paracrine effects)[17,18]. Parathyroid hormone (PTH) and fibroblast growth factor 23 (FGF23) influence the expression of CYP27B1[19] *(see section 1.2)*. Mutations in the CYP27B1 gene can cause inactivation in the 1a(OH)ase resulting in vitamin D dependent rickets type 1, which occurs despite normal vitamin D intake or status, hereby indicating the importance of the enzyme for vitamin D function[20].

$1,25(OH)_2D$ is catabolized by 24 hydroxylase (24(OH)ase)[21]. This enzyme hydroxylates both 25(OH)D and $1,25(OH)_2D$[11] but it has been shown that the preferred substrate is $1,25(OH)_2D$[21]. 24-hydroxylase is expressed in the kidney and many other organs and tissues. The main role of 24(OH)ase is to reduce the amount of $1,25(OH)_2D$ in plasma and tissues by promoting the catabolism of $1,25(OH)_2D$ to $1,24,25(OH)_3D$ and further downstream metabolites, leading to the formation of calcitroic acid. It also hydroxylates 25(OH)D into 24,25-dihydroxy vitamin D ($24,25(OH)_2D$)[21]. This results in decreasing the pool of $25(OH)_2D$ available for 1a-hydroxylation, in that way preventing conditions of toxicity from 1,25(OH)D[21] which can cause impaired bone mineralization[22].

Most of the $1,25(OH)_2D$ actions require the activation of a high affinity receptor, vitamin D receptor (VDR)[23]. The initial step to activate the receptor is binding to a molecule of calcitriol which induces or suppresses the expression of gene regulate a wide range of physiological processes including maintaining mineral homeostasis, skeletal health, immune and cardiovascular function[24].

All the vitamin D metabolites circulate bound to proteins predominantly (vitamin D binding protein; DBP)[11]. Small fractions are free in the circulation[11].

1.2. Regulation of vitamin D hydroxylases

1.2.1. Parathyroid hormone and calcitriol

Calcitriol and Parathyroid hormone regulate the expression of CYP27B1 and CYP24A1 and through this vitamin D metabolism[25]. Indirectly, low dietary calcium (Ca) enhances the activity of 1a(OH)ase[11]. This positive feedback occurs with the stimulation of PTH secretion which

mediates the production of 1,25(OH)$_2$D in the kidney through increasing the transcription of CYP27A1[26,27]. On the other hand, a negative feedback loop occurs (**Figure 4**) through an increase in intestinal calcium absorption. That suppresses PTH and 1,25(OH)$_2$D[11]. 1,25(OH)$_2$D suppresses PTH production, 1a(OH)ase gene expression[28] and stimulates 24(OH)ase expression[29] (*see section 6.3.1*).

1.2.2. Fibroblast growth factor-23

Increased levels of FGF23 suppress the expression of 1a(OH)ase and promotes catabolism of 1,25(OH)$_2$D through increased 24(OH)ase expression in the kidney. This leads to a decrease in FGF23 expression in bone creating a negative feedback loop[30]. FGF signaling requires Klotho, a protein involved in calcium and phosphate homeostasis, as a cofactor *(see section 4*). Loss of Klotho leads to an increased expression of 1a(OH)ase although the exact mechanism of this is still unknown[31]. Fibroblast growth factor-23 (FGF23) it is produced mainly by osteocytes and osteoblasts[32]. It belongs to the FGF19 subfamily that function through an endocrine mechanism[33]. FGF23 is a phosphaturic hormone which stimulates renal excretion of phosphate by inhibiting its reabsorption in the proximal tubule in response to an elevated plasma phosphate concentration[34]. It is also a regulatory factor for vitamin D metabolism as described above[30,34]. 1,25(OH)$_2$D stimulates the production of FGF23 in bone, independent to phosphate serum levels[35].

Elevated FGF23 is linked to pathological conditions such as hypophosphatemia, low serum 1,25(OH)$_2$D, rickets and osteomalacia[30]. High FGF23 has been identified as the cause of autosomal dominant hypophosphatemic rickets (ADHR), X-linked hypophosphatemic rickets (XLH) and tumor-induced osteomalacia (TIO) mainly due to excessive phosphate excretion[36–38].

1.2.3. Other hormones regulating vitamin D metabolism

It has been reported that oestrogen alone or with progesterone or androgens stimulate 1,25(OH)$_2$D production[39–41]. Also, oestrogen suppresses 24,25(OH)$_2$D synthesis[39]. Studies showed a direct effect of calcitonin on renal 1a(OH)ase transcription increasing circulating 1,25(OH)$_2$D in case of increased calcium requirements[42]. This is particularly important during

phases of growth or lactation when calcitonin protects against excessive resorption and promotes calcium accretion into the skeleton[42]. Prolactin, a hormone that increases during lactation, is related with the rate of 1a(OH)ase transcription and can stimulate $1,25(OH)_2D$ production[42]. Therefore, calcitonin and prolactin have a stimulatory effect on calcium absorption and inhibit bone resorption and through this protect the maternal skeleton during lactation[13,43].

Under conditions with high calcium demand, calcitonin decreases osteoclast activity in order to inhibit bone reabsorption and calcium release in the circulation[39]. Elevated calcitonin is correlated with smaller size osteoclast[39]. These actions of calcitonin are very important during lactation when the requirement for calcium is increased and both $1,25(OH)_2D$ and calcitonin levels are elevated.

2. Bone structure and physiology

2.1. Bone anatomy

The human skeleton has various functions. The bones of the skeleton provide structural support for the body, protect vital organs, regulate mineral homeostasis and provides the environment for hematopoiesis[44]. There are four categories of bones: the short bones, the long bones, the irregular bones and the flat bones[45].

Macroscopically, the bone has two components: cortical and trabecular bone. Cortical bone forms the shaft of long bones and the outer surface of flat bones. The texture of cortical bone is very dense and it has mechanical and protective functions[46]. Trabecular bone is found at the epiphysis (**Figure 1**) of long bones, in the pelvis, ribs, skull and vertebrae. It consists of a latticework of trabeculae[46] and is mineralised in a highly regulated process[41]. Trabecular bone has a major metabolic role in mineral homeostasis[46].

The main component of the extracellular mineralised matrix of bone consists of hydroxyapatite crystals bound to proteins, mostly collagen[46]. New bone is initially synthesised as osteoid (unmineralised bone matrix) which consist of type I collagen and glycosaminoglycans[47]. Matrix maturation is directly associated with alkaline phosphatase

expression and some calcium- and phosphate-binding non-collagenous proteins such as osteopontin, osteocalcin and sialoprotein (which are incorporated in the osteoid)[45].

2.2. Bone cells

There are number of cell types: osteoblasts, osteoclasts, osteocytes, bone lining cells and cells of vascular and nervous supply. Osteoblasts are known for their function as bone forming cells[39,40]. They originate from mesenchymal stem cells (MSC) (**Figure 1**). Many factors affect osteoblast differentiation such as components of the Wnt-singlling pathway, PTH, $1,25(OH)_2$ vitamin D, FGF18 and connexin 43[48,49]. Osteoblasts synthesise organic bone matrix and regulates its mineralization during the process of bone formation[50,51]. Mature osteoblasts can undergo apoptosis or differentiate to become bone lining cells or osteocytes [52,53].

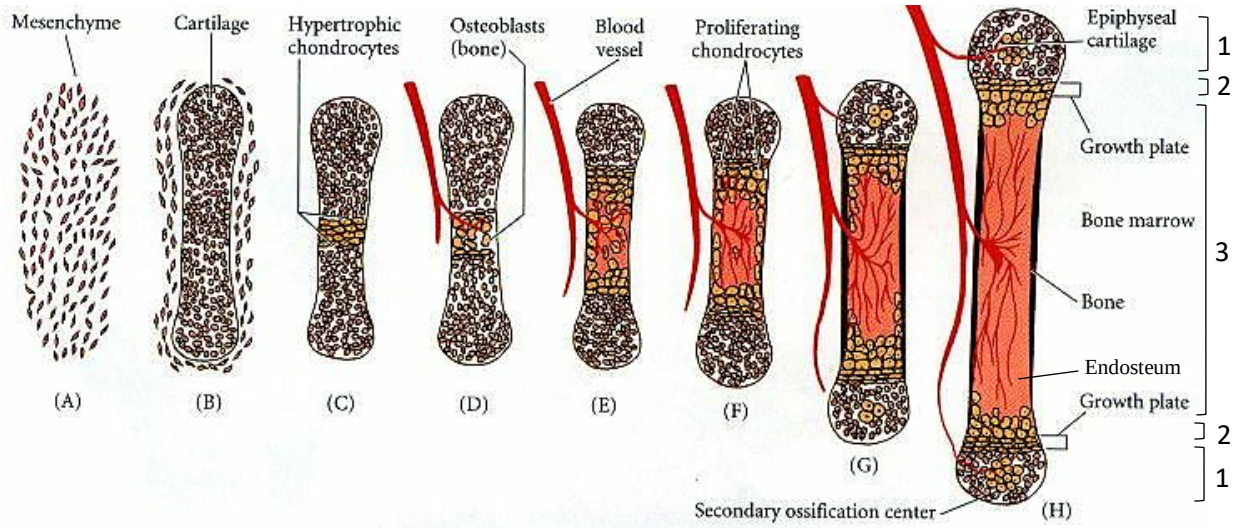
Bone lining cells are flat-shaped osteoblasts on the bone surface[53]. Their function is not completely clear. They have a protective function against bone resorption[53]. Bone lining cells prevent the direct interaction between bone matrix and osteoclasts[53]. Bone lining cells also produce the receptor activator of nuclear factor kappa-B ligand (RANKL) and osteoprotegerin (OPG) which regulate osteoclast differentiation (*see section 2.3*)[54,55]. It is also suggested that RANKL and OPG are produced by osteoblasts and osteocytes[54,55].

Osteocytes are the most abundant bone cells and are located in lacunae within mineralized bone matrix[53]. In contrast with the osteoblasts, osteocytes have a dendritic cell morphology, although this depends on the bone type[56]. Osteocytes are derived from MSCs through osteoblast differentiation. Osteocyte differentiation can be divided in four stages: osteoid-osteocyte, preosteocyte, young osteocyte and mature osteocyte[56]. They are close to vascular supply from where they get oxygen and nutrients for their survival[56]. Osteocytes have an important role in osteoblast-osteoclast communication[57]. They sense mechanical loading through fluxes of the interstitial fluid[57]. Through this mechanism, osteocytes control bone remodelling by regulating osteoblasts and osteoclasts activity in order for bone to adapt to mechanical loading[58].

Osteoclasts are the only resorbing bone cells[45]. Osteoclastogenesis is regulated by many factors. receptor activator of nuclear factor kappa-B RANKL is the main factor and is produced by bone lining cells, osteoblasts and osteocytes. RANKL binds to its receptor receptor

activator of nuclear factor kappa-B (RANK) in osteoclast precursor cells and induces osteoclast differentiation[59]. This can be inhibited by OPG which can also bind to RANK receptor there by preventing the RANKL/RANK interaction[60]. This mechanism is regulated by other factors (*see section 2.3*) and are directly related with the rate of bone remodelling.

Figure 1. Endochondral ossification



A, B: Mesenchymal cells condense and differentiate into chondrocytes and form the cartilaginous model of the bone. **C**: Chondrocytes while change and mineralize their extracellular matrix undergo hypertrophy and apoptosis. Their apoptosis open access for blood vessels to enter. **D, E**: Blood vessels bring in osteoblasts, which bind to the degenerating cartilaginous matrix and deposit bone matrix. **F-H**: Bone formation and growth consist of an ordered arrangement of proliferating, hypertrophic, and mineralizing chondrocytes. Secondary ossification also occurs when blood vessels enter near the tips of the bone[61].
1: epiphysis, **2**: metaphysis, **3**: diaphysis[62].

2.3. Bone modelling and remodelling

Bone constantly undergoes resorption and formation in order to adjust to the mechanical demands of the body (bone modelling) and to repair damages (bone remodelling). Modelling is a process during which the shape and the size of the bone is changed in contrast with remodelling where new bone replaces old bone at the site of resorption[46]. During childhood and adolescence bone modelling is necessary for longitudinal and radial growth and to increase bone mass.

Bone remodelling starts before birth and continues until death. This procedure is required in order to maintain bone strength and mineral homeostasis by removing discrete pockets of old bone and replacing them with newly formed bone[45]. Under physiological conditions the amount of resorbed bone is equivalent to the newly formed bone[45]. However, with ageing less bone is formed and loss of bone mass occurs[45]. In healthy people osteoclasts and osteoblasts work in a tightly coupled group during bone remodelling. The remodelling cycle can be divided into four phases: activation, bone resorption, reversal and bone formation[45] (**Figure 2**). Bone resorption lasts about two to four weeks in every bone remodelling cycle. The remodelling cycle is regulated from a variety of factors such as the ratio of RANKL/OPG, cloning-stimulating factor (CSF), PTH, $1,25(OH)_2D$ and calcitonin[63,64]. During the activation phase, mononuclear monocyte-macrophage and osteoclast precursors are recruited from the circulation[45]. Bone lining cells move away from the bone surface exposing the bone matrix to preosteoclasts[45]. Osteoclasts are formed to start the resorption process[45] (**Figure 2**). They secrete hydrogen ions into the resorbing cavity[45]. The hydrogen ions acidify the environment, mobilizing bone minerals and degrading type-I collagen[45]. The end products of bone resorption, collagen fragments, phosphate and calcium are released in the circulation. Part of the removed calcium and phosphate is re-incorporated in the matrix[65]. The remainder is transported through the blood to kidneys for excretion[65]. Some of these end products that are released in the circulation, such as, collagen fragments and matrix proteins. These are used as bone turnover markers and can be measured in the blood or urine depending on the marker tested[66] (**Table 1**). The reversal phase is the intermediate stage between resorption and formation where the newly formed bone cavity is being prepared for osteoblast formation. Transforming growth factor beta (TGF-β) is released from the resorbed bone matrix and inhibits osteoclast resorption through RANKL[67,68]. Bone formation is a much longer process than bone resorption and it takes approximately four to six months[45]. Osteoblasts produce new collagenous organic bone matrix on the resorbed cavity and regulate the mineralization of the newly synthesised bone[51] (**Figure 2**). At the end of the bone formation phase half or more of the osteoblasts undergo apoptosis and the rest of the cells differentiate into osteocytes and bone lining cells[45]. Perimenopausal and early postmenopausal women have an increased bone remodelling rate. This decreases with further ageing but it is still higher than premenopausal women and both pre- and post-andropausal men[45] (**Figure 3**).

The rate of bone modelling is increased under pathological conditions such as hyperparathyroidism[69] and renal osteopathy which lead to bone loss[70].

Figure 2. Bone remodelling cycle[45]

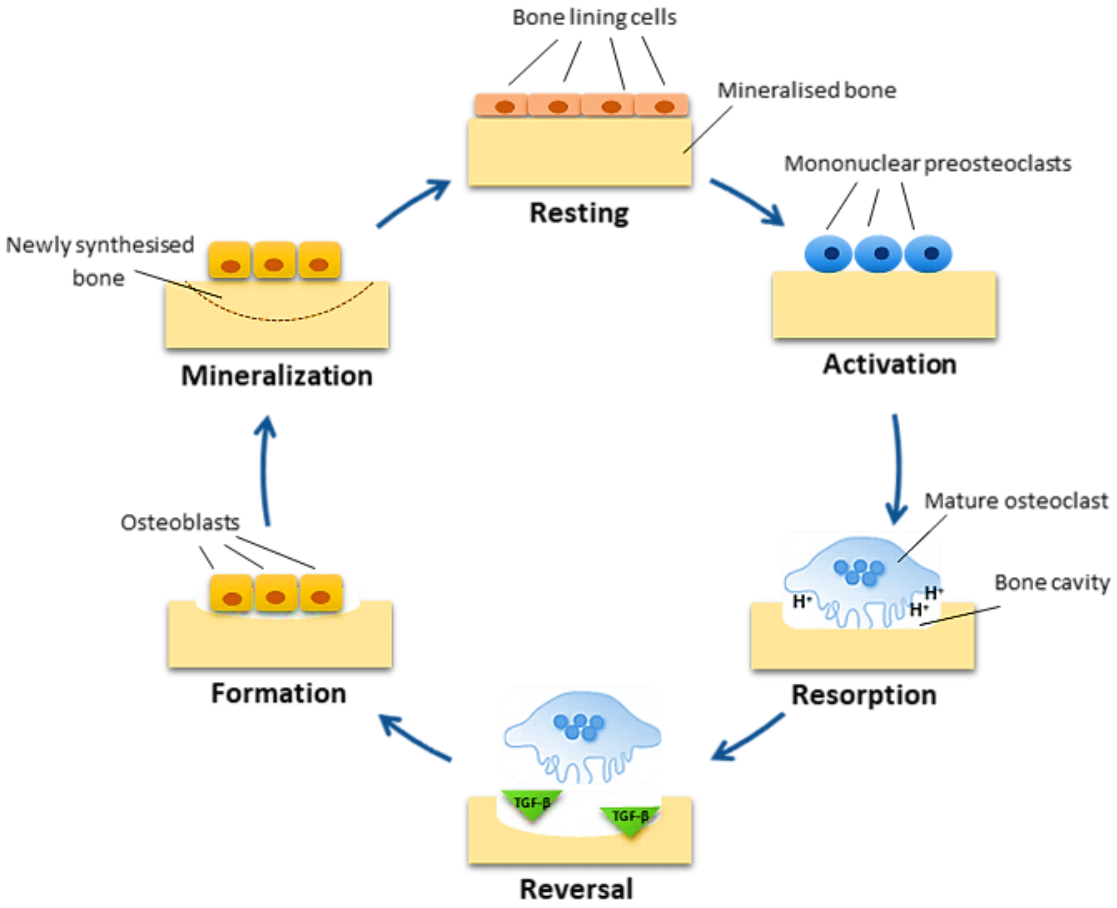
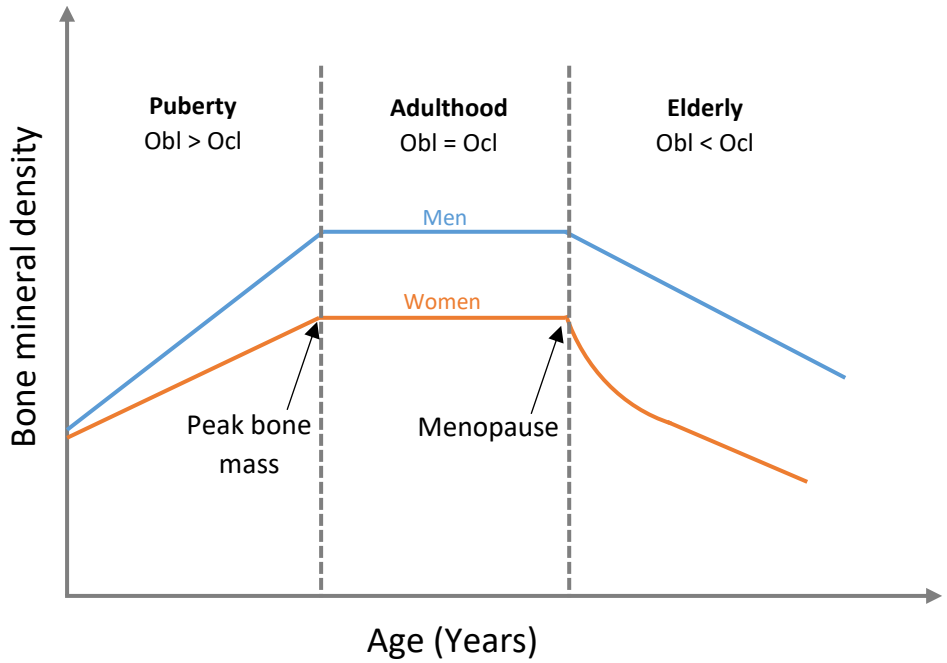


Figure 3. Bone mineral density during the life cycle



Bone mineral density (BMD) differs between male and females throughout the life cycle. Females have a lower peak bone mass compared to men. During menopause BMD rapidly declines in contrast with men where the decrease is more linear. Bone cells ratio changes as well, as shown on the graph, in each phase and is independent of sex[45].
Obl: osteoblasts, **Ocl**: osteoclasts

2.4. Bone mineral density and bone biochemical markers

Bone mineral density (BMD) and bone mineral content (BMC) are the diagnostic criteria for osteoporosis[71]. BMD is measured with dual-energy X-ray absorptiometry (DXA). A DXA scan compares measured bone density with a reference bone density value for young healthy adults (30 years) of the same gender and ethnicity[72]. The difference of the measured value and the reference value is calculated as a standard deviation (SD) score[72]. This is known as the T-score[72]. BMD may also be expressed as the Z-score. This is the difference of measurement and reference values for healthy adults with the same characteristics (age, gender and potentially ethnicity)[72]. According to UK NHS guidelines, above -1 SD is normal, below -2 is low bone density, between -1 and -2.5 is mildly reduced BMD and at or below -2.5 is defined as osteoporosis[72].

The biochemical measurement of bone turnover markers can support the differential diagnosis of osteoporosis at they indicate the rate of bone formation and resorption. Bone

markers can be classified as an indicators of bone resorption and bone formation (**Table 1**). Increased rates of bone turnover markers are correlated with higher fracture risk in older adults in western populations[66]. Assessment of bone turnover markers (**Table 1**) in combination with other classical risk factors are suggested to improve the assessment of fracture risk[73]. However, the interpretation of these markers is complicated due to the biological variability and they are influenced by different physiological and pathological conditions. The International Federation of Clinical Chemistry and Laboratory Medicine recommend the use of C-terminal telopeptide of type-I collagen (CTX) and procollagen type-I N peptide (PINP) as reference analytes[74] (**Table 1**).

Table 1. Biochemical markers of bone turnover[75–77]

Markers of bone resorption	Molecule type	Tissue of origin	Analytical source	Comments
N- terminal telopeptide of type-I collagen (NTX)	Telopeptide	All tissues containing type-1 collagen (bone, skin, connective tissue)	Urine†/serum	NTX is generated by osteoclasts during bone resorption and released in the circulation. Serum or urine NTX measurement reflects the rate of bone resorption.
C- terminal telopeptide of type-I collagen (CTX)	Telopeptide	All tissues containing type-1 collagen (bone, skin, connective tissue)	Urine†/serum	During ageing of the molecule, the C-terminal telopeptides a1 chain undergoes β-isomerization. Serum β-CTX represents breakdown of mature collagen.
Deoxypyridinoline (DPD)	Protein	Bone, connective tissue	Urine*	PYD cross-links is a combination of three hydroxylysine side-chains and DPD is a combination of two hydroxylysine side-chains and one lysine chains. DPD is more bone specific than PYD. These two molecules' pyridinium ring connects the collage molecules and stabilizing the structure of type-1 collagen. During bone resorption and collagen breakdown PYD and PDP are released into the circulation.
Pyridinoline (PYD)	Protein	Bone, connective tissue	Urine*	
Bone sialoprotein (BSP)	Glycoprotein	Bone, dentin, cementum and calcified cartilage	Serum/ plasma/ synovial serum fluid	Is one of the most abundant proteins in bone. It is involved in mineralization of new bone matrix and the calcification of extra-skeletal tissues. It is a highly acidic protein with very strong affinity to hydroxyapatite.
Tartrate-resistant acid phosphatase (TRAP)	Glycosylated monomeric metalloprotein enzyme	Osteoclasts, macrophages, dendritic cells and other cell types	Serum	Is expressed in osteoclasts and it is involved in skeletal development, collagen synthesis and degradation and bone mineralisation. TRAP can degrade skeletal osteoproteins.
Markers of bone regulation	**Molecule type**	**Tissue of origin**	**Analytical source**	**Comments**
Sclerostin (SOST)	Glycoprotein	Bone (osteocytes)	Serum	Negative regulators of bone formation. They inhibit osteoblast activity via blocking Wnt pathway. DKK1 is recommended to be measured in plasma instead of serum since it is present in blood platelets. Both are new biomarkers and their exact function is still unclear.
Dickkopf-related protein 1 (DKK1)	Protein (cytokine)	Bone (osteocytes)	Plasma	
Markers of bone formation	**Molecule type**	**Tissue of origin**	**Analytical source**	**Comments**

Alkaline phosphatase (ALP)/ Bone specific alkaline phosphatase (BAP)	Glycosylated protein	In most tissues (ALP)/ Specific originated from bone tissue (BAP)	Serum/ plasma	Serum alkaline phosphatase consists of liver and bone isoforms. In people with normal liver function total alkaline phosphatase can be used to assess bone metabolism. BAP is found on the surface of osteoblast cells and reflects their biosynthetic activity.
Osteocalcin (OC)	Non-collagenous protein (with hormonal functions)	Bone, dentin, calcified cartilage	Serum	Is synthesized by mature osteoblasts. Fragments of OC are released in the circulation during bone resorption.
Type-1 procollagen C-terminal propeptide (PICP)	Propeptide	Soft connective tissues and bone	Serum	Osteoblasts synthesize procollagen a precursor of type-1 collagen in bone. Procollagen is cleaved and the C-terminal and N-terminal peptides (PICP and PINP) are released in the circulation. The serum levels are mainly from skeletal sources due to its high rate of turnover.
Type-1 procollagen N-terminal propeptide (PINP)	Propeptide	Soft connective tissues and bone	Serum	

†Second morning void without dietary restrictions, *Random urine sample

3. *The kidney: Renal physiology and glomerular filtration*

The kidneys are a pair of organs located in the posterior wall of the abdomen[78]. The outer region of the kidney is the cortex and the inner region is the medulla[78]. Both consist of nephrons, blood vessels, lymphatics and nerves. The medulla is divided in different renal pyramids[78]. These are placed between the corticomedullary border and the minor calyx[78]. The minor calyces collect urine which then through the major calyces is transferred to the ureter and then the urinary bladder for excretion[78].

Nephrons are the functional unit of kidneys. The nephron is a single cell layer tube and consists of renal corpuscle, proximal tubule, loop of Henle, distal tubule and a collecting duct system[78]. The renal corpuscle consists of glomerular capillaries and Bowman's capsule[78]. Each nephron segment has unique suited cells which perform specific transport functions (*see section 4.3*).

The blood flow of the kidneys is approximately 25% of the cardiac output (in resting stage)[78]. The blood supply in the kidney has a key role in various functions[78]. It determines the glomerular filtration rate (GFR), modifies the rate of reabsorption in the proximal tubule, influences the concentration of urine, delivers oxygen, nutrients and hormones to renal cells and returns carbon dioxide and the reabsorbed fluid to the general circulation and delivers substrate for excretion[78]. The first step in the formation of urine is the production of an ultrafiltrate from the plasma across the renal filtration barrier[78] of the glomerulus. The composition of the ultrafiltrate is determined by the pore size of the restricting the size of the molecules filtered[78]. In healthy individuals the plasma ultrafiltrate does not contain cellular elements e.g. blood cells, platelets and the protein concentration is very low[78]. This contrasts with the organic molecules (e.g. glucose and amino acids) and salts which have similar concentrations as in the plasma[78].

The second step of urine formation is the reabsorption of water and other molecules from the ultrafiltrate[78]. That occurs across the different segments of the nephron through diverse transepithelial mechanisms specific for each segment (*see section 4.3 and 5.1*). The reabsorption procedure regulates and limits urinary losses of key molecules as necessary for homeostasis e.g. calcium, phosphate and magnesium[78]. The last step of the urine formation is the secretion of selected molecules into the tubular fluid[78]. The tubules regulate the volume, the composition, the osmolality and the pH of the intracellular and extracellular

fluid[78]. Under pathological conditions these processes may be altered, resulting in various changes in the urine composition and molecules retained and accumulating in the human body as a result or causing disease (*see section 7*).

4. Calcium and Phosphate metabolism

The tight regulation of the plasma and intracellular calcium and phosphate concentration is very important since they have major physiological functions. Homeostasis of these two minerals is mainly regulated by 1,25$(OH)_2$D, PTH and FGF23 through their actions on the specific target organ systems: the intestine, bone and kidney.

4.1. Intestine

The intestine has an important role in calcium homeostasis. It is the organ where dietary calcium is absorbed. Vitamin D (1,25$(OH)_2$D) regulates calcium absorption in the intestine in order to maintain the calcium homeostasis[79] (**Figure 4**). There are two pathways, active and passive[80]. The passive, paracellular pathway of calcium absorption becomes dominant when calcium intake is high and it is regulated by transepithelial electrochemical gradients[81]. The active pathway of calcium absorption is upregulated by 1,25$(OH)_2$D in cases of high tissue requirements or low dietary intake of calcium. In more detail, 1,25$(OH)_2$D stimulates the absorption of calcium ions by the epithelial cells through the upregulation of transient receptor potential vanilloid type 6 (TRPV6) calcium channel and calbindin (calbindin-D_{9k}), an intracellular binding protein[79,80]. Calcium ions are translocated through the TPRV6 channel in the enterocyte and binds to calbindin-D_{9k} in order to be transferred to the basolateral space[82]. Via Na^+/Ca^{2+} exchanger and the intestinal plasma membrane Ca^{2+} ATPase 1b (PMCA1b), calcium ions are transferred into the circulation[82]. Most calcium is be absorbed in the duodenum and jejunum but also small amounts of calcium can be absorbed in the ileum and colon[79]. Active calcium absorption in the intestine decreases with age[79]. That is a result of age related decline in oestrogen and decreased intestinal sensitivity to 1,25$(OH)_2$D[83].

Intestinal absorption of phosphate takes place mainly in the duodenum and jejunum through paracellular pathways[84]. Active absorption take place through the type-2 sodium-phosphate

co-transporter (NaPi-2b) which is expressed in the intestine and is regulated by 1,25$(OH)_2$D and dietary phosphate intake[84]. From the enterocyte, phosphate molecules pass into the circulation through an unknown basolateral transporter[82]. Active phosphate absorption, regulated by 1,25(OH)D accounts for up to 30%[82] and the remaining 70% of phosphate is absorbed through passive paracellular absorption[82]. Therefore phosphate absorption is highly dependent on phosphate intake[82]. Maintaining phosphate balance occurs predominantly in the kidney through the regulation of renal phosphate reabsorption and excretion[82]. Any damage or renal impairment can therefor affect phosphate homeostasis[82].

4.2. Bone

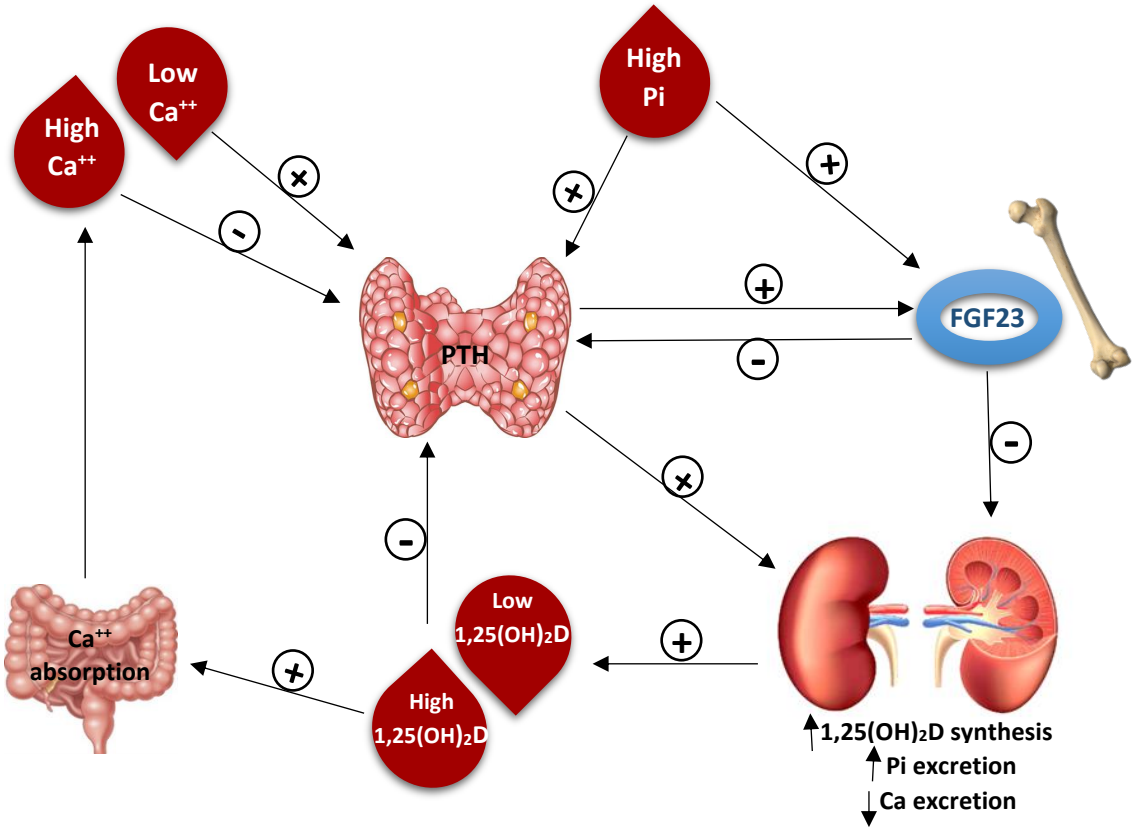
In response to a low circulating calcium and phosphate concentration, PTH and 1,25$(OH)_2$D increase[19] (**Figure 4**). PTH stimulates bone resorption for calcium and phosphate to be released in the circulation[19]. However, PTH does not act directly on osteoclasts because they have no PTH receptors[19]. PTH signaling occurs through osteoblasts through the activation of expression of RANKL and Macrophage colony-stimulating factor[19]. This in return activates osteoclasts differentiation and through this, stimulate osteoclastic mediated bone resorption *(see section 2.2*). Also 1,25$(OH)_2$D increases the expression of RANKL gene[47].

4.3. Kidney

Renal reabsorption of calcium occurs through a passive (paracellular) route or an active (transcellular) route[85]. Approximately 80% of calcium is reabsorbed through the passive route[84]. Passive reabsorption of calcium takes place in the proximal tubule of the nephron together with sodium and water. Active renal calcium reabsorption mainly occurs in the distal tubule. PTH stimulates both active and passive routes. It activates the apical Na^+-K^+-$2Cl^-$ cotransporter which stimulates paracellular calcium reabsorption[86]. Transcellular calcium reabsorption involves activation of transient receptor potential cation channel subfamily V member 5 (TPRV5) which allows calcium ions to enter the cell, bind to calbindin-D_{28k} and be transported to the basolateral side through a sodium-calcium exchanger (NCX1) and PMCA1b transporter[87,88] (*see section 4.1.2)*.

The kidneys regulate phosphate homeostasis through its reabsorption and excretion. Approximately 85% of phosphate reabsorption occurs in the proximal tubule[89]. Phosphate is transferred from the luminal filtrate into the cell though an energy dependent process which requires sodium[90]. There are three phosphate cotransporters: Npt2a, Npt2c and PiT-2 all on the apical brush border membrane of the proximal tubule[90]. The amount of reabsorbed phosphate depends on the number of phosphate cotransporters on the cell membranes[90]. An increase in the dietary intake of phosphate leads to internalization and degradation of Npt2a, Npt2c and PiT-2 from the proximal renal tubule in order to decrease renal reabsorption. In contrast, dietary restriction of phosphate leads to an increase in expression and in the abundance of Npt2a, Npt2c and PiT-2[84]. PTH and FGF23 act on the kidney and increase phosphate excretion by decreasing the number of phosphate cotransporters on the brush border membrane of proximal tubule cells[91,92] *(see section 4.1.1)* (**Figure 4**).

Figure 4. Parathyroid hormone regulation signalling



Hypercalcemia reduces PTH secretion and promotes PTH fragment release in contrast with hypocalcemia which stimulates intact PTH secretion. High Pi stimulates PTH secretion and. $1,25(OH)_2D$ inhibits the production of PTH either directly by affecting the transcription of the PTH gene or indirectly by increasing the intestinal calcium absorption which then activates the calcium sensing receptor (CaSR). FGF23 is stimulated by high serum Pi or $1,25(OH)_2D$. FGF23 decreases PTH secretion and renal $1,25(OH)_2D$ synthesis and increase urinal excretion of Pi[93].

5. Parathyroid hormone synthesis and functions

PTH is a single chain hormone (84 amino acids) mainly produced by the chief cells in the parathyroid gland[93]. Pre-pro-PTH (115 amino acids) breaks down to pro-PTH (90 amino acids) in the endoplasmic reticulum and finally it converted into (1-84)PTH by the Golgi complex[94]. (1-84)PTH is stored in a secretory granules until it is released in the blood stream[94]. Catabolism of PTH occurs in liver and fragments are cleared from the circulation through the kidneys[94]. Therefore any renal impairment might lead to accumulation of PTH fragments[94]. In the circulation, PTH is present in its intact form (the full length 1-84 PTH) and different fragments of PTH[93]. PTH fragments include carboxyl terminal PTH (C-PTH), amino terminal PTH and mid length PTH[94,95]. C-PTH includes (7-84)PTH, (10-84)PTH and (15-84)[96,97]. PTH metabolism and secretion from the parathyroid gland is mainly regulated by extracellular

concentration of calcium[93], through its binding and activation of calcium-sensing receptor (CaSR) on the parathyroid cells[93]. Other factors that regulate PTH secretion include plasma phosphate, $1,25(OH)_2D$, FGF23 in a system of negative and positive feedback loops[93] (**Figure 4**). Accordingly, plasma PTH increases in response to impaired $1,25(OH)_2D$ production (e.g. with CKD or severe vitamin D deficiency) and low calcium absorption. Also increased resistance of the kidneys and bone to PTH, due to a downregulation of its receptor[98] leads to an increase in PTH. An increase in plasma phosphate and FGF23 further stimulate PTH secretion.

As set out above, PTH regulates the activation of vitamin D in the kidney and renal calcium and phosphate reabsorption and excretion.

Intact PTH (iPTH) regulates bone remodelling through a direct pathway acting on osteoblasts and osteocytes or indirectly through osteoclasts[93] (*see section 2.3*). The effect of PTH can be anabolic (intermitted exposure) or catabolic (sustained high PTH concentrations)[93]. The anabolic effect of PTH acts via downregulation of sclerostin expression in osteocytes thus Wnt-signalling pathway can proceed[93] (*see section 6.3.2*).

The actions of PTH are predominantly through the PTH-receptor 1 (PTHR1). PTH binds to the PTH-receptor which activates the protein kinase A (PKA) and C (PKC), resulting in phosphorylation of Na+/H+ exchange regulatory factor (NHERF-1) and this results in phosphate excretion[97] (**Figure 5**).

Intact 1-84 PTH is the most biological active form, but (1-34)PTH has also been suggested to stimulate the synthesis of $1,25(OH)_2D$. It is suggested that (7-84)PTH is an antagonist of the biological activation of (1-84)PTH in bones and kidneys[99,100]. (7-84)PTH binds the C-PTH receptor (C-PTHR) which is mainly expressed in osteoblasts and osteocytes but it can also bind PTHR1, and through this, antagonize the effect of PTH on the PTHR1[93]. The (7-84)PTH fragment has also been reported to antagonize the synthesis of $1,25(OH)_2D$[100]. With CKD, the skeleton becomes resistant to the action of PTH. This is thought to be partly mediated through the increased concentrations c-terminal fragments and reduction of receptor expression.

6. FGF23 and Klotho signaling

FGF23 is a phosphaturic hormone. Its main function is to reduce phosphate reabsorption from the glomerular filtrate through downregulating the available sodium phosphate cotransporters[32,36]. FGF23 can also downregulate the expression of 1a(OH)ase in the kidney suppressing the production of active vitamin D and increasing the catabolism of $1,25(OH)_2D$. This indirectly suppresses plasma phosphate through decreasing $1,25(OH)_2D$ mediated active intestinal absorption[32]. Osteoblasts and osteocytes are the main sources of FGF23 as mentioned above[32]. High extracellular phosphate and $1,25(OH)_2D$ stimulate the production of FGF23 resulting in a feedback loop between bone and kidney[101,102]. FGF23 receptor binding requires a formation of a complex consisting of FGF receptors (FGFRs) and the transmembrane protein αKlotho (Klotho)[103,104]. There are four types of FGFRs (FGFR1, 2, 3 and 4). It has been suggested that Klotho can bind to FGFR1, 3 and 4 but not FGFR2[104]. There is only one known Klotho gene expressed in mammalians, αKlotho, but there are 2 isoforms of the Klotho protein (the full length protein 130-kDa and an alternative smaller protein 62 kDa)[105]. Klotho is mainly expressed in proximal and distal renal tubules, the choroid plexus and the parathyroid glands[106,107].

6.1. FGF23 and Klotho signalling in the kidney

6.1.1. Renal proximal tubule

Kidney is one of the major target organs for FGF23 and Klotho. As mentioned above, FGF23 increases excretion of phosphate in urine by decreasing its reabsorption through suppression of the apical membrane abundancy of Npt2a and Npt2c[32,108] (**Figure 5**). In more detail, FGF23 downregulates the expression of phosphate cotransporters (NaPi-2a) in renal proximal tubule membrane by phosphorylation of the NHERF-1[107]. This occurs through a Klotho dependent mechanism that involves extracellular signal regulated kinase-1 (ERK1/2) and serum glucocorticoid regulated kinase-1 (SGK1)[107] (**Figure 5**). Phosphorylation of Na+/H+ exchange regulatory factor-1 (NHERF-1) results in degradation and internalization of NaPi-2a[109,110]. Mice with a deletion of the FGFR1 gene (but not FGFR3 and FGFR4) in the proximal renal tubule are resistant to the phosphaturic actions of FGF23 which suggest that FGFR1 is

essential for the FGF23 signalling in proximal renal tubule[111], although very little is known about the role of different FGFRs in the proximal renal tubule.. *In vivo* studies suggest that FGF23 predominantly acts in the distal renal tubule[111].

PTH and FGF23 have similar physiological effects on phosphate reabsorption and there is interaction between these two hormones in the regulation of renal phosphate handling.[107,110]. It has been suggested that PTH has a permissive role in FGF23 mediated renal phosphate[112]. Similar to FGF23, PTH is also involved in the phosphorylation of NHERF-1[97].

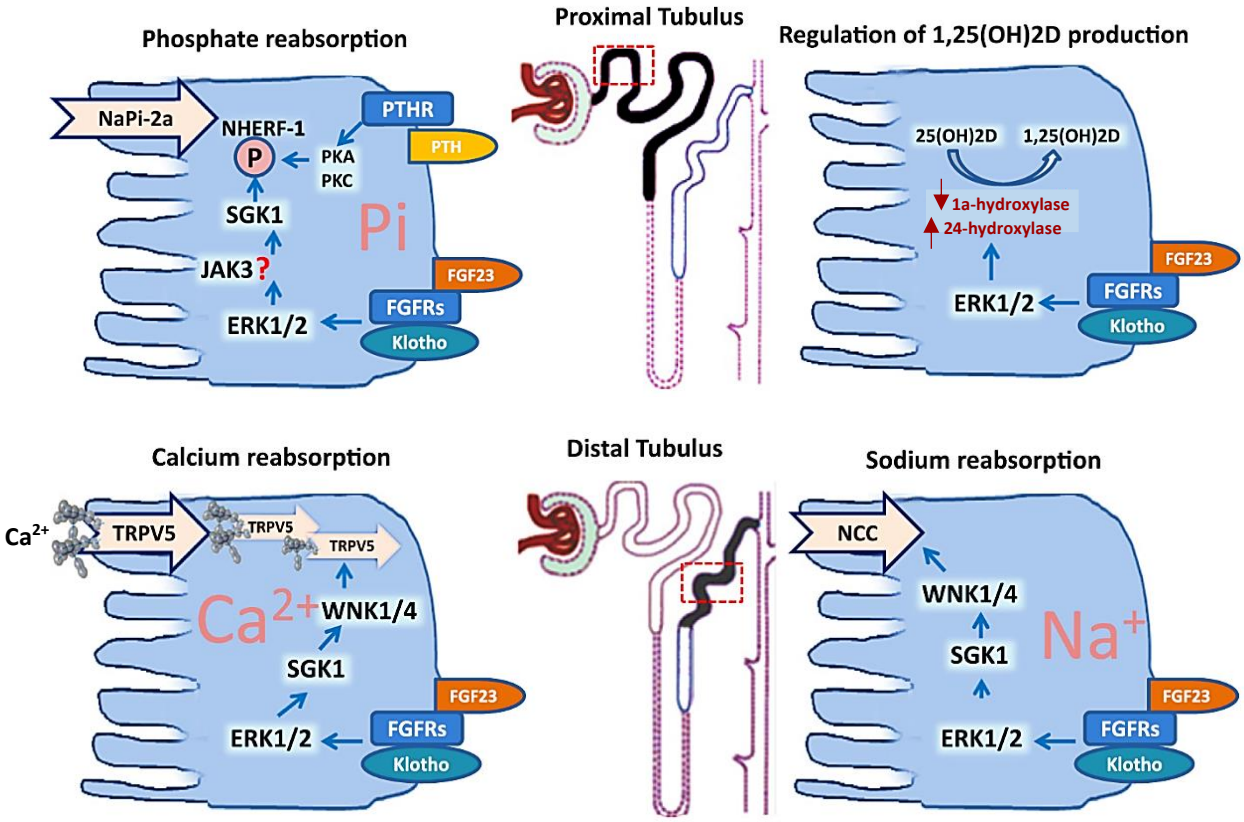
FGF23 and PTH have important roles in the regulation of the $1,25(OH)_2D$ production in the kidney. 1a(OH)ase is mainly expressed in the proximal tubule and is tightly regulated by PTH, FGF23 and $1,25(OH)_2D$[113]. The activity of this enzyme is suppressed by FGF23 and $1,25(OH)_2D$ but stimulated by PTH[114]. A study showed that functional loss of FGF23 and Klotho cause tumoral calcinosis a result of high $1,25(OH)_2D$, hyperphosphatemia, hypercalcemia, calcification of blood vessels and soft tissues[115,116]. Studies on phenotypes of knock out Klotho (-/-) and FGF23 (-/-) suggest that FGF23 regulates 1a(OH)ase expression through a Klotho and ERK1/2 dependent mechanism[117] (**Figure 5**). These knock out mice are characterised by an elevated serum level of FGF23 which is correlated with high ERK1/2 signalling. Activation of the ERK1/2 by FGF23[118] can subsequently suppress 1a(OH)ase transcription[119] (**Figure 5**). Blockage of ERK1/2 pathway improved hypophosphatemia, bone mineralization defects and lower $1,25(OH)_2D$ concentration[119].

6.1.2. Renal distal tubule

Klotho is expressed in the distal renal tubule (**Figure 5**), where calcium reabsorption occurs[120]. Calcium reabsorption requires the action of both Klotho and FGF23[120]. FGF23, through the FGFR/Klotho receptor complex activates ERK1/2, SCK1 and the kinase with-no-lysine kinase 4 (WNK4)[97]. WKN kinases are main regulators of intracellular protein transportation which act in a complex of WKN1, 3 and 4[121,122]. *In vivo* studies suggest that plasma FGF23 is positively associated with active calcium reabsorption in the renal distal tubule[97], where calcium is reabsorbed through the TPRV5 channel (**Figure 5**)[87]. FGF23 and Klotho regulate the abundance of the TPRV5 on the apical membrane[87]. This pathway is independent of $1,25(OH)_2D$ concentration[97]. Studies in mice showed that injections with FGF23 upregulates TPRV5 membrane expression and reduce urinary excretion of calcium[97]. Another study

confirmed this, by showing renal calcium wasting in knockout mice with *fgfr1* deletion in distal tubule[111].

Figure 5. Renal FGF23-Klotho signalling[97]



Proximal renal tubules: FGF23 binds to FGFRs and αKlotho (Klotho) receptor complex and activates a signalling pathway involving ERK1/2 and SGK1. Activated SGK1 in turn phosphorylates NHERF-1, resulting in internalization and degradation of NaPi-2a. FGF23 signalling may also involve Janus kinase-3 (JAK3) but this is still unclear. PTH binds to the PTH receptor (PTHR), which activates PKA and PKC, and subsequent phosphorylation of NHERF-1. Phosphorylation of NHERF-1 reduces the membrane abundance of NaPi-2a and leads to increased urinary phosphate excretion. The FGF23 signalling activates downstream of ERK1/2 which in return suppresses the transcription of 1α-hydroxylase in proximal renal tubule cells, regulating plasma $1,25(OH)_2D$. **Distal renal tubules**: circulating FGF23 binds to the FGFRs-Klotho receptor complex, activates ERK1/2, SGK1, and then WNK1/4 complex. Therefore, stimulates the luminal membrane abundance of glycosylated TRPV5 and of Na+-Cl− cotransporter (NCC), resulting to increased distal tubular Ca^{2+} and Na^{+} reabsorption[97].

6.2. The interaction of parathyroid hormone and the FGF23-Klotho axis

Sustained elevated PTH levels stimulate the secretion of FGF23 in bone[123]. FGF23 inhibits the secretion of PTH, forming a negative feedback loop between the bone and the parathyroid

gland[123]. The regulatory mechanism by which regulates PTH secretion by FGF23 is not yet fully clarified[124]. Klotho and FGFRs are expressed in parathyroid gland[125,126]. The expression of FGFRs and Klotho on the parathyroid gland suggest a direct effect of FGF-Klotho signaling[126]. However, an *in vivo* study using a PTH specific Klotho (-/-) mice model showed that Klotho deficiency was not associated with functional changes in the parathyroid gland[127]. In contrast, other *in vivo* and *in vitro* experiments showed that the regulation of PTH secretion by FGF23 is Klotho dependent and involves calcineurin[127]. Also, a study with chronic kidney disease patients (CKD) showed a resistance of parathyroid gland to FGF23 action as a result of downregulation of Klotho and FGFR[128].

As described in *section 6.1*, both PTH and FGF23 regulate renal P handling, renal $1,25(OH)_2D$ and through this intestinal calcium and phosphate absorption. Through this, PTH and FGF23 have indirect mutual effects on their expression and secretion.

6.3. Bone

Fgf23 mRNA expressed at its highest levels in bone compared to other tissues[129]. It is thought that osteocytes and osteoblasts are the main source of circulating FGF23[32,125]. However, Klotho expression in bone is very low compared to it expression in the kidney[125,130]. Despite that, Klotho plays an important role in bone metabolism. Klotho and FGF23 influence bone mineralization and their deficiency impairs this[32,125,129]. This was confirmed in various studies[32,125]. Klotho deficient mice are characterized by osteomalacia, elevated plasma $1,25(OH)_2D$, phosphate and osteopontin. Although this might be a result of lack of renal Klotho which leads to an increase of plasma phosphate and decrease of plasma calcium due to the reduced sensitivity to FGF23, decreasing renal clearance of phosphate and retention of calcium. A study in *Fgf23 -/-* mice showed that also lack of FGF23 is associated with impaired mineralization [129]. In the absence of FGF23, tissue nonspecific alkaline phosphatase (*Tnap*) and osteopontin expression are increased[129]. This appears to be independent of Klotho[129]. The increase in *Tnap* together with a high plasma phosphate lead to an increase in osteopontin which inhibits bone mineralization[129].

7. Age-related changes

Ageing is a progressive natural process caused by oxidative stress and cellular damage[131] in combination with a decline in cellular and organ function and structural changes in several organ systems[131].

7.1. Bone metabolism during ageing

Ageing is associated with various physiological changes, including an increase in bone catabolism and reduced tissue sensitivity to regulating hormones (PTH, $1,25(OH)_2D$ and FGF23)[83]. Also the prevalence of vitamin D insufficiency and deficiency increases with age[132,133]. Low vitamin D status, combined with age-related changes, results in changes in the renal-bone axis and altered bone metabolism and bone loss (*see section 8.1*). More specifically Wnt-signalling is affected during ageing. Plasma concentrations of SOST and FGF23 increase with age, while the FGF23 receptor co-factor αKlotho, declines[134] (*see section 8.3.2*). There is also some evidence that there is an increase in circulating fragments of PTH and FGF23[135,136] (*see section 8.3.1*). These changes may lead to a reduction in Wnt-signalling and eventually to loss of bone mass and integrity[83]. The latter may particularly be detectable in trabecular bone, the partition that is the most metabolically active.

7.2. Hormonal changes during ageing

With ageing, the secretion and sensitivity to many hormones decrease[137]. Also circadian rhythms change[137]. This affects various endocrine systems regulating adrenal function (leading to adrenopause) and reproductive system (leading to menopause and andropause)[137]. The reproductive function declines in both males and females. However, females have more rapid changes in their reproductive capacity than men[137]. Ovulation frequency decreases by the age of 40[83] and 90% of the circulating oestrogen is lost by the time of complete ovarian failure[137]. Follicle-stimulating hormone (FSH) and Luteinizing hormone (LH) are increased with age progression, causing a decrease of estrogen concentrations[138]. Andropause is mainly caused by the decrease of free testosterone due to the decrease of its production and increase of sex- hormone-binding globulin with ageing[139–141]. During andropause FSH and LH are also increased[83].

Both in men and women loss of sex hormones contributes to changes in body-mass and musculoskeletal system[137]. Menopause is characterised by a phase of rapid bone loss[142] (**Figure 3**). 5-15% of age related bone loss appears during the perimenopausal period of which 80% is trabecular bone[83]. With age progression a moderate rise of serum calcium occurs without changes in PTH levels[83]. That indicates a decrease in PTH sensitivity in older age[83].

7.3. The ageing kidney

Kidney is also affected by age progression. These changes can be either functional or structural or both[131]. Decline in renal function is predominantly a result of a reduction of the number of functional nephrons. Changes also occur in the capillary wall of glomeruli. This results in a decline in GFR, changes in tubular reabsorption of components in the glomerular filtrate, urinary concentration and production of the kidney derived hormones[131,143–145]. The volume of kidneys and GFR are strongly correlated with age[146]; the volume is estimated to decline by 1% per year after the age of 50 and eGFR declines by approximately 1 mL/min/m^2 from the age of 30[142,143].

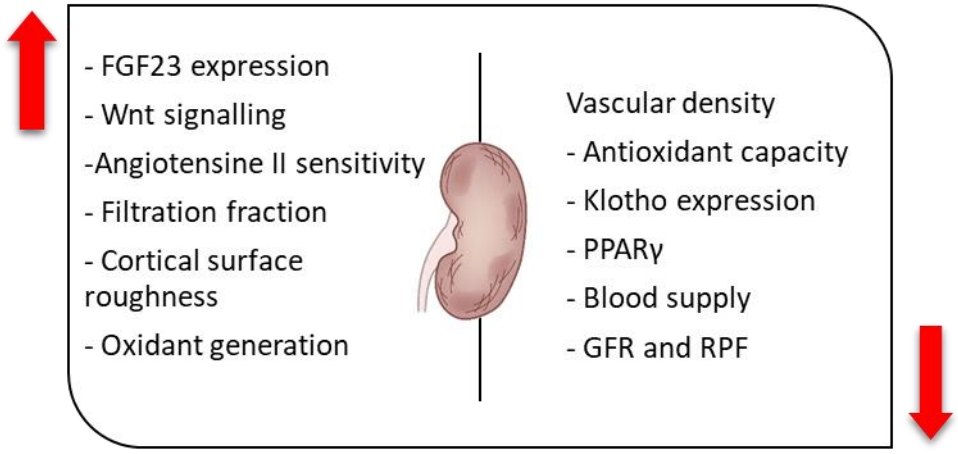
Structural changes can be identified by renal biopsy or imaging procedures such as computerized tomography (CT) scans[131]. Macro-anatomical changes include nephrosclerosis and nephron hypertrophy[131]. Ageing is mostly associated with nephrosclerosis rather than nephron hypertrophy[147]. Nephrosclerosis is caused by arteriosclerosis of small arteries in the kidney and results in glomerulosclerosis, interstitial fibrosis and tubular atrophy[131]. On the other hand, micro-anatomical changes mainly affect kidney volume and the development of kidney cysts and tumors[131].

Age progression it is related to low blood supply because of age-related cortical atrophy which leads to progressive loss of nephrons[148,149]. The most important functional age-related changes are a decline of GFR and renal plasma flow (RPF)[131]. It is suggested that this begins at the age of 30[149]. Filtration fraction (FF) is the ration of GFR to RPF (FF=GFR/RPF) which represents the proportion of fluid that reaches the kidney and passes into renal tubules[149]. With age progression this proportion increases due to the rapid change of the RPF[150].

7.3.1. FGF23-Klotho signalling in the ageing kidney

With age progression, Klotho decreases and FGF23 increases[151.] This is associated with an increased risk of chronic kidney disease (CKD), atherosclerosis[105,152] and mortality[153]. Klotho can suppress FGF and regulate of Wnt-signalling[154,155] (**Figure 6**). With a decrease in Klotho expression, Wnt-signalling increases resulting in increased fibrosis and vascular calcification[156]. Peroxisome proliferator-activated receptor-γ (PPARγ) also decreases during ageing and is suggested to influence FGF23/Klotho signalling[151]. PPARγ protects against oxidative stress, the main cause of ageing[157]. It is suggested that PPARγ agonists increase Klotho expression[158,159].

Figure 6. Functional and structural alterations in ageing kidney[131,151]



Functional and structural alterations occur in the kidneys during ageing affecting gene expressions, homeostasis and kidney function. In response to that is development of pathologic conditions.

From the very early stages of renal impairment, plasma FGF23 increases before an increase in plasma phosphate is detectable[160]. With the progression of CKD, FGF23 continues to increase [161]. In parallel, the expression of Sclerostin (SOST) and RANKL, both inhibitors of the Wnt/β-catenin signalling pathway increase, stimulating osteoclast activity[161]. This leads to a decrease of bone integrity, increased fracture risk together with calcification of soft tissues[162], the hallmarks of CKD-Mineral Bone Disease (MBD).

8. Chronic kidney disease and hormonal changes

Alterations in plasma concentration of FGF23 play a central role in the changes observed with CKD. Multiple pathways are involved and described in detailed in *sections 7.3* and *8.2*

Approximately 50% of adults over 70 years old are diagnosed with CKD[163,164]. CKD is more prevalent in older men than in women[165]. The most common causes are diabetes, inflammation of the glomerulus and nephron (glomerulonephritis), inherited kidney structure abnormalities (e.g. polycystic kidney disease), obstruction of urine flow, hypertension and blockage of kidney blood supply[165]. CKD can be diagnosed with routine tests before clinical symptoms are apparent[165]. The most important features of kidney impairment are: a reduced estimated Glomerular Filtration Rate (eGFR), based on plasma creatinine and/or cystatin, excess protein and/or blood in urine and abnormal appearance of the kidney[165]. The disease has five stages depending on its progression and symptoms (**Table 2**). According to the Kidney Disease Improving Global Outcome (KDIGO) (2012) guidelines, CKD is defined as a eGFR less than 60 ml/min/1.73m^2 for 3 months or more[166] and additionally considers albuminuria.

For further detail on markers of renal function, *see section 8.5*.

Table 2. Stages of chronic kidney disease defined by NHS guidelines[165]

Stage	Kidney function (eGFR)	Characteristics	Medical approach
Stage G1 A1-3	>90ml/min	Normal kidney function but kidney damage may be found	Look for complications. Provide treatment. Make lifestyle changes (e.g. nutrition)
Stage G2 A1-3	60-89ml/min	Normal to mildly reduced kidney function and/or an addition damage was found in the Kidney	
Stage G3A A1-3	45-59ml/min	Mild to moderate reduction of kidney function	
Stage G3B A1-3	30-44ml/min	Moderate to severe reduction of kidney function	
Stage G4 A1-3	15-29ml/min	Severe reduced of kidney function	Dialysis or kidney transplant
Stage G5	<15ml/min	Kidney failure	Dialysis or kidney transplant

The stages are defined from 1 to 5 depending to eGFR range and the second parameter (A) is the amount of protein (albumin) lost in urine.
A1: Hardly any protein in the urine (<30 mg/g).
A2: Small amount of protein in the urine (30-299 mg/g).
A3: Significant amount of protein in the urine (≥300 mg/g)

8.1. Vitamin D metabolism in chronic kidney disease

The prevalence of vitamin D deficiency/insufficiency is higher in CKD patients than in the general population[167]. 70-80% of CKD patients are vitamin D insufficient (<25-50 nmol/l), according to the US IOM in Full here plus abbreviation guidelines[167]. Many factors contribute to the high prevalence of vitamin D deficiency in CKD patients. CKD patients, especially on hemodialysis (HD) have restricted sun light exposure[167]. In addition, HD patients are characterized by skin hyperpigmentation which reduces the cutaneous synthesis of vitamin D[167]. Nutritional factors also contribute to insufficient status of vitamin D in CKD patients. Some of the disease symptoms affect overall food intake of patients, such as reduced appetite and capacity of physical activity caused and uremia[168]. *In vivo* studies showed low jejunal absorption of vitamin D in uremic rats[168]. CKD patients usually have to follow a lot of dietary restrictions, dependent on the stage of the disease. These include a reduction/limitation of protein and phosphate intake[169]. Foods that typically contain vitamin D, are also high in protein and phosphate and therefore, these specific dietary restrictions affect vitamin D intake.

As mentioned in the beginning, vitamin D is converted to its active form in the kidney. With renal impairment, the functional kidney mass decreases and with that the ability of converting 25(OH)D to 1,25$(OH)_2$D decreases[167]. Also proteinuria contributes to vitamin D deficiency[170]. DBP and 25(OH)D are filtered and reabsorbed in glomerulus through a megalin/cubilin mediated internalization process[171]. With CKD, megalin and cubilin expression[171] is reduced. As a result, patients with proteinuria excrete DBP and vitamin D metabolites bound to this excreted DBP in urine[171]. in addition, the reduction in megalin/cubili reduces the internalization of 25(OH)D into tubular cells, required for the hydroxylation into 1,25$(OH)_2$D[172.] This, together with the reduced expression of CYP27B1, impairs the capacity to generate 1,25$(OH)_2$D. Further, when 25(OH)D falls below 15nmoL/L, this results in a reduction of 25(OH)D levels in the glomerular ultrafiltrate[15,] reducing the substrate for hydroxylation.

The importance of megalin was shown in a study with megalin-null mice. These mice have a phenotype of severe vitamin D deficiency despite their normal renal function[15]. This is due to the loss of DBP and vitamin D metabolites in urine. There is also a strong correlation between vitamin D deficiency and albuminuria since megalin also regulates renal albumin (a second binder of 25(OH)D) reabsorption and through that, 25(OH)D reabsorption[173]. Renal megalin is stimulated by $1,25(OH)_2D$[15] therefore, the decreased $1,25(OH)_2D$ production capacity with CKD may lead to a further megalin mediated functions[173].

Specific deletion of renal megalin expression also reduces the phosphaturic response to PTH[174]. Megalin regulates sodium-phosphate internalization through NaPi2a cotransporter which is required for the inhibition of renal phosphate reabsorption by PTH[175]. In CKD patients, the decline in megalin and VDR expression might be therefore partially responsible for not only albuminuria but also phosphate retention despite elevated serum PTH[174].

8.1.1. Medication use and its relationship with vitamin D deficiency

Many drugs used by older people to manage symptoms of CKD and cardiovascular disease (CVD) influence vitamin D metabolism and synthesis[176,177] but mechanisms are still largely unclear[178]. The most commonly used therapies for CKD patients involve angiotensin inhibitors (ACE), aldosterone receptor antagonists (ARAs) and receptor blockers[178]. They inhibit the Renin-Angiotensin-Aldosterone System (RAAS)[179]. Statins are also often used [24]. However, data on their correlation with vitamin D status are conflicting. Yuste *et al*. found significant lower 25(OH)D concentrations in patients treated with statins compared to patients treated with ACE inhibitors or ARAs[178]. In the same study, higher 25(OH)D concentrations were found in patients treated with xanthine oxidase inhibitors[178]. On the other hand, another study showed no significant association between low vitamin D status and treatment with statins, ACE inhibitors and/or ARAs[170]. Differences in the study population or statistical analyses might explain the outcome variations of the different studies. Also, the influence of proteinuria was considered whereas Yuste,[178] did not.

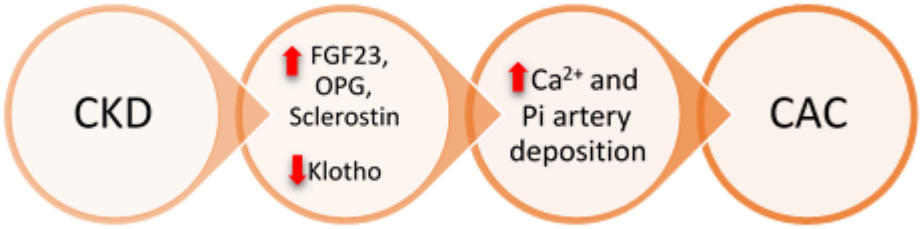
Vitamin D analogs (paricalcitriol, calcitriol) are commonly used on CKD patients (*see section 8.1*) to treat secondary hyperparathyroidism (SHPT) and CKD-MBD. Vitamin D analogs have been shown to significantly reduce proteinuria[180].

8.2. Chronic kidney disease hormonal phenotype and mortality risk factors

In CKD patients, PTH and FGF23 levels are chronically elevated because of decreased renal $1,25(OH)_2D$ production and phosphate retention[181]. Plasma FGF23 concentration is positively correlated with CKD progression, heart failure, vascular calcification, left ventricular hypertrophy and mortality in CKD patients[182]. In addition, calcium retention caused by increased concentrations of FGF23 and PTH is highly associated with vascular calcification[181] (**Figure 7**). Aldosterone is usually elevated in CKD patients due to activation of RAAS[183]. High circulating aldosterone may enhance the effect of FGF23 on sodium retention in CKD patients due to their synergetic effect in sodium metabolism (*see section 4.1.2*)[123] . Sodium and volume retention further contributes to the risk of vascular calcification[181] (**Figure 7**).

The plasma concentration of FGF23 increases in early stages of CKD, before an increase in plasma phosphate is detectable[184]. FGF23 is a phosphaturic hormone which is predominantly produced by osteocytes and acts in the kidneys to increase phosphate excretion[184]. It requires the co-factor αKlotho, the expression of which decreases with ageing and renal impairment, thereby decreasing FGF23 receptor activation. As described in *section 7.3*, FGF23 also has other functions. FGF23 stimulates the catabolism of both 25(OH)D and $1,25(OH)_2D$. FGF23 also downregulates the expression of 1a(OH)ase, suppressing the production of renal $1,25(OH)_2D$. Further, FGF23 can stimulate PTH secretion[184], although the mechanism of this FGF23-PTH interaction is not well understood. An increased plasma FGF23 concentration is associated with soft tissue calcification, increased risks of CVD and the promotion of CKD-MBD[184].

Figure 7. Chronic kidney disease as a risk factor of Coronary artery calcification development[433]

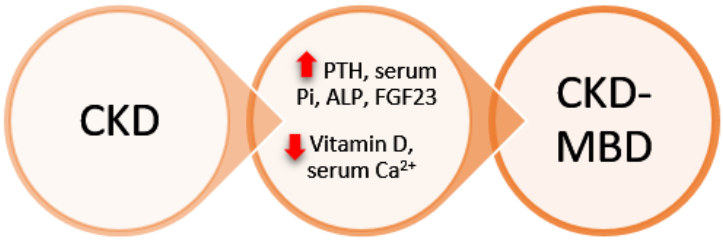


Increased FGF23, sclerostin and OPG in serum are risk factors for vascular calcification progression in CKD patients. Klotho is an inhibitor of coronary artery calcification (CAC) and its deficiency reduces this protective function.

8.3. Chronic kidney disease and bone health

Renal impairment leads to abnormalities in hormones and the regulation of bone and mineral metabolism[162] causing CKD-MBD (**Figure 8**). As described in earlier sections, renal impairment causes a decline in $1,25(OH)_2D$ production by the kidney, phosphate and calcium retention, increased PTH, FGF23 and alkaline phosphatase[184,185]. In CKD, the kidney and bone become resistant to actions of PTH due to the downregulation of the expression of PTHR1 and downstream signals[98]. CKD-MBD leads to a decrease of bone integrity, increased fracture risk and decreasing bone health and calcification of soft tissues[162] (**Figure 8**).

Figure 8. Development conditions of Chronic Kidney Disease-Mineral Bone Disorder[433]



Renal impairment in CKD patients results in hyperphosphatemia, high alkaline phosphatase (ALP), low vitamin D and hypocalcemia. PTH and FGF23 are used for the diagnosis of CKD-MBD.

8.3.1. Parathyroid hormone in renal impairment

The elevation in PTH is one of the main causes of CKD-MBD (for actions of PTH, *see section 5*). This might be the result of the accumulation of PTH fragments, in particular (7-84)PTH[186]. An *in vivo* study suggested that (7-84)PTH decreases the effect of (1-84)PTH on plasma calcium[187]. It has also been suggested that (7-84)PTH could inhibit the expression of PTHR1 in the skeleton or could inhibit the osteoclastic activity[188].

PTHRs are expressed in cardiovascular system and may explain why CVD is a common complication in CKD (*see section 5.2*)[189]. The different PTH fragments have diverse actions in cardiovascular system. Myocardial fibrosis is very common and observed mainly during the final stages of CKD and has been suggested to be a result of elevated PTH levels and calcium retention[189]. A study in a CKD rat model showed that intermittent (1-34)PTH dose decreased

vascular calcification in comparison with a (7-84)PTH dose, which only showed to have a minor effect[190]. Taken together, the above findings indicate that PTH fragments have various functions in the skeleton, kidney and cardiovascular health.

8.3.2. Sclerostin (SOST) as a negative regulator of bone health

CKD-BMD is associated with changes in osteocyte morphology and function[191]. Osteocytes have a dendritic morphology (*see section 2.2*) which allows them to regulate the communication between osteoblasts, osteoclasts and other osteocytes. Through this, they regulate bone remodeling and mineral metabolism[192]. The wnt/β-catenin pathways plays a key role and is regulated by FGF23, Sclerostin and Dickkopf-1 (DKK1)[192] (*see section 2.2*). The effect of wnt/β-catenin signaling is mainly anabolic[193] as it increases osteoblast differentiation and osteocyte function[192]. Wnt/β-catenin signalling also inhibits osteoclasts differentiation through upregulation of OPG (*see section 2.2*).

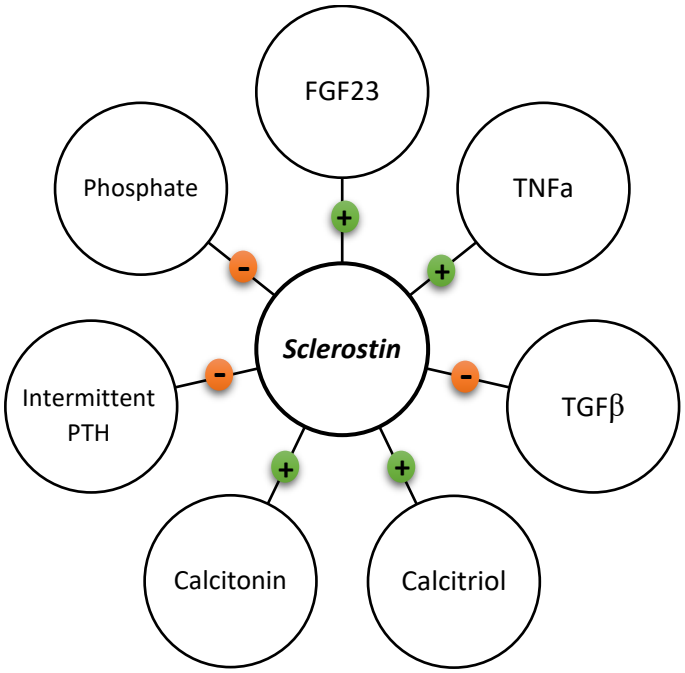
Sclerostin is expressed by the SOST gene[192]. Sclerostin is mainly expressed in osteocytes[194], in contrast to DKK1 which is more widely expressed in the human body[195]. Sclerostin acts as an inhibitor of Wnt/β-catenin pathway causing bone loss[192]. Sclerostin production is regulated by a variety of factors such as TGF-β and PTH[192] (**Figure 9**). TGF-β (*see section 2.3*) is suggested to suppresses osteoblast maturation through stimulation of sclerostin[193]. In contrast, PTH down-regulates sclerostin[192].

Signal transduction of Wnt occurs upon binding to the low density lipoprotein receptor (LRP)[195]. Cytoplasmic β-catenin acts a second messenger, which enters the nucleus and activates the transcription of Wnt target genes[192]. The regulation of this pathway can be inhibited by molecules that bind to Wnt itself or to the low-density lipoprotein receptor-related protein (LRP) coreceptors e.g. Sclerostin or DKK1[192]. PTH, upon binding to PTHR1 stimulates phosphorylation of LRP6, which promotes β-catenin expression in a Wnt independent way[196]. Thus, sclerostin, expression is controlled by β-catenin targeted transcription[192]. Further, it has been suggested that sclerostin negatively regulates bone mineralization through the regulation of the expression of FGF23[197]. High phosphate concentrations, intermittent secretion of PTH and TGFβ are inhibitors of sclerostin. However, FGF23, tumor necrosis factor-α (TNFα), calcitriol and calcitonin can stimulate the expression of sclerostin[192].

A genetic model of progressive CKD suggests that the anabolic Wnt/β-catenin pathway is suppressed due to an increase in sclerostin which occurs already in the early stages of CKD[198]. My study showed that even mild renal impairment was associated with an increase of the sclerostin plasma concentration[198]. Since sclerostin depends on clearance through the kidney, lower filtration rate and clearance may also be responsible for the high levels of sclerostin in serum with renal impairment[199]. An alternative explanation for the high serum sclerostin concentrations may also be the presence of inactive sclerostin fragments that are measured in the assays used[192]. This has to date not been examined or confirmed.

In cross-sectional studies, a positive association between plasma sclerostin and rate of bone mass loss is found. Higher plasma concentrations are also associated with an increased risk of CVD and increase in mortality[200].

Figure 9. Interactions and factors regulating Sclerostin expression[192]



8.4. Inflammation and iron status and kidney impairment

The risk of iron deficiency and increased plasma concentrations of markers of inflammation increase both with ageing and with CKD[201,202]. It has been suggested that iron deficiency[203]

and inflammation[203] mediate part of the multiple pathways that lead to alterations in the renal-bone axis with renal impairment Preclinical studies suggest that increased inflammation and a decrease in iron status may play a role in the regulation of FGF23. In healthy people, plasma phosphate is the main regulator of plasma FGF23, but from early stages of CKD, FGF23 increases before an increase in CKD associated plasma phosphate is observed[204]. Preclinical studies suggest that these factors may influence FGF23 transcription and post-translational modification[205]. Iron deficiency and the pro-inflammatory cytokines TNFα and Interleukin 6 (IL6) have been shown to be associated with increased osteocytic FGF23 transcription and cleavage, resulting in increased plasma concentrations of particularly c-terminal (FGF23) and to a lesser extent intact-FGF23 (iFGF23)[206,207]. Also, hepcidin, the main hormonal regulator of systemic iron homeostasis, may play a direct or indirect role. Hepcidin increases in response to inflammation and inhibits the incorporation of iron into erythrocytes[208,209].

High concentrations of FGF23, iron deficiency and increased inflammation have been reported to be association with poor skeletal integrity, low BMD and bone loss[210–212]. Increases in FGF23 and inflammation are also associated with alterations of regulators of Wnt-signaling, SOST and RANKL[154,213]. This suggest that these factors may mediate the development of CKD-MBD.

Vitamin D deficiency, frequently found in CKD[214] as reported in detail in *section 8.1.*, has been reported to be associated with elevated concentrations of c-reactive protein (CRP) and pro-inflammatory cytokines[215–219] but evidence is conflicting[220] and regulatory mechanisms are only partly elucidated. However, states of acute inflammation and infection are reported to increase catabolism of 25(OH)D[221]. Also iron status has been reported to be negatively associated with vitamin D status in some but not all reports[222–224].

Supplementation with Vitamin D may increase erythropoiesis through the reduction of hepcidin and inflammatory cytokines, but human studies are limited and have provided mixed results[209,222,225]. We (this thesis) and others reported that vitamin D supplementation was associated, an increase in iron status and the anti-inflammatory cytokine interleukin 10 (IL10) and a reduction in TNFα. We found (this thesis) that this effect was dependent on renal function[226,227]. Our findings suggest that an effect of vitamin D supplementation on

inflammation and iron status may particularly be found in those with lower renal function. We and others also found that an increase in iFGF23 and cFGF23[214,226–228], which was accompanied by a decrease in plasma phosphate in those with normal renal function, but not in those with renal impairment. The physiological significance of this increase in FGF23 remains unclear.

Further detail regarding the effect of vitamin D supplementation in CKD populations is described in *section 9.1* and Chapter 2 of this thesis.

8.5. Kidney function markers

A range of biochemical markers are used to assess renal function (**Table 3**)[165]. Plasma concentrations of creatinine and urea are routinely used[229]. The measurement of the clearance of inulin is considered to be the golden standard measurement of kidney function[230]. This method assesses the renal clearance of inulin after an intravenous single dose[229]. Other methods use an isotope as a tracer.

Renal clearance is expressed in the GFR. GFR is a measure of how many milliliters (ml) of fluid the kidneys can filter from blood in one minute (ml/min)[165]. A normal GFR is defined as >90ml/min[165]. However, direct measurement of GFR by a tracer clearance method is expensive and not commonly used due to its strict protocol[230]. In practice, urinary and plasma creatinine concentrations are more commonly used, which is a relatively easy and inexpensive marker to measure. The creatinine clearance rate is calculated from concomitant (timed) urinary and plasma measurements. In clinical practice, kidney function is mainly assessed on the basis of plasma concentrations of creatinine or and/or Cystatin C from which the eGFR[165] is calculated. Several formulae are used which include different parameters and characteristics of the patient[165]. These are given in **Table 3.** The most commonly formulae used are: Cockroft Gault (CG), 4-modification of diet in renal disease (MDRD-4) and Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) (2009)[230] (**Table 3**). All formulae have their limitations and were developed and validated in populations with specific characteristics[230]. The MDRD-4 and CKD-EPI are the formulae most commonly used clinically in the UK and elsewhere[165]. CKD-EPI was developed in large population based cohort[230] and is recommend for the classification of CKD for the majority of patients[231]. CG and MDRD-4 are

more accurate when eGFR is <60ml/min[165] since they were developed in populations with that characteristic. In addition to the eGFR, various other biochemical markers are used to aid the differential diagnosis of patients, including serum creatinine and urea, blood urea nitrogen[229] (**Table 3**). Also, imaging tests and kidney tissue biopsy might be necessary in some cases.

UK National Institute of Health and Care Excellence (NICE), National Kidney foundation (NKF), Kidney Disease Improving Global Outcomes (KDIGO) and the Caring for Australasians with Renal Impairment (CARI) have established guidelines on the use of these different formulae. This is explained in more detail in Chapter 2b.

Table 3. Biochemical markers[230] of renal function and eGFR calculations[232–234]

Biomarker	Tissue of expression/origin	Analytical source	Part of the kidney processed
Creatinine (Cr)	Muscles	Urine/ Serum	Significant percentage of creatinine in the urine derived from proximal tubular secretion. It is used to calculate GFR.
Urea	Liver	Serum	Filtered from blood by glomeruli.
Blood urea nitrogen (BUN)	Liver	Urine	Filtered from blood by glomeruli.
Cystatin C	Produced by all nucleated cells	Serum	Filtered in proximal tubules.
β-trace protein (BTP)	N/a	Urine	Filtered in proximal tubule.
Inulin or isotope tracer	Non metabolised molecule (given as single bolus)	Blood	Measurement of glomerular filtration rate.
B2-microglobulin (B2-M)	All nucleated cells in the body	Blood	Filtered in proximal tubule.
Urinary liver-type fatty acid-binding protein	Kidney, liver	Urine	Filtered in proximal tubule.
Urinary N-Acetyl-b-O-glucosaminidase	Proximal tubule cells	Urine	Filtered in proximal tubule.
Urinary connective tissue growth factor	Connective tissues	Urine	Filtered in proximal tubule.
Urinary CD14 mononuclear cells	Bladder epithelial cells	Urine	Kidney cells.
Neutrophil gelatinase associated lipocalin	Produced by injured nephron epithelial	Urine / serum	Filtered in proximal and distal tubule.
Kidney injury molecule-1 (KIM-1)	Not normally present in what? but it is expressed in	Urine/serum	Filtered in proximal tubule.

	proximal tubule apical membrane		
Fibroblast growth factor-23 (FGF23)	Osteocytes, osteoblasts	Serum	Filtered in proximal and distal tubule. It responds to decline in phosphate clearance.
Urinary retinol binding protein 4 (uRBP4)	Synthesised in liver	Serum	Filtered in proximal tubule.

Type of formula	Equation	Abbreviations/units
Cockroft Gault (CG)	C_{Cr}={((140–age) x weight)/(72xSCr)}x 0.85 (if female)	C_{cr} (clearance creatinine) in ml/min Age (years) Weight in kg SCr in mg/dL
4-modification of diet in renal disease (MDRD-4)	GFR= 175 × $(Scr)^{-1.154}$ × $(Age)^{-0.203}$ × (0.742 if female) × (1.212 if African American)	GFR (mL/min/1.73 m^2)
6-modification of diet in renal disease (MDRD-6)	GFR = 198 × $[sCr]^{-0.858}$ × $[age]^{-0.167}$ × [0.822 if patient is female] × [1.178 if African American] × $[\text{serum urea nitrogen concentration}]^{-0.293}$ × $[\text{urine urea nitrogen excretion}]^{0.249}$	SCr (mg/dL) Age (years) serum urea nitrogen concentration (mg/dL) urine urea nitrogen excretion (g/d)
Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) (2009)	GFR = 141 × $\min(Scr/\kappa, 1)^{\alpha}$ × $\max(Scr/\kappa, 1)^{-1.209}$ × 0.993^{Age} × 1.018 (if female) × 1.159 (if black) κ= 61.9 (for females) and 79.6 (for males) α= -0.329 (for females) and -0.411 (for males)	Scr in µmol/L Age (years)
CKD-EPI with CC	GFR=133 x $\min(S_{cys}/0.8, 1)^{-0.499}$ x $\max(S_{cys}/0.8, 1)^{-1.328}$ x 0.996^{Age} x 0.932 [if female] min = indicates the minimum of Scys/0.8 or 1 max = indicates the maximum of Scys/0.8 or 1	Scys in mg/L Age (years)
CKD-EPI with CC and Cr	135 × $\min(S_{Cr}/\kappa, 1)^{\alpha}$ × $\max(S_{Cr}/\kappa, 1)^{-0.601}$ × $\min(S_{cys}/0.8, 1)^{-0.375}$ × $\max(S_{cys}/0.8, 1)^{-0.711}$ × 0.995^{Age} × 0.969 [if female] × 1.08 [if black] κ = 0.7 for females or 0.9 for males; α = -0.248 for females or -0.207 for males; min(SCr/κ or 1) = indicates the minimum of SCr/κ or 1 max(SCr/κ or 1) = indicates the maximum of SCr/κ or 1 min(Scys/0.8, 1) = indicates the minimum of Scys/0.8, 1 max(Scys/0.8, 1) = indicates the maximum of Scys/0.8, 1	S_{cr} (mg/dL) Scys (mg/L) Age (years)

9. Vitamin D supplementation and the renal-bone axis

International guidelines provide recommendations for the prevention, management and correction of vitamin D deficiency, secondary hyperparathyroidism and metabolic bone disease. These depend on stage of CKD and were subject to review in Chapter 2 and are briefly outlined here.

In the early stages of CKD (G1 &G2), The Kidney Disease Outcomes Quality Initiative (KDOQI), NFK and KDIGO recommend the same approach to prevent vitamin D deficiency/insufficiency and treatment strategies to correct deficiency for the general population[235,236]. These recommendations vary by age-group and by country or continent. Specific guidelines for patients with more advanced renal impairment consider altered vitamin D metabolism and increased requirements. The target concentration range of 25(OH)D is usually higher than defined in population guidelines. For patients with CKD 3-4, it is recommended that with vitamin D supplementation, plasma calcium and phosphate should be monitored and supplementation dose should be adjusted when required[237] (**Figure 14**). The use of vitamin D analogues[237] may be recommended on the basis of progressive SHPT and CKD-MBD. Detailed discussion can be found in Chapter 2b of this thesis.

9.1. The effect of vitamin supplementation on PTH, 1,25(OH)$_2$D and clinical outcomes in CKD patients

In patients with CKD and dialysis, native (i.e. the naturally occurring form) vitamin D supplementation improves their biochemical profile and clinical symptoms: (i) increased serum levels of 25(OH)D and 1,25(OH)$_2$D[238–240], (ii) decreased PTH serum levels[241–243], (iii) reduced proteinuria[244], (iv) improvement on endothelial cardiovascular markers[245] and (v) decrease inflammation markers[246]. However, the effect depends on the stage of the disease[247,248]. Vitamin D supplementation may also be required to prevent or reverse CKD associated bone disease. Vitamin D supplementation efficiently increases serum 25(OH)D and 1,25(OH)$_2$D production in stage 3 and 4 of the disease[247,248], in contrast to the small effect reported in stage 5 patients[249]. Only are few randomized controlled trials evaluating the effect of vitamin D supplementation on bone health of CKD patients have been conducted and results are conflicting. Also, vitamin D supplementation does not consistently result in

normalization of serum calcium and phosphate[250]. Vitamin D supplementation is not as efficient as active vitamin D (i.e. 1,25$(OH)_2$D or its analogues) in decreasing PTH[251,252] and reversing or preventing SHPT associated with renal impairment. For the treatment of SHPT is calcitriol or vitamin D analogs (or vitamin D receptor activator-VDRA) are recommended[253]. VDRAs have high efficacy in decreasing PTH serum levels although they have adverse effects such as, adynamic bone disease, toxicity risk and hypercalcemia[254,255]. In most cases of reported adverse effects, this occurred in patients with serum PTH values which were not significantly above the reference range[254,255]. Vitamin D analogs are not recommended as a routine treatment in pre-dialysis due to these adverse effects[193]. The use of active vitamin D or analogs are also associated with increased FGF23 levels which may result in adverse effect on vascular health[256].

9.2. Vitamin D catabolism in CKD

As described in *section 8.1* renal production of 1,25$(OH)_2$D is impaired and supplementation does not uniformly lead to a decrease in PTH in CKD patients. In addition, studies show much lower concentration of 24,25(OH)D in CKD patients compared to healthy individuals[257,258] and a progressive decline of 24,25(OH)D concentration as renal failure progresses[259]. It was initially thought that is due to lower 25(OH)D availability as a substrate for 24-hydroxylation[260]. This hypothesis has been challenged since it has been showed that correction of serum 25(OH)D with supplementation did not increase serum 24,25(OH)D in CKD patients[260]. This suggests that reduced 24,25$(OH)_2$D in CKD patients involves different mechanisms. It has been suggested that these may include: (i) the majority of circulating 24,25$(OH)_2$D is produced in the kidney and *CYP24A1* enzyme expression declines with kidney mass despite the upregulation in *CYP24A1* mRNA due to an increase in FGF23[260], (ii) the increase in PTH with CKD down-regulates *CYP24A1* expression or activity[261,262].

9.3. Vitamin D supplementation and regulators of Wnt-signalling

Plasma concentrations of OPG and sclerostin are increased and DKK1 decreased in CKD patients[263]. Low Vitamin D status has been shown to be associated with increased plasma concentrations of SOST in healthy people and in patients with CKD[264]. Cross sectional studies also identified inverse correlations between serum sclerostin and biochemical markers of the

renal-bone axis, such as iFGF23, iPTH and serum alkaline phosphatase[264–267]. In addition, sclerostin has been shown to be inversely associated with uric acid and eGFR[264].

Similar to eGFR, increased concentrations of SOST and OPG are associated with the risks of the development of coronary artery calcification (CAC) and CVD[209,210] (**Figure 7**).

Supplementation studies and correction of vitamin D deficiency in CKD patients have however yielded conflicting results[238,239,274–278,240,264,268–273]. Some studies showed that correction of vitamin D status results in reduction of sclerostin[279,280], while others reported an increase in sclerostin after supplementation with native[281] or active forms[282] of vitamin D in healthy people and CKD patients. Other trials reported no effect of vitamin D_3 supplementation on sclerostin levels despite a decrease in serum PTH levels[283,284].

Also data on the effect of vitamin D supplementation on RANKL are conflicting. Several ex-vivo, in-vitro studies reported that 1,25$(OH)_2$D decreased the expression of RANKL and upregulate of the OPG/RANKL ratio. This is partly mediated through the inhibitory effect of 1,25$(OH)_2$D on inflammatory factors[285,286]. However, another study suggests that 1,25$(OH)_2$D increases the expression of RANKL and decreased OPG and enhanced osteoclast formation[287].

10. Summary

Bone and renal metabolism are regulated by common factors and there is extensive cross-talk between these two organs. PTH, FGF23 and vitamin D metabolites are among the main regulatory factors of the renal-bone axis. They also have regulatory functions in other organ systems such as cardiovascular system and intestine. Ageing is associated with a range of physiological changes, including reduced bone mass, renal impairment and reduced sensitivity to regulating hormones which results in changes in this cross-talk. Also, the prevalence of vitamin D insufficiency and deficiency increases with age. Together, these age-related changes lead to changes in the hormonal regulation of the renal-bone axis.

Chapter 1b: PhD Research

The PhD research described in this dissertation aimed to investigate the effect of vitamin D supplementation and renal impairment on the renal-bone axis in older adults with a particular focus on FGF23 and markers of the Wnt-signalling pathway.

The central hypothesis addressed in this thesis is:

Vitamin D supplementation and renal impairment influence the renal-bone axis.

Aims

I. To provide a comprehensive review of guidelines for adult, pre-dialysis renal patients for the management of vitamin D status and SHPT. Generate a road map and tabulate guidelines on the form and dosages of vitamin D recommendations for the prevention or correct of vitamin D deficiency and target values of plasma 25(OH)D concentrations, according to the different stages of CKD (*Chapter 2a*).

II. To conduct a systematic review of recent RCTs with different forms of vitamin D in CKD patients with a focus on CKD-MBD related outcomes and a meta-analyses of the effectiveness of supplementation on plasma PTH concentrations (*Chapter 2b*).

III. To investigate changes in regulators and markers of bone metabolism, BMD and BMC in response to different dosages of vitamin D supplementation in older people for 12 months. We investigated four categories of markers: (a) calcium metabolism and renal function, (b) vitamin D metabolites, (c) Wnt-signalling and (d) bone parameters and bone metabolism. Further, we investigated their associations with total 25(OH)D and free 25(OH)D at baseline and after 12 months of supplementation (*Chapter 3*).

IV. To identify differences in vitamin D metabolism, bone turnover and Wnt-signalling markers between adults categorised on the basis of kidney function (eGFR ≥60 mL/min/1.73m^2 (representing CKD G1 and G2) versus eGFR 30-60 mL/min/1.73 m^{2}representing CKD3a and 3b; mild to moderately and moderately to severely impairment, respectively). Differences were investigated before after 12 months of vitamin D supplementation (*Chapter 4*).

V. To investigate the differences in the response to vitamin D supplementation by category of CKD (with markers defined in aim III) (*Chapter 3 & 4*).

VI. To investigate markers of inflammation, iron status and regulators of Wnt-signalling and bone metabolism and their associations with FGF23 in older adults with an eGFR <60 ml/min/1.73m^2 (CKD stage G3a and G3b) and eGFR >90 ml/min/1.73m^2 (CKD stage G1; normal renal function) and their response to vitamin D supplementation (*Chapter 5*).

Outcomes

The following markers were selected for study:

- Calcium and Phosphate metabolism: plasma calcium, phosphate, cFGF23 and iFGF23
- Kidney function: plasma albumin, creatinine, cystatin C and Klotho
- Vitamin D metabolism: plasma 25(OH)D, 1,25$(OH)_2$D, 24,25$(OH)_2$D, DBP and iPTH
- Wnt-signalling: OPG, DKK1, SOST and RANKL
- Bone mineral density and metabolism: hip BMD, hip BMC, FN BMD, FN BMC, plasma BAP, β-CTX and PINP
- Iron status: plasma iron and hepcidin
- Inflammation markers: serum CRP, IL10, plasma IL6 and TNFα

CHAPTER 2: Vitamin D supplementation for patients with chronic kidney disease

Chapter 2 is based on the following publication[214].

Christodoulou M, Aspray TJ, Schoenmakers I. Vitamin D Supplementation for Patients with Chronic Kidney Disease: A Systematic Review and Meta-analyses of Trials Investigating the Response to Supplementation and an Overview of Guidelines. Calcif Tissue Int. 2021 Aug;109(2):157-178. doi: 10.1007/s00223-021-00844-1.

Summary

CKD patients are often vitamin D deficient (25(OH)D <25 or 25-30nmol/L per UK and US population guidelines). CKD-MBD due to secondary hyperparathyroidism and vitamin D deficiency is common in these patients. There are still many gaps in the literature for the management of vitamin D status in relation to CKD–MBD hindering the formulation of comprehensive guidelines.

A systematic review of 22 RCTs using vitamin D or analogues treatment and a meta-analysis for PTH was carried out. An overview of current guidelines on vitamin D status management for pre-dialysis CKD patients is provided.

The effect of vitamin D on PTH concentrations was inconsistent but meta-analyses showed a borderline significant reduction. Calcidiol consistently reduced PTH. Calcitriol and paricalcitol treatment were associated with a consistent greater suppression of PTH. Increase FGF23 after analogue treatment was observed in all studies reporting this outcome but was unaltered in studies with Vitamin D or 25(OH)D. Few RCTs reported markers of bone metabolism and variations in the range of markers prevented direct comparisons.

Current guidelines for the first CKD stages (G1-G3a) follow general population recommendations for the prevention of vitamin D deficiency. Use of calcitriol or analogues is restricted to stages G3b-G5 and depends on patient characteristics.

In conclusion, vitamin D administration in CKD patients has an inconsistent effect on PTH although meta-analysis showed a significant overall effect. Calcifediol and analogues consistently suppress PTH, but the reported increase in FGF23 with $1,25(OH)_2D$ analogues warrants caution.

CHAPTER 2a: A systematic review and meta-analyses of randomized controlled trials investigating the response of CKD patients on vitamin D treatment

The physiology of altered vitamin D and bone metabolism with CKD

The alterations in in vitamin D metabolism[167], calcium and phosphate homeostasis and bone metabolism[167] with CKD is multifactorial and associated with CKD-MBD[162].

A high proportion, 70-80% of CKD patients have a plasma 25(OH)D concentration below 50nmol/L[167] and the majority well below the concentration recommended for patients with renal impairment (>75nmol/L), if not treated. Many factors contribute to the high prevalence of vitamin D deficiency in CKD patients and changes in vitamin D metabolism occur at several levels.

Supply is decreased as a result of lower cutaneous vitamin D production due to skin hyperpigmentation, ageing, sun avoidance and dietary restrictions[169]. Losses are increased with proteinuria, when vitamin D binding protein and albumin and vitamin D metabolites bound to these proteins are lost in urine[169]. Hepatic conversion of vitamin D into 25(OH)D is reported to be suppressed in CKD patients[12,13,288]. Accordingly, the dose-response appears to be lower than in healthy individuals, although this is poorly characterized.

With the loss of functional renal tissue the capacity to convert 25(OH)D to $1,25(OH)_2D$ (1,25 dihydroxy vitamin D or Calcitriol) is reduced leading to a decline in plasma $1,25(OH)_2D$[13]. Also, the renal capacity to internalise 25(OH)D may be impacted, reducing its availability of 25(OH)D. In healthy people, plasma 25(OH)D concentrations between 15-40nmol/L are thought to be required to ensure there is no substrate limitation for renal $1,25(OH)_2D$ production[289]. In people with CKD, higher concentrations may be required.

Plasma PTH increases in response to impaired $1,25(OH)_2D$ production and calcium malabsorption in combination with increased resistance of the kidneys and bone to PTH, due to a downregulation of PTHR1[98]. An increase in plasma phosphate and FGF23 further stimulate PTH secretion.

The plasma concentration of FGF23 increases in early stages of CKD, before an increase in plasma phosphate is detectable[184]. FGF23 is a phosphaturic hormone which is predominantly produced by osteocytes and acts in the kidneys to increase phosphate excretion[184]. It requires the co-factor αKlotho, the expression of which decreases with ageing and renal impairment, thereby decreasing FGF23 receptor activation. FGF23 also has other functions. FGF23

stimulates the catabolism of both 25(OH)D and 1,25$(OH)_2$D. FGF23 also downregulates the expression of 1a(OH)ase, suppressing the production of renal 1,25$(OH)_2$D. Further, FGF23 can stimulate PTH secretion[184], although the mechanism of this FGF23-PTH interaction is not well understood. An increased plasma FGF23 concentration is associated with soft tissue calcification, increased risks of CVD and the promotion of CKD-MBD[184].

CKD-MBD has a heterogeneous phenotype due to the involvement of several underlying mechanisms, in which SHPT plays an important role[290]. CKD-MBD can either be characterised by an increase or a decrease in bone turnover, but may also be normal. An increase can lead to osteomalacia, which is characterized by the excessive presence of undermineralised bone tissue and osteoporosis defined as a low bone mineral density (Z-score -2.5 SD) and loss of bone integrity. A decrease in bone remodelling leads to adynamic bone disease increasing fracture risk[291]. CKD-MBD is therefore generally characterized by a decrease of bone integrity, increased fracture risk and calcification of soft tissues[162] and alterations in bone turnover markers[291]. In the management of SHPT and CKD-MBD, maintenance of a sufficient vitamin D status is recognized as an important target.

Clinical trials of vitamin D in CKD patients and gaps in the evidence-base

There are gaps in the evidence base for the management of vitamin D status in relation to CKD–MBD, i.e. SHPT, altered bone metabolism, bone density and integrity and fracture risk. These include the dose- response to vitamin D supplementation and the response in PTH. The optimal concentration ranges of PTH and 25(OH)D for the management and prevention of CKD-MBD are not well established for each stage of CKD. This is reflected in the guidelines for the management of vitamin D status in CKD-MBD.

The limited number of randomized controlled trials reporting the effects of treatment with vitamin D or its analogues on CKD-MBD related outcomes provided conflicting results[238,239,274–278,240,264,268–273]. Several studies reported the effect of supplementation on renal function and proteinuria[244] and markers of endothelial and cardiovascular function and inflammation[245,246]. The effects were shown to depend on the stage of the disease[247,248]. Adverse treatment effects were reported, particularly with active vitamin D and analogues and includes hypercalcemia[254,255], adynamic bone disease and increased FGF23 levels[292].

In this systematic review we aimed to summarize the findings of the most recent randomized controlled trials reporting the effects of vitamin D or its analogues, conducted with pre-dialysis CKD patients and that report 25(OH)D, PTH, markers of calcium and phosphate and/or bone metabolism. Where provided, we also summarized adverse effects and other outcomes that may be relevant for vitamin D metabolism (e.g. proteinuria) or the effects of interventions on markers of vascular health. Findings are grouped according to form of vitamin D given, preceded by a short description of their characteristics. Meta-analyses were conducted to provide an estimate of the effectiveness of supplementation on plasma PTH concentrations.

Methods

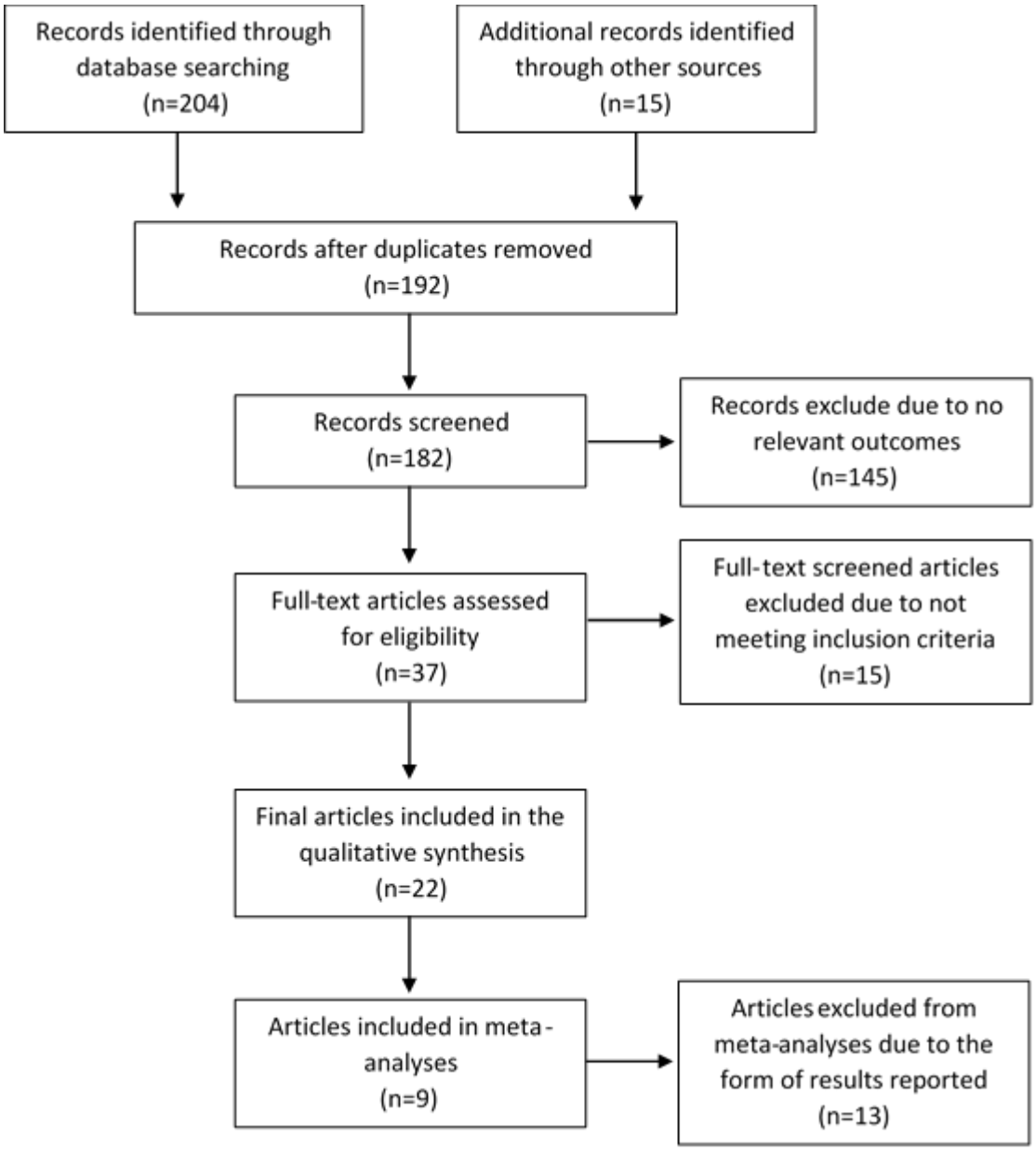
Search strategy

We searched for published studies indexed in MEDLINE, EBSCO, Science direct and PubMed from inception of 2003, the year that NKF KDOQI guidelines were published, to October of 2020. Search terms used: vitamin D, oral vitamin D, vitamin D supplementation, vitamin D analogues, paricalcitol, calcifediol, calciferol, ergocalciferol, chronic kidney/renal disease, renal/kidney impairment, impaired kidney/renal failure, RCT, randomized controlled trials. Outcomes search terms were not used in order not to limit search results. Papers were selected on basis of relevant outcomes. English was applied as a language limitation. Only full text published manuscripts were included. References were hand searched for additional publications.

Inclusion criteria

The detailed process of study selection presented on **Figure 10**. Only randomized controlled trials (RCT) were included that used any type of vitamin D in non-dialysis CKD patients (>18 years old) and studies at any stage of the disease were considered. Studies were included if placebo controlled, compared 2 or more treatments or were randomized cross-over studies. Studies had to include a definition of dosage and duration of the vitamin D administered and outcomes related to CKD-MBD. This systematic search provided 22 RCTs (**Table 4**).

Figure 10. Flow chart of systematic search and literature selection



Adjusted from PRISMA protocol 2019.

Table 4. Study characteristics and outcomes included in the systematic review

Authors	Country	Study population	Intervention	Outcomes
Vitamin D*				
Dogan et al. 2008[293]	Slovakia	40 predialysis CKD patients (stage 3 and 4), PTH <200pg/ml. No use of phosphate binders	300 000 IU D_3/month for 1 month or placebo Route: oral	Treatment group: ↑ 25(OH)D, ↓ iPTH, ↔ serum calcium, ↔ serum phosphorus, ↔ ALP
Oksa et al. 2008[294]	UK	87 CKD patients (stage 2-4) (including hypertensive, diabetic and vitamin D insufficient/ deficient CKD patients). Use of phosphate binders (calcium-based). No calcimimetics use	5 000 or 20 000 IU/week D_3 for 12 months Route: oral	↑ 25(OH)D, ↓ iPTH in both treatment groups, ↔ serum calcium, ↔ serum phosphate, ↔ urinary calcium, ↔ urinary phosphate
Petchey et al. 2013[270]	USA	28 CKD patients (stage 3-4), 25(OH)D <150 nmol/L	2000 IU/day D_3 or placebo for 6 months Route: oral	↑ 25(OH)D, ↑ $1,25(OH)_2D$, ↔ PTH, ↔ serum calcium, ↔ serum phosphate, ↔ insulin sensitivity
Dreyer et al., 2014[275]	USA	38 CKD patients (stage3-4), non-diabetic, 25(OH)D <40 nmol/L	50 000 IU/week D_2 for 1 month followed by 50 000 IU/month D_2 for 5 months or placebo for 6 months Route: oral	↑25(OH)D, ↔ PTH, ↔ calcium, ↔ phosphate, ↔ blood pressure, ↔left ventricular mass index. Improvement in endothelium dependent microcirculatory vasodilation
Chandra et al. 2008[295]	Turkey	34 CKD patients (stage 3 and 4), 25(OH)D <75 nmol/L and SPTH (PTH >70 pg/mL). No calcimimetic use	50 000 IU/week D_3 or placebo for 12 weeks Route: oral	↑ 25(OH)D, ↔ $1,25(OH)_2D$, ↔ PTH, ↔ serum calcium, ↔ BAP, ↔ TRAP5b, ↔ CTX
Westerberg et al. 2018[296]	Sweden	95 CKD patients (stage 3–4), 25(OH)D <75nmol/L, PTH >64.1pg/ml. No calcimimetic use	8000 IU/day D_3 or placebo for 12 weeks Route: oral	↑25(OH)D, ↑$1,25(OH)_2D$, ↔ PTH but it was significantly lower than the mean value of the placebo group. ↑ calcium, ↔ phosphate and ↔ FGF23
25 (OH) Vitamin D - calcifediol				
Sprague et al. 2014[238]	USA	78 CKD patients (stage 2-4), 25(OH)D <75 nmol/L, iPTH>70pg/ml	ER calcifediol (30, 60 or 90 µg/day) or placebo for 6 weeks	Dose dependent ↑ 25(OH)D and ↓ iPTH, serum ↔ calcium, ↔ serum phosphorus ↔FGF23

			Route: oral	
Petkovich et al. 2015[297]	USA	29 CKD patients (stage 3-4), 25(OH)D <75 nmol/L, SHPT	Single oral dose of ER calcifediol (450mg or 900mg) or a single bolus IV injection of calcifediol (448mg) and monitoring for 42 days Route: oral and iv	*ER calcifediol (450mg or 900mg):* ↔ 25(OH)D and ↔ $1,25(OH)_2D$, ↔ PTH compared to IV group *ER calcifediol (900mg)* ↔ 25(OH)D and ↔ $1,25(OH)_2D$, ↔ $24,25(OH)_2D$, ↓iPTH after 72h compared to IV group. *IV injection group*: ↑25(OH)D, ↑$1,25(OH)_2D$, ↑$24,25(OH)_2D$, ↔iPTH. ↔calcium in all treatment groups.
Sprague et al.2016[298]	USA	429 CKD patients (stage 3-4), 25(OH)D 25-75 nmol/L, SHPT (≥ 85 and <500pg/ml)	ER calcifediol (30 or 60 µg/day) or placebo for 26 weeks Route: oral	↑25(OH)D, ↓PTH in both treatment groups compared to placebo, ↔ serum calcium, ↔ serum phosphorus, ↔FGF23
$1,25(OH)_2$ vitamin D or Vitamin D analogues**				
Coyne et al. 2006[299]	USA	220 CKD patients (stages 3 and 4) with PTH (>70 pg/mL)	Paricalcitol capsules (Dosing was based on serum iPTH, calcium, and phosphorus levels) 3/week or 1/day or placebo for 24 weeks Route: oral	↓ iPTH compared to placebo, ↔ urinary calcium, ↔ urinary phosphorus, ↓ BAP, ↓ osteocalcin, ↓ urinary pyridinoline compared to baseline.
de Zeeuw et al. 2010[300]	USA	281 patients with type 2 diabetes, nephropathy (stages 1-4) and PTH 35–500 pg/mL	1µg paricalcitol/day, 2 µg paricalcitol/day or placebo for 24 weeks Route: oral	↑25(OH)D, ↓iPTH in both treatment groups
de Boer et al., 2013[271]	USA, Poland	22 non-diabetic CKD patients (stage 3-4). No phosphate binders use	Cross-over study with paricalcitol or placebo for 8 weeks (washout 8 weeks between arms) Route: oral	↓ 25(OH)D, ↓ 1,25(OH)2D, ↑ 24,25(OH)D, ↓ PTH, ↑ serum calcium, ↔ serum phosphorus, ↑ FGF23, ↔ insulin sensitivity
Coyne et al. 2014[301]	Germany, Greece, Italy, Netherlands, Poland, Portugal,	110 CKD patients (stage 3-4), PTH >120 pg/ml. Use of phosphate binders	0.25 µg/d $1,25(OH)_2D$ or 1 µg/day of paricalcitol for 24 weeks Route: oral	↓ iPTH, ↔ serum calcium, ↔ serum phosphorus, ↓ALP compared to baseline

	Spain, USA, Taiwan			
Larsen et al., 2013[273]	Sweden	26 CKD patients (stage 3-4), non-diabetic, albuminuria (urine albumin >30 mg/L)	paricalcitol (2 µg/day) or placebo for 6 weeks (crossover design) with a 2-week washout period Route: oral	↔ 25(OH)D, ↓ iPTH, ↑ plasma calcium, ↑ plasma phosphate, ↑ urinary calcium (24h), ↑ FGF23, ↓ ALP, ↓ albumin excretion rate, ↓ creatinine clearance, ↔ renin, ↔ angiotensin II, ↔ aldosterone
Lundwall et al. 2015[269]	Denmark	36 non-diabetic CKD patients (stage 3-4), PTH 35-500 pg/mL	Paricalcitol (1µg or 2 µg/day) or placebo for 3 months Route: oral	↔ 25(OH)D, ↓ PTH, ↔ calcium, ↔ phosphate in both treatment groups. ↔ albuminuria, ↔ pulse wave velocity, ↔ muscle sympathetic nerve activity, ↓ endothelial function at the placebo and 1µg treatment group, ↑ blood velocity in both treatment groups
Thadhani et al. 2012[276]	USA	227 CKD patients (stage 3-4), iPTH 50-300 pg/ml and mild-moderate left ventricular hypertrophy	Paricalcitol (2 µg/day) or placebo for 48 weeks Route: oral	↓ PTH, ↑ calcium, ↔ phosphate, ↔ left ventricular mass index
Riccio et al. 2015[302]	Italy	60 CKD patients (stage 3b-5), PTH 20-300 pg/mL and anaemia (Hb levels: 10-12.5 g/dL), including use of calcium supplements and phosphate binders.	Paricalcitol (1 µg/ day) or $1,25(OH)_2D$ (0.5 µg/ every other day) for 6 months Route: oral	↔PTH, ↔calcium, ↔phosphate compared to baseline, ↓GFR in the paricalcitol group. The paricalcitol group had a significant ↑Hb where in $1,25(OH)_2D$ group was significantly decrease.
Zoccali et al. 2014[303]	Italy	88 CKD patients (stage 3 to 4), PTH >65 pg/mL. Use of phosphate binders	Paricalcitol (2 µg/ day) or placebo for 12 weeks Route: oral	↑25(OH)D, ↓$1,25(OH)_2D$, ↓ PTH, ↑ serum calcium, ↑ serum phosphate, ↑ FGF23, ↓ GFR
Kovesdy et al. 2012[304]	USA	80 CKD patients (stage 3-4), 25(OH)D <75 nmol/L and SHPT. Use of phosphate binders.	50 000 IU/week D_2 titrated to achieve serum 25(OH)D 75 nmol/L or paricalcitol (1 µg/day) for 16 weeks Route: oral	↑25(OH)D in both groups compared to baseline, ↓PTH in paricalcitol group, ↔ serum calcium, ↔ serum phosphorus
Moe et al. 2010[305]	Australia	47 CKD stages 3 and 4 with 25(OH)D < 50 nmol/L and SPTH (>100 to 150pg/mL for stage 3	4 000 IU/day D_3 for 1 month, then 2 000 IU/day D_3 for 2 months or doxercalciferol (1 µg/day) for 3 months	↑ 25(OH)D in both treatment groups, ↓ PTH in doxercalciferol group, ↔ serum calcium, ↔ serum phosphorus, ↔ urinary calcium

		and >150 to <400pg/mL for stage 4). No calcimimetic use	Route: oral	
Levin et al.2017[306]	USA	87 CKD patients (stage 3b-4). Use of phosphate binders (calcium-based)	Calcifediol (5000 IU) or $1,25(OH)_2D$ (0.5 µg) or placebo, thrice weekly for 6 months Route: oral	↑25(OH)D in the calcifediol group. ↔ $1,25(OH)_2D$, ↓ PTH, ↔ serum calcium, ↔ serum phosphate, ↔ FGF23 in calcifediol and $1,25(OH)_2D$ group
Combination treatment				
Susantitaphong et al. 2017[278]	Thailand	68 CKD patients (stage 3-4), 25(OH)D <75 nmol/L with proteinuria	40 000 IU/week D_2 plus placebo or 40 000 IU/week D_2 plus $1,25(OH)_2D$ (5µg two times/ week) for 12 weeks Route: oral	↑ 25(OH)D in both treatment groups (higher in the combined group), ↔ iPTH in D_2 group, ↓ iPTH levels in the combined treatment group, ↔ serum calcium ↔ serum phosphate. ↓ urine protein-creatinine ratio in both treatment groups

*3 further studies with vitamin D (Moe et al. 2010, Kovesdy et al. 2012 and Susantitaphonget al. 2017) and ** further study with calcifediol (Levin et al. 2017) are listed below.

Conversion factor plasma concentration 25(OH)D in nmol/L to ng/mL: divide by 2.5

Conversion factor Vitamin D in IU to µg: divide by 4

Quality assessment

The 22 studies were assessed for their methodological quality using the Van Tulder et al. criteria list[307] by two independent investigators (**Table 5**). High quality studies were defined with score ≥8 and low quality with score ≤7. Eighteen studies were categorized as high quality and five as low quality. Scores are provided in **Table 5**.

Table 5. Quality assessment of the RCTs included in the systematic review according to Tulder et al, 2003[307]

	Van Tulder et al. 2003 criteria list											
Studies included	**1.**	**2.**	**3.**	**4.**	**5.**	**6.**	**7.**	**8.**	**9.**	**10.**	**11.**	**SCORE**
*Dogan et al. 2008**	+	−	+	−	−	−	+	?	?	−	+	4
Oksa et al. 2008	+	?	−	?	?	?	+	?	?	+	−	3
*Moe et al. 2009**	+	+	+	−	+	+	+	+	+	+	+	10
Petchey et al., 2013	+	+	−	+	+	+	+	+	+	+	−	9
*Dreyer et al., 2014**	+	+	n/a	+	+	+	+	+	+	+	+	10
Chandra et al. 2008	+	+	+	+	+	+	+	?	+	+	+	10
*Westerberg et al. 2018**	+	+	+	+	+	+	+	+	+	+	+	11
Sprague et al.,2014	+	+	+	+	+	?	+	?	?	+	+	6
Petkovich et al. 2015	+	−	?	−	−	+	+	+	+	+	?	6
Sprague et al.2016	+	+	+	+	+	+	+	?	+	+	+	10
Coyne et al. 2006	+	+	+	+	+	+	+	?	+	−	+	9
de Zeeuw et al. 2010	+	+	+	+	+	+	+	?	−	+	+	9
de Boer et al., 2013	−	+	+	+	+	+	n/a	+	+	+	+	9
Coyne et al. 2014	+	+	−	−	−	−	+	+	+	+	+	7
Larsen et al., 2013	+	+	?	+	+	+	+	+	+	+	?	9
*Lundwall et al., 2015**	+	+	−	+	+	+	+	?	+	+	+	9
Thadhani et al., 2012	+	+	+	+	+	?	+	?	+	+	+	9
Riccio et al. 2015	+	+	+	+	+	+	+	?	+	+	?	9
Zoccali et al. 2014	+	+	+	+	+	+	+	?	+	+	?	9
*Susantitaphong et al., 2017**	+	+	+	+	+	?	+	?	+	+	?	8
*Kovesdy et al. 2012**	+	−	+	−	?	?	+	?	+	+	+	6
*Levin et al.2017**	+	+	+	+	+	+	+	?	−	+	+	9

Green: Yes, Red: No, Grey: Unknown

1. Was the method of randomization adequate?
2. Was the treatment allocation concealed?
3. Were the groups similar at baseline regarding the most important prognostic indicators?
4. Was the patient blinded to the intervention?
5. Was the care provider blinded to the intervention?
6. Was the outcome assessor blinded to the intervention?
7. Were co-interventions avoided or similar?
8. Was the compliance acceptable in all groups?
9. Was the drop-out rate described and acceptable?
10. Was the timing of the outcome assessment in all groups similar?
11. Did the analysis include an intention-to-treat analysis?

*Studies included in the meta-analyses

Meta-analyses

A semi-formal meta-analyses were conducted to provide an estimate of the effectiveness of supplementation on PTH. PTH plasma concentrations were converted into uniform units (pg/ml). Separate analyses were conducted for studies using the precursor of $1,25(O)_2D$, i.e. vitamin D or 25(OH)D and with $1,25(OH)_2D$ or analogues. All analyses were conducted on the basis of the reported mean and standard deviations, either between treatment groups and/or placebo as appropriate. Not all studies reported sufficient detail for inclusion. Also, some studies were excluded from the meta-analyses due to the non-normally distributed PTH and the form of the reported values. In view of the limited number of placebo-controlled studies, for some studies baseline data were used as comparator. Four studies reported baseline and post-placebo treatment data and none of these individual studies reported a significant change in PTH. This was confirmed by meta-analysis showing a non-significant change (results not shown).

Heterogeneity was assessed using the I^2 test. Significant heterogeneity was present with I^2 >50%. A fixed effect model was used. Meta-analyses were performed using RevMan (version 5.4; non-Cochrane Collaboration). A p-value of ≤0.05 was considered significant.

Results

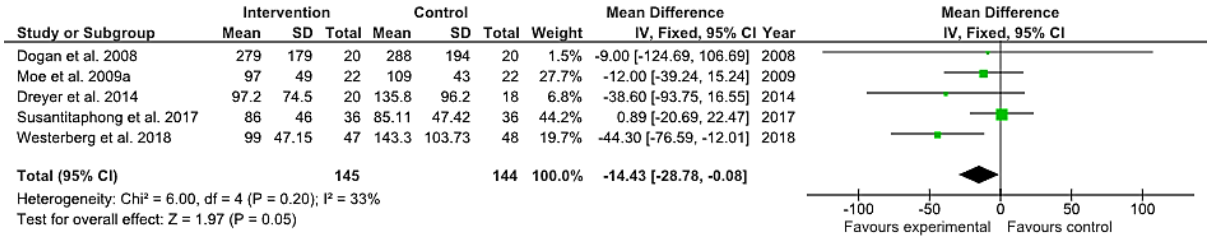
RCTs with Vitamin D supplementation

Vitamin D is the inactive precursor of $1,25(OH)_2D$ and exists in two forms, vitamin D_3 (cholecalciferol) and D_2 (ergocalciferol). Vitamin D is the most commonly used form for the prevention and treatment of deficiency in both the general population and patient groups. Vitamin D_3 supplementation leads to a longer sustained increase of 25(OH)D, but otherwise the metabolism of D_3 and D_2 is identical.

Nine of the RCTs included in this systematic review used vitamin D_3 (six studies)[270,293–296,305] or D_2 (three studies)[275,278,304] supplementation. In some of these studies the group that received Vitamin D served as the reference group. They were all conducted with CKD patients stages 3-4. The doses varied from 2 000-4 000 IU daily or 40 000-50 000 IU weekly (**Table 4**) and the duration was between 1 and 12 months. All nine studies found a significant increase

in plasma 25(OH)D concentrations. Four[293,294,304,305] of these studies observed a significant reduction of PTH, while in the remaining five, no significant change of PTH in response to the vitamin D supplementation was found[270,275,295,296]. The differences in dosages and duration of supplementation may explain these inconsistent results; higher doses show to be more effective in suppressing PTH. Only five of these studies provided sufficient detail for inclusion in meta-analysis. This showed a borderline significant effect on PTH (**Figure 11**). When also two studies with the $1,25(OH)_2D$ precursor calcifediol were considered, the effect on PTH was highly significant ($p< 0.0001$), but heterogeneity was substantial (I^2 60%) (not shown).

Figure 11. Forest plot showing the effect of Vitamin D_2 or D_3 on PTH



Only three of the studies[270,295,296] measured changes in $1,25(OH)_2D$, with two showing a significant increase with supplementation. Most of the studies reporting no effect of the supplementation on PTH, also noted no effect on calcium and phosphate concentrations. Only one study measured FGF23 and found no change. Bone turnover markers were only reported in two studies. Alkaline Phosphatase (ALP), CTX and Tartrate-resistant acid phosphatase 5b (TRAP5b) were shown not to change with supplementation. One study reported a significant improvement on the endothelium dependent microcirculatory vasodilation and other markers of vascular function.

RCTs with Calcifediol supplementation

In recent years, preparations of the 25 hydroxylated form of vitamin D, i.e. 25(OH)D or calcifediol were developed for oral administration. There are three forms; calcifediol and the extended release (ER) formula, provided as capsules and the immediate release (IR), provided as a liquid or capsule. The pharmacokinetic profile differs from the parent compound vitamin

D. Intestinal absorption of 25(OH)D is known to be more efficient and is not dependent on fat absorption, the increase in plasma 25(OH)D is more rapid and the dose-response higher than that of the parent compound. The IR calcifediol formulation was approved in the US in 1980 for treatment of CKD-MBD in dialysis patients[308]. However, it was withdrawn from the market in 2002, since it failed to show meaningful reduction of PTH (≥30%) in patients with CKD G3-4. IR calcifediol is still available in Europe and licensed for use in various conditions including vitamin D deficiency rickets, renal osteopathy and hypocalcaemia[308]. ER formulations of 25(OH)D are only available in the US at the moment[308]. In 2016 ER calcifediol was approved in the US to treat SHPT in adult CKD patients G3-4 and vitamin D insufficiency[308]. Studies in CKD patients showed that ER calcifediol results in a slower increase of 25(OH)D levels, more significant suppression of iPTH and less of an increase of 24,25(OH)D compared to IR-calcifediol[297].

Three ER calcifediol and one calcifediol study in CKD patients (G2-4) published since 2003 were found in our systematic search[238,297,298,306] (**Table 4**). Dosages and durations of treatment varied but a significant dose-dependent increase in 25(OH)D and a decrease in iPTH was seen after oral administration in all studies[238,297,298]. A meta-analysis could not be conducted due to study and data limitations.

ER calcifediol showed a significant but gradual increase of serum 25(OH)D in all three of the studies in contrast to the sharp increase with intra-venous administration of calcifediol. Intra-venous administration did not significantly suppress iPTH but there was an increase in $24,25(OH)_2D$, a catabolic product of 25(OH)D. ER calcifediol administration was associated with unaltered plasma calcium and phosphate concentrations in all studies and FGF23 concentrations in the 2 studies that included this measurement.

<u>RCTS with calcitriol and vitamin D analogues</u>

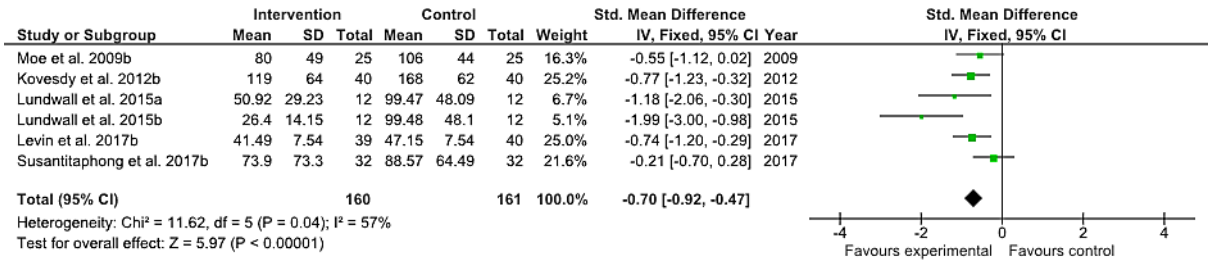
The active form of vitamin D and its analogues used in the treatment of CKD patients include calcitriol and the vitamin D analogues paricalcitol (19-nor-$1,25(OH)_2D_2$) and the $1,25(OH)_2D$ precursor alfacalcidol ($1a(OH)D_3$)[309]. $1,25(OH)_2D_3$ is identical to the endogenous activated form of calcifediol ($25(OH)D_3$). Paricalcitol and alfacalcidol, the latter of which requires hepatic hydroxylation at the 25 position, are synthetic analogues of vitamin D[309] and are also referred to as VDRA. VDRAs have been used for the management of SHPT in CKD patients for

a few decades[310] and show to have reno-protective properties such as reducing albuminuria, renal damage and dysfunction[273,300,311]. Paricalcitol suppresses PTH secretion while it has a lower stimulatory effect on intestinal absorption of calcium and phosphate compared to $1,25(OH)_2D$[273]. Paricalcitol is also associated with reduction of cardiovascular events[276], although sufficient studies with CKD patients are still lacking.

All twelve studies included CKD patients G3-4, however there were differences between studies in patient characteristics. For example, diabetes, established SHPT, use of phosphate binders and proteinuria.

All twelve RCTs showed a significant reduction in plasma PTH after administration of paricalcitol or $1,25(OH)_2D$[269,271,305,306,312,273,276,299–304] (**Table 4**). This was also seen in a study that combined $1,25(OH)_2D$ and Vitamin D_2[278]. The duration of administration varied from 2 weeks to 48 weeks and the dosages used in these studies from 0.25µg to 2µg. Meta-analysis of the five studies for which sufficient detail was available, confirmed this finding (**Figure 12**). The effect of paricalcitol on serum 25(OH)D and $1,25(OH)_2D$ concentrations were inconsistent. Two of the studies with paricalcitol[271,303] reported a reduction of $1,25(OH)_2D$ after supplementation.

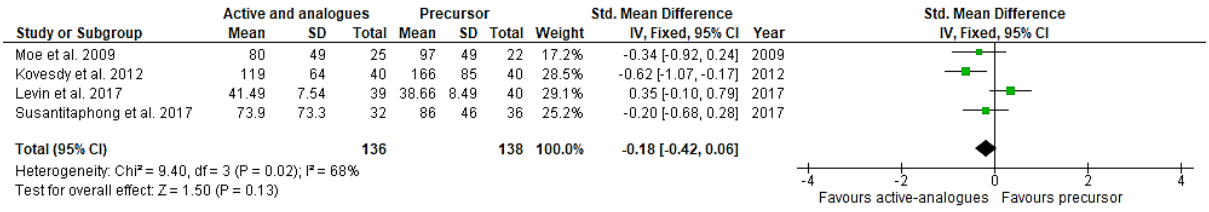
Figure 12. Forest plot showing the effect of calcitriol or analogues on PTH



Several studies compared different forms of supplementation. Kovesdy et al. compared the effects of vitamin D_2 compared to paricalcitol in vitamin D deficient CKD patient with SHPT[304]. In both treatment groups, 25(OH)D significantly increased, but only the paricalcitol group there was a significant decrease in PTH. Coyne's study compared the effects of $1,25(OH)_2D$ with paricalcitol[301]. In both treatment group there was a significant decrease of PTH.

However, paricalcitol appeared to be suppress PTH more compared to 1,25(OH)$_2$D (-52% and -46% PTH reduction, respectively). Susantitaphong et al. examined the effect of Vitamin D2 with or without 1,25(OH)$_2$D and found a suppression of PTH only in the combined group[278]. Levin et al.[306] compared the effects of oral calcifediol with 1,25(OH)$_2$D. Both treatment groups had a significant reduction of PTH, which was larger in the calcifediol group. A significant increase in 25(OH)D was shown in the calcifediol group, while there were no differences between the groups in the 1,25(OH)$_2$D. Meta-analysis comparing the effects of calcitriol or vitamin D analogues versus the administration of the precursor calcidiol or vitamin D on PTH, showed no significant difference between these forms. However, this analysis was limited to 4 studies for which sufficient detail was available (**Figure 13**).

Figure 13. Forest plot showing the effect of calcitriol or analogues (active) versus Vitamin D2 or D3 (precursors) on PTH



Inconsistent results were reported for serum/plasma calcium (four increase; six unchanged) and phosphate (two increase; eight unchanged) concentrations. Most of the studies reported no cases of hypercalcemia. Only one study reported significant higher incidence of hypercalcemia in the intervention group compared to placebo (22.6% and 0.9% respectively)[276]. An increase in FGF23 after vitamin D analogues administration was found in all three studies reporting this outcome and in two of these studies, also a decrease in renal function was reported[271,273,303]. An increase in FGF23 was not reported in the four studies with Vitamin D or 25(OH)D and one with 1,25(OH)$_2$D. Meta-analysis of the FGF23 in response to vitamin D or analogue administration could not be conducted due to the low number of studies and lack of sufficient information provided (e.g. not all manuscripts reported whether iFGF23 or cFGF23 was measured).

All four studies that measured either ALP or bone specific (BAP) after paricalcitol treatment reported a decrease in this marker of bone metabolism.

Further outcomes included markers of endothelial and cardiovascular function, insulin sensitivity and proteinuria and were inconsistent between studies.

Discussion

As expected, 25(OH)D increased after supplementation, but the vitamin D -25(OH)D dose-response appeared to be lower than in healthy people. Individual participant data (IPD)-level meta-analysis to characterize this relationship in CKD patients would be helpful to underpin the evidence base of vitamin D requirements to prevent and correct vitamin D deficiency in CKD patients.

The effect of vitamin D on PTH concentrations was inconsistent but meta-analysis showed a borderline significant suppressive effect. Comparing these studies with a meta-analysis of the PTH response in studies with participants not selected on the basis of their renal function[313,314], results in CKD patients are less consistent. In these meta-analyses in pre-dominantly non-renal participants, a suppression of PTH was found in the majority of individual studies and meta-analyses of results found an overall reduction in PTH after vitamin D supplementation with generally lower dosages of vitamin D supplementation compared to dosages used in CKD patients. To address this, IPD-level meta-analysis of existing data and a vitamin D dose ranging study in CKD patients with and without SHPT to characterize the response of PTH is urgently required. This research would facilitate the identification of patient groups that do and do not respond with a decrease in PTH to supplementation.

Administration of calcifediol was reported in four studies and all reported suppression of PTH. This may seem surprising since also this form requires renal activation to $1,25(OH)_2D$. Although it is difficult to compare these results to the administration of vitamin D, the use of calcidiol to suppress PTH in CKD patients holds promise. Inclusion in and/or comparison with the outcomes of the above mentioned IPD-level meta-analysis may clarify its relative effect. This may aid the incorporation of calcidiol in guidelines and provide alternative options for patients that might be prescribed calcitriol and paricalcitol. We found that treatment with calcitriol and paricalcitol was associated a consistent and greater suppression of PTH but an

increased risk of hypercalcaemia. In addition, an increase FGF23 after treatment with vitamin D analogues was observed in all three studies reporting this outcome, but was unaltered in 4 studies with Vitamin D or 25(OH)D. This warrants further attention. FGF23 is a relatively novel biomarker of phosphate metabolism with multiple functions. FGF23 is a phosphaturic hormone. Its main function is to reduce phosphate reabsorption from the glomerular filtrate through downregulating the available sodium phosphate cotransporters[32]. FGF23 also downregulates the expression of 1a(OH)ase, suppressing the production of renal 1,25(OH)2D and upregulates 24(OH)lase increasing $1,25(OH)_2D$ catabolism[32]. FGF23 is concentration-dependent and positively correlated with CKD progression, heart failure, vascular calcification, left ventricular hypertrophy and mortality in CKD patients[182]. This is partly thought to be caused by increased calcium retention caused by increased FGF23 and PTH concentrations[182], together with elevated aldosterone concentrations found in CKD patients due to activation of the RAAS[183]. High circulating aldosterone may enhance the effect of FGF23 on sodium retention in CKD patients. Sodium and volume retention further contributes to the risk of vascular calcification[182].

An increase in both iFGF23 and cFGF23 with vitamin D supplementation is also reported in a meta-analyses of trials with deficient and healthy individuals[228], but so far not linked to increased risk of CVD events[228]. It was also earlier reported in CKD patients[292]. The increase in FGF23 may indicate an undesirable side effect of administration of vitamin D and its analogues. A better understanding of its effects with different forms of vitamin D is required especially in CKD patients in relation to their already increased FGF23 concentrations, alterations in vitamin D metabolism and increased risk of CVD.

Few RCTs reported the effect of vitamin D on markers of bone metabolism and variations in the range of markers prevented direct comparisons. However, all four studies that measured either ALP or BAP after paricalcitol treatment reported a decrease in this marker indicative of increased bone turnover in CKD patients.

Several factors may have influenced the overall findings. These include form, frequency and dosages of vitamin D and its analogues used. The selection of patient population, co-morbidities and their use of other medication will have influenced the response to supplementation. Further, the method used for estimating renal function may have influence the selection of study participants.

Methods for estimating renal function

All studies included in this review used eGFR as a measure to assess kidney function. None applied a direct method. Most studies used creatinine based algorithms, and the majority the MDRD-4 equation, except Zoccali et al.[303] who used the CKD-EPI Creatinine-Cystatin C algorithm, that incorporates both creatinine and cystatin C. Plasma creatinine is significantly affected by age, nutrition, gender, physical activity and muscle mass[315,316]. Also, Agarwal et al showed that short-term paricalcitol treatment in CKD patients can increase serum creatinine and creatinine excretion without altering creatinine clearance[315,316]. MDRD-4 is the most commonly used formula for eGFR in medical practice. However, there are differences between guidelines. The UK NICE recommends the use of CKD-EPI for the majority of patients[231], but for the assessment of eGFR in those with CKD stage 3a (eGFR 45–59 mL/min/1.73m^2) and no proteinuria, the use of cystatin C-based equations is recommended[317]. Also, the KDIGO, NKF and CARI guidelines recommend the use of CKD-EPI. The CG algorithm may be the preferred option for the older population since this incorporates body size[318].

Cystatin C is a relatively new biomarker. Cystatin C based eGFR has been reported to correlate better with mortality risk factors in CKD patients than creatinine based eGFR[315,316]. Cystatin C is filtered by the glomerulus, is not secreted by the renal tubules and it is generated at a constant rate by all cells in the body[315,316]. There are two formulae based on cystatin C; the CKD-EPI using cystatin C and CKD-EPI using a combination of cystatin C and creatinine[315,316].

There are considerable differences in the resulting eGFR value and CKD classification[318] and accordingly, the choice of the method may have influenced the characteristics of patients included in studies.

Medication use and vitamin D

Many drugs used by CKD patients to manage symptoms of CKD and CVD influence vitamin D metabolism and synthesis, although the mechanisms are still largely unknown[178]. The most commonly used therapies for CKD patients involve angiotensin inhibitors (ACE), ARAs and

receptor blockers[178]. They are used to inhibit the RAAS[179]. Statins are also frequently used[24]. There are conflicting results on the association between these medications and vitamin D status. Yuste et al. found significant lower 25(OH)D levels in patients treated with statins compared to the patients treated with ACE inhibitors or ARAs[178]. In the same study they found higher 25(OH)D levels in patient treated with xanthine oxidase inhibitors (medication for hyperuricemia)[178]. On the other hand, a different study showed no significant associations between concentrations of 25(OH)D and treatment with statins, ACE inhibitors and/or ARAs[170]. The use of these medications is however seldomly reported in RCTs.

Medication use or dietary strategies to manage hyperphosphatemia in CKD can also have influenced the findings and the reported side effects in these studies. Hyperphosphatemia is usually managed by dietary restriction of phosphate intake or prescription of phosphate binders[319] in order to decrease the availability of phosphate for intestinal absorption. There are three types of phosphate binders available: containing calcium, aluminum or non-calcium containing binders. Calcium based binders can be used as the initial binder therapy in CKD patients but are not preferred in case of hypercalcemia and/or when plasma PTH concentrations are <150 pg/ml on two consecutive blood tests[320]. Calcimimetics are allosteric activators of the calcium sensing receptor of parathyroidal cells[309]. Calcimimetics are usually prescribed in CKD patients with SHPT in order to activate calcium receptors and thus suppressing PTH[309] and are used when vitamin D analogues have failed to reduce SHPT. Calcimimetics in combination with active vitamin D therapy are used to reduce the risk of developing hypercalcemia and hyperphosphatemia[321].

A secondary analysis of a large study showed that the choice of phosphate binders may significantly impact vitamin D metabolism and may influence the safety profile of vitamin D administration. In the Phosphate Normalization Trial on CKD patients[322], participants were randomized to receive either sevelamer carbonate, lanthanum carbonate, calcium acetate for 9 months after which vitamin D metabolites were measured. In the group taking calcium acetate, 24,25$(OH)_2$D, the vitamin D metabolite ratio [24,25$(OH)_2$D:25(OH)D] increased and 1,25$(OH)_2$D decreased[322]. Also, the group taking sevelamer carbonate had an increased 24,25$(OH)_2$D and vitamin D metabolite ratio, but this was lower than in the calcium acetate group[322]. No changes in 1,25$(OH)_2$D were reported[322]. In the group taking lanthanum there we no changes in vitamin D metabolite concentrations.

In the studies included in this systematic review, patients were included that did or did not use phosphate binders and/or calcimimetics. No clear pattern in the response or incidence of hypercalcemia appeared among these studies.

CHAPTER 2b: An overview of existing guidelines on vitamin D treatment for CKD patients

Guidelines for dietary Vitamin D intakes and supplementation for population health and patient management

General population requirements and recommendations

Population guidelines for dietary vitamin D intakes are partly based on the required intakes to prevent vitamin D deficiency or to achieve pre-defined target ranges of 25(OH)D or sufficiency. Also, evidence from RCTs and other research designs linking vitamin D intake and health outcomes are considered. Thresholds for deficiency, i.e. the plasma concentration of 25(OH)D below which the risk of disease increases, are predominantly defined on the basis of skeletal health outcomes, but in some guidelines also other health outcomes are considered. In most guidelines, minimal contribution of vitamin D synthesis in the skin is assumed. There is considerable variation in the definition and thresholds or ranges of 25(OH)D concentration that encompasses vitamin D sufficiency or target ranges of 25(OH)D between health authorities. In addition, strategies (e.g. type of data considered) to set requirements differ between health authorities. Review of approaches are beyond the scope of this review and are provided in Bouillon 2017[323].

Dietary reference values (DRV) or equivalents, defined by different public health institutes vary as a consequence of differences in the defined threshold for deficiency or target values for 25(OH)D and by age group or physiological state and are summarized in **Table 6** [8,324,325]. The umbrella term DRV is used in Europe and World Health Organization/ Food and Agriculture Organization (WHO/FAO) and similarly, dietary reference intake (DRI) in the US that comprise a range of nutrient values that apply to the general population or specific population groups[8,324–326]. The DRV includes the Reference Nutrient Intake (RNI) which is the intake to meet the requirements of 97.5% of the population. There where this cannot be established, an Adequate Intake (AI) is defined. Similarly in the US, RDA represents the requirements of 97.5% of the population[327]. Population guidelines differ from clinical guidelines as the latter consider altered dietary requirements associated with underlying conditions.

Table 6. Population daily Reference Nutrient Intake (RNI) or Recommended Dietary Allowance (RDA) or equivalents for vitamin D according to different countries and organizations

Country/ Organization	Adults (µg/day)	Older 65 y (µg/day)	Deficiency of 25(OH)D[323]
Nordic countries[328]	10	10-20	25-30 nmol/L
UK[2]	10	10	<25 nmol/L
Ireland[329]	0–10	10	<30nmol/L
Netherlands[330]	0–10	20	25-30 nmol/L
Belgium[330]	10–15	15	-
France[330]	5	10-15	-
DACH[330]	20	20	25-30 nmol/L
Spain[330]	15	15	-
Australia and New Zealand[331]	15	15-20	25-30 nmol/L
EFSA 2017[326*]	15	15	<50nmol/L*
Institute of Medicine[327]	15	20	25-30 nmol/L
WHO/FAO	5	5	25 nmol/L

After Lips P et al. 2019[324] and Bouillon R. 2017[323]
*Adequate intake set for vitamin D intake based on target value of 25(OH)D.

Although vitamin D deficiency is one of the most common nutritional deficiencies in the world, no routine screening for the general population and most patient groups is recommended[231,326,327,332]. Only people at high risk or with clinical features of vitamin D deficiency are recommended to be tested[333]. For example, KDIGO working group and NKF recommend annual screening for vitamin D deficiency for CKD patients[237]. CKD patients are classified as a high-risk group due to their dietary modification (restricted protein intake), advice to restrict sunlight exposure and reduced cutaneous synthesis associated with common CKD comorbidities and renal losses.

Guidance for patient management and CKD patients

Clinical guidelines for patient management or for specific patient groups provide guidance for the prevention and correction of vitamin D deficiency. These are partly based on population guidance or define specific target values for 25(OH)D and vitamin D intakes, based on altered supply or bioavailability, metabolism and/or requirements. The NICE[231] and the US Endocrine Society[332] offer guidelines for prevention and treatment of vitamin D deficiency in patients, including CKD patients. The UK Royal Osteoporosis Society (ROS)[334] provides guidelines that focus on patients with osteoporosis and for osteomalacia and osteoporosis prevention (**Table**

7). Specific guidance for the management of CKD-MBD were developed by the NKF, the KDIGO CKD-MBD Guideline[237] and CARI[331,335,336]. Approaches and criteria used are discussed in more detail below.

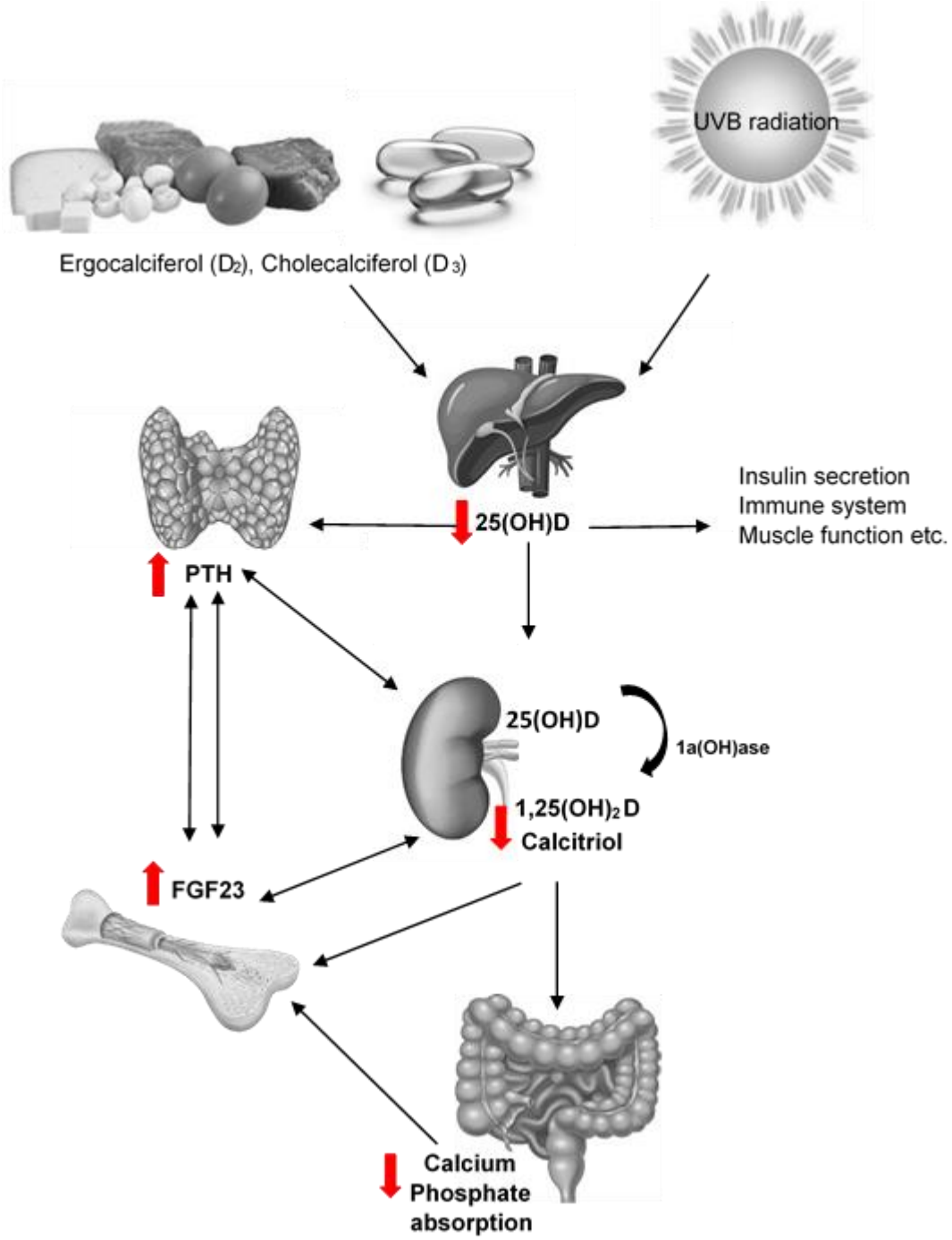
Table 7. Guidelines for the correction of vitamin D deficiency for patient management (general and for specific groups)

DOSAGE SCHEMES FOR THE CORRECTION OF VITAMIN D DEFICIENCY		
Endocrine society (2011)[332]	**NICE (2018)[231]**	**ROS (2018)[334]**
Sufficient: >75nmol/L Insufficient: 50-75nmol/L Deficiency: <50nmol/L	Sufficient: >50nmol/L Insufficient: 25-50nmol/L Deficiency: <25nmol/L	Sufficient: >50nmol/L Insufficient: 25-50nmol/L Deficiency: <25nmol/L
• Dietary intake for patients at risk: 19-70y 600 IU/day; >70y 800 IU/day • Treating vitamin D deficiency in adults: 50,000 IU/week for 8 weeks or 6000 IU/day. • Followed by maintenance therapy of 1500-2000 IU/day.	• Vitamin D_3 is the preferred form of supplementation to treat vitamin D deficiency. • Vitamin D deficiency treatment: Fixed loading dose of vitamin D up to total of 300,000 IU, split dose either weekly or daily. • Followed by lifelong maintenance treatment of 800 IU/day.	• Vitamin D_3 is recommended for treating vitamin D deficiency. • Vitamin D deficiency treatment: fixed loading up to a total of 300,000 IU given either as weekly or daily split doses. • Maintenance therapy: started one month after loading with doses equivalent to 800- 2000 IU/day (maximum 4000 IU/day) given either daily or intermittently.

No specific guidelines for CKD patients are included. Endocrine society and ROS are led by clinical experts and NICE is a government led committee.

The NKF and KDIGO guidelines cross-refer to guidelines for the general population, especially for patients in early stages of CKD. Specific guidelines for patients with advanced renal impairment include the use of vitamin D analogues[237]. These guidelines, including the daily recommended vitamin D intakes are detailed in **Table 5** and **Table 6** by CKD category. Additional recommendations are in place for patients with CKD 3-4. For these patients, it is recommended that with vitamin D supplementation, plasma calcium and phosphate should be monitored and supplementation dose should be adjusted when required[237] (**Figure 14**).

Figure 14. Changes in Vitamin D metabolism and the renal-bone axis with CKD



Arrows indicate direction of changes with CKD. With chronic kidney disease the combination of limited vitamin D intake and reduced renal capacity to activate 25(OH)D to $1,25(OH)_2D$ results to a chain reaction of changes in metabolism. A decrease in $1,25(OH)_2D$ results in a decrease in intestinal absorption of calcium. This stimulates PTH secretion which in turn increases bone resorption. Phosphate retention due to reduced kidney filtration capacity further stimulates PTH and the production and release of FGF23 from bone cells in order to increase renal phosphate excretion.

The US NKF developed the KDOQI, a specific CKD-MBD guideline (Clinical Practice Guidelines for Bone Metabolism and Disease in Chronic Kidney Disease), published in 2003[320]. This guideline includes recommendations for the management of vitamin D deficiency and SHPT, calcium and phosphate metabolism. Also, the effects of vitamin D deficiency and supplementation on bone metabolism and disease were considered (**Table 8**).

The guideline was predominantly based on evidence on the prevention and management of vitamin D deficiency and the progressive increase of PTH. It is acknowledged that there is a lack of high-quality evidence for bone health or patient orientated clinical outcomes, such as mortality and cardiovascular disease risk. Part of this guidance was revised by NKF in 2016[237] focusing on vitamin D deficiency and SHPT in CKD stages 3-4. In this updated guidance, targets for iPTH thresholds by CKD category, as defined in the 2003 guideline were removed[237], due to lack of evidence of benefit. Specific dosage schemes for the prevention of vitamin D deficiency for CKD patients are not given in the NKF guidelines 2003[320] (**Table 8**), but for the correction of deficiency, recommendations on dosages and duration are provided (**Table 9**). Vitamin D analogues are recommended only for patients with a progressive increase in PTH and for the treatment of SHPT. The 2016 update highlighted the potential benefits of the use of oral ER 25(OH)D in CKD patients. Few studies were available at that time to support this. In our systematic review, additional studies using this form of vitamin D are included. In 2017, a commentary was published, reflecting the views of the KDOQI CKD-MBD work group which were mostly in agreement with the updated KDIGO guidelines discussed below[337].

Table 8. Guidelines for monitoring and correction of vitamin D deficiency in CKD patients

	G1	G2	G3	G4	G5
	≥90mL/min	60-89mL/min	30-59mL/min	15-29mL/min	<15mL/min
NKF KDOQI (2003)[320] (2016)[237]	• Follow guidelines for general population • Target thresholds as general population.		• If plasma/serum 25(OH)D concentration is <75nmol/L D_2 supplementation should be given with dosages dependent on baseline values (**Table 4**) with monitoring of 25(OH)D and calcium and phosphate homeostasis† (**Fig. 1**) • Maintenance: continue supplementation with a vitamin-D containing multi-vitamin and an annual reassessment of 25(OH)D†. • ER calcifediol can be used with vitamin D deficiency and SHPT.		• Vitamin D analogue therapy should be given when SHPT is progressive and persistent higher from the upper limit of the assay used.
KDIGO (2009)[291] (2017)[236]	• Follow guidelines for general population • Target thresholds for 25(OH)D as for general population • Monitor plasma/serum 25(OH)D concentrations once a year. If normal no treatment is required. If deficient, treat per general population‡		• Monitoring of plasma/serum 25(OH)D concentrations at intervals dependent on CKD stage, baseline values and therapeutic interventions*, with monitoring of calcium and phosphate homeostasis* Vitamin D deficiency and insufficiency be corrected using treatment strategies recommended for the general population. • In non-dialysis patients with progressively rising PTH concentrations above the upper limit of normal for the assay, despite correction of modifiable factors the use $1,25(OH)_2D$ or vitamin D analogues is recommended. • With severe and progressive SHPT and CKD-MBD in G4-5 $1,25(OH)_2D$ or vitamin D analogues is recommended.		
Kidney Health Australia (CARI 2012-2013) [331,335]	• Vitamin D deficiency (<37.5 nmol/L) and insufficiency (37.5-75 nmol/L) should be corrected using treatment strategies recommended for the general population. • Vitamin D therapy for early CKD patients with SHPT is recommended with monitoring of markers of calcium and phosphate homeostasis and bone metabolism§ • 25(OH)D and PTH levels should be monitored regularly while on vitamin D therapy§.				

† Monitor of phosphorus and corrected total calcium every 3 months. See figure 1 for more details [237,320]; ‡Monitor calcium and phosphate levels. Provide instructions to reduced dietary phosphate intake [236,291]; * Monitoring intervals: G3: serum calcium and phosphorus: every 6-12 months; intervals for PTH based on baseline concentration and CKD progression; G4: serum calcium and phosphorus: every 3-6 months; PTH: every 6-12 months; G5 to 5D: serum calcium and phosphorus: every 1-3 months; PTH: every 3-6 months [236,291]; §CKD patients on vitamin D therapy: regular monitoring of plasma calcium, phosphate and alkaline phosphatase levels [331,335].

Table 9. Correction and monitoring of vitamin D deficiency in patients with CKD G3-4

Serum 25(OH)D nmol/L [ng/ml]	Definition	Vitamin D_2 dose	Duration (months)	Comment
<12 [<5]	Severe vitamin D deficiency	50 000 IU/w orally x 12w; then monthly	6 months	Measure 25(OH)D levels after 6 months
		500 000 IU as a single I.M. dose	n/a	Assure patient adherence; measure 25(OH)D at 6 months
12-37 [5-15]	Mild vitamin D deficiency	50 000 IU/w x 4 w; then 50 000 IU/m orally	6 months	Measure 25(OH)D levels after 6 months
40-75 [16-30]	Vitamin D insufficiency	50 000 IU/m orally	6 months	n/a

After NKF KDOQI (2003): Guideline 7;Table 26[320]

In 2003 the first detailed guidelines by KDIGO based on the available literature was published[291]. Since then, updates of those guidelines occur due to new literature available. That is the reason of carrying out a systematic search of RCTs dating back to 2003. In 2017 KDIGO issued their updated guideline for CKD-MBD[236]. In parallel to the NKF guideline, there are no specific recommendations (type of vitamin D, dose and duration) for the prevention of vitamin D deficiency and the treatment of SHPT due to a lack of sufficient high-quality evidence specific to CKD patients. This guideline emphasizes the importance of managing other factors influencing PTH, including a high plasma phosphate and low calcium. This recommendation also considered reported adverse effects (since the previous review) of vitamin D analogues and calcitriol on the development of hypercalcemia, while clinically relevant outcomes did not substantially improve. It was therefore concluded that the risk-benefit ratio of treating an elevated PTH with these forms of vitamin D is no longer favorable for the majority of patients[320]. Therefore, in this guideline, the use of vitamin D analogues and $1,25(OH)_2D$ are recommended only for patients with severe and progressive SHPT (**Table 8**). The Kidney Health Australia, CARI guidelines of 2012-2013[331,335] and the CKD guidelines for general practice issued in 2015[336] recommend that vitamin D deficiency in CKD patients should be corrected following guidelines for the general population (**Table 6**; **Table 8**). Vitamin D therapy on prescription is recommended for early stages of CKD with SHPT, with regular monitoring of plasma calcium, phosphate, PTH, ALP and 25(OH)D. Treatment with $1,25(OH)_2D$ is only recommended in later stages of CKD for the treatment of SHPT.

Thresholds and correction of vitamin D deficiency

Thresholds for the definition of vitamin D deficiency for the general population differ between advisory bodies (summarized in **Table 6**). Some, but not all also provide thresholds of sufficiency Recommended 25(OH)D target concentrations and thresholds for deficiency for specific patient groups may be higher than for generally healthy people (**Table 7**; **Table 9**). Guidance for the correction of vitamin D deficiency is not provided in population guidance, but instead relies on country-specific clinical guidance for patient management. **Table 7** and **Table 8** provides an overview of the recommendations from 4 authorities, including the NKF[331,332].

There is considerable between-person variation in the dose-response to vitamin D because of the numerous factors that can affect plasma 25(OH)D and its increment after intake. Reported increases in plasma 25(OH)D in apparently healthy populations (including those that were deficient at baseline) range from 1.1-5.75nmol/L per 100IU/d[338–341]. This dose-response relationship is influenced by baseline concentrations of 25(OH)D, vitamin D dose, frequency of administration, body composition and a number of medical conditions[308]. Population guidance for the prevention of deficiency allows for these variations, but specific at-risk groups may need higher intakes to prevent and correct vitamin D deficiency. For the correction of deficiency, higher intakes are required and often loading dosages are recommended, followed by maintenance therapy (**Table 7**). Clinical monitoring is required with these loading dosages.

Population guidance considered at what concentration range of 25(OH)D, the risk of SHPT was increased and this is incorporated in the assessment of vitamin D requirements[8,327]. Also, this relationship is characterized by a large variability and target values for PTH were not formulated for the general population.

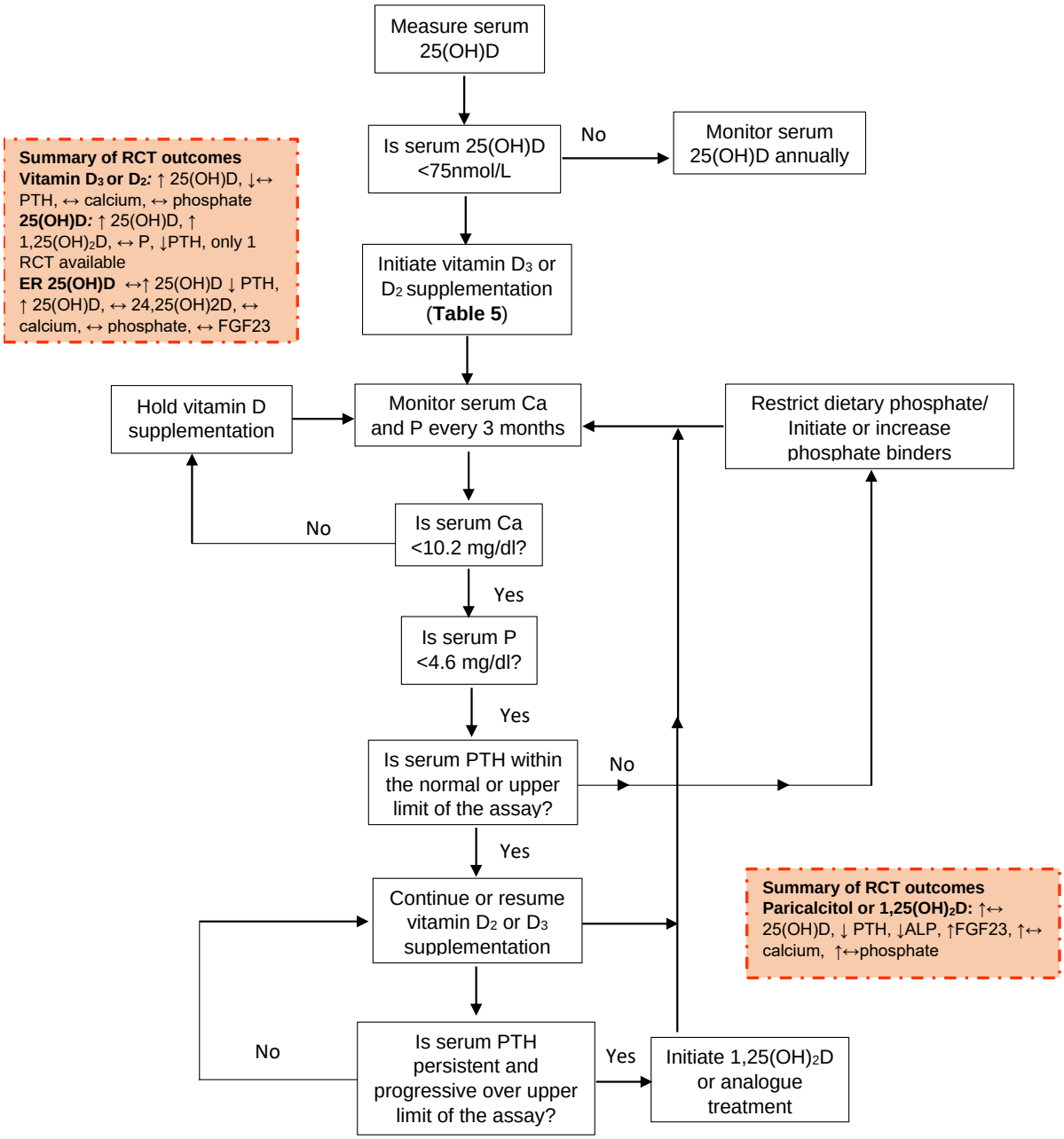
In CKD patients, the threshold for vitamin D sufficiency and the vitamin D intake to achieve and maintain sufficiency is less well established than in generally healthy people. This partly due to the fact that few high-quality studies relating 25(OH)D with clinical outcomes, such as fragility fractures or bone mineral density, were conducted in CKD patients. Also, the heterogeneity in this patient group plays a role. In these patients, the vitamin D dose-response and relationship between 25(OH)D and PTH is dependent of CKD category, renal

capacity to produce 1,25$(OH)_2$D and the degree of PTH resistance. Also, diversity in clinical presentation of CKD-MBD, influences the response in bone metabolism.

For CKD patients, a higher target concentration (>75 nmol/L) than for the general population is recommended in the NKF and CARI guidelines [237,331] with regular monitoring and correction as required on a 6-12 monthly basis (**Table 7**; **Table 8**; **Table 9**; **Figure 15**). Target values for PTH could not be set on the basis of the evidence available. In addition, the PTH-25(OH)D relationship (used formulation of in many population guidelines) could not be used in the assessment of vitamin D requirements for CKD patients due to their altered relationship. Current recommendations advise regular monitoring and management if PTH progressively increases. In view of the variability in clinical presentation, patients are managed on a case-by-case basis with monitoring of calcium and phosphate homeostasis (**Figure 15**).

Guidance for management of vitamin D status and deficiency, population recommendations and patient management are summarized in **Table 6**; **Table 7** and **Figure 15**.

Figure 15. Guidance for monitoring of vitamin D status and supplementation and monitoring of calcium and phosphate metabolism in CKD stages G3-4



Based on recommendations from the different organizations summarized in Table 5. Adapted from: NKF KDOQI (2003)[320]. Finding in recent RCTs (2003-2019) included in the systematic review are summarized in yellow boxes.

Conclusion of Chapters 2a & 2b

Major gaps remain in the evidence base for the management of vitamin D status in relation to CKD–MBD, i.e. SHPT, altered bone metabolism, bone density and integrity and fracture risk. Recent studies included in this systematic review varied in design, vitamin D form used there was a high degree of heterogeneity with regard to duration, dose, and population characteristics. Our systematic review and meta-analyses showed that although the effect of vitamin D on PTH concentrations was inconsistent between studies, meta-analyses showed a borderline significant reduction. This is in contrast to findings in studies in patients not selected on basis of pre-existing CKD. This is likely explained by the fact that in some but not all patients with CKD, other drivers are predominant and prevent a decrease of PTH secretion. More consistent effects on PTH were found with calcidiol; all four studies that used this form reported a reduced PTH. Treatment with calcitriol and paricalcitol was associated with a consistent and a greater suppression of PTH but an increased risk of hypercalcaemia. An increase FGF23 after treatment with vitamin D analogues was observed in all three studies reporting this outcome, but was unaltered in four studies with Vitamin D or 25(OH)D. The increase in FGF23 with analogue administration warrants attention as this hormone is already elevated in CKD patients and is a predictor of vascular calcification and CVD. Its increase may indicate an undesirable side effect of administration of these forms of vitamin D. Few RCTs reported the effect of vitamin D on markers of bone metabolism and variations in the range of markers prevented direct comparisons. However, all four studies that measured either ALP or BAP after paricalcitol treatment reported a decrease in this marker indicative of increased bone turnover in CKD patients.

Guidelines for the first stages (G1-G3a) follow general population recommendations for the prevention of vitamin D deficiency. For the correction of deficiency, general or CKD specific patient guidelines provide recommendations. These are summarized in a tabulated format to facilitate their use in clinical practice.

CHAPTER 3: Vitamin D supplementation for 12 months in older adults alters regulators of bone metabolism but does not change Wnt-signalling pathway markers

Chapter 3 is based on the following publication[342].

Christodoulou M, Aspray TJ, Piec I, et al. Vitamin D Supplementation for 12 Months in Older Adults Alters Regulators of Bone Metabolism but Does Not Change Wnt Signaling Pathway Markers. JBMR Plus. March 2022:e10619. doi:10.1002/JBM4.10619

Summary

Vitamin D status and supplementation regulates bone metabolism and may modulate Wnt-signalling.

In this chapter the study aimed to investigate changes in regulators and markers of bone metabolism, BMD and BMC in response to different dosages of vitamin D supplementation in older people for 12 months (VDOP study: 12,000IU, 24,000IU, 48,000IU/month for 1 year; men and women >70y; n=379; ISRCTN35648481). We investigated four categories of markers: (a) calcium metabolism and renal function, (b) vitamin D metabolites, (c) Wnt-signalling pathway markers and (d) bone parameters and bone metabolism. Further, we investigated their associations with total 25(OH)D and free 25(OH)D at baseline and after 12 months of supplementation.

Baseline vitamin D status was (mean ± SD) 25(OH)D: 40.0 +/- 20.1 nmol/L. Supplementation dose-dependently increased total and free 25(OH)D concentrations and decreased plasma phosphate and iPTH (all $p<0.05$). The PINP:CTX ratio, cFGF23 and iFGF23 significantly increased with no between-group differences, while Klotho was unchanged. $1,25(OH)_2D$ and PINP significantly increased in the 24 and 48,000IU groups. SOST, OPG, RANKL, BMD, BMC and CTX remained unchanged. Subgroup analyses with baseline 25(OH)D<25nmol/L (n= 94) provided similar results.

Baseline total and free 25(OH)D concentrations were positively associated with $1,25(OH)_2D$, $24,25(OH)_2D$ ($p<0.001$), DBP ($p<0.05$), BMD and BMC ($p<0.05$). Associations with iPTH

(p<0.001), cFGF23 (p<0.01) and BAP (p<0.05) were negative. After supplementation, total and free 25(OH)D concentrations remained positively associated only with $24,25(OH)_2D$ (p<0.001), DBP (p<0.001) and negatively with eGFR (p<0.01). iPTH and SOST were significantly associated only with free 25(OH)D. There were no significant relationships with BMD and BMC after supplementation.

The decrease in iPTH and increase in PINP:CTX ratio suggest a protective effect of supplementation on bone metabolism although no significant effect on BMD or pronounced changes in regulators of Wnt-signalling were found. The increase in FGF23 warrants caution due to its negative association with skeletal and cardiovascular health. Associations of total and free 25(OH)D with biomarkers were similar and known positive associations between vitamin D status and BMD were confirmed. The change in associations after supplementation might suggest a threshold effect.

Methods

Study design

This study is a secondary analyses utilising plasma samples collected as part dose-ranging randomised vitamin D intervention trial in older people (VDOP)[343] (ISRCTN35648481 and EudraCT 2011-004890-10). In brief, this RCT included 379 adults aged ≥70 y (48% women; mean age 75 y) from the northeast of England. Participants were randomly allocated to 1 of 3 doses of vitamin D_3 [12,000 international units (IU), 24,000 IU, or 48,000 IU] given once a month for a year. The 12,000 and 24,000 IU dosages correspond to the UK Dietary RNI of 400 IU/day (10 µg/day)[8] and the North American RDA of 800 IU/day (20 µg/day) for people over 70 years old.

This study was powered to detect a change in BMD at the hip in response to 12,000, 24,000, or 48,000 IU vitamin D_3/m for 1 year, using 12,000 IU as the reference dose. The power calculation was based on findings in an earlier, similar study in the North of the UK[343,344]. Detailed description of the design, methods and primary outcomes of VDOP were earlier published[343,345]. Results for bone mineral density and bone area (at hip and femoral neck), plasma concentrations of 25(OH)D, iPTH, albumin, calcium and creatinine were earlier

reported but are also included here as part of secondary analyses and to support data interpretation.

Additional methods used for these secondary analyses are provided below. These explorative secondary analyses were not pre-specified in the original trial design and analyses plan.

The study was conducted in accordance with guidelines laid down in the Declaration of Helsinki. A favourable opinion was obtained from the Tyne & Wear South Research Ethics Committee (REC, 12/NE/0050) with Research and Development approval from the sponsor, Newcastle upon Tyne Hospitals NHS Foundation Trust. All participants provided written informed consent.

Measurements

Measurements of BMD and BMC at the hip and femoral neck (FN), height and weight were taken [343]. Early morning fasting blood samples were collected from all participants at baseline and after 12 months of supplementation. Plasma calcium, albumin and creatinine were measured by Newcastle upon Tyne hospitals NHS Foundation Trust (NUTH) laboratories and the analysis was carried out immediately after sample collection.

The remaining blood samples were placed on ice and separated within 30 minutes of collection in a refrigerated centrifuge at 1,800 g for 20 minutes. Plasma was transported on dry ice and stored at −80°C. Biochemical analysis took place at Medical Research Council (MRC) Human Nutrition Research, Cambridge, UK. The assays specifications was as described before [343]. Analyses specific for this secondary study were conducted at Bioanalytical Facility of University of East Anglia (UEA), UK and are specified below.

In brief, analyses conducted at MRC Human Nutrition Research included 25(OH)D (LC-MS/MS), DBP (ImmunDiagnostik AG ELISA), iPTH (Immulite 2000, SIEMENS), PINP (UniQ, RIA), β-CTX (Immunodiagnostic) and BAP (DiaSorin LIAISON). All assays were performed in duplicate except for PTH. Assay performance was monitored using kit and in-house controls and under strict standardisation according to ISO 9001:2000. External quality assurance of 25(OH)D and iPTH assays were performed as part of the Vitamin D External Quality Assessment Scheme (www.deqas.org) and the National External Quality Assessment Scheme

(**www.ukneqas.org.uk**). Measurements of 25(OH)D were harmonised against NIST standards as part of the Vitamin D harmonisation program [343].

Measurements conducted at UEA included serum phosphate (Phosphate (Inorganic) ver.2, Cobas, Roche), αKlotho (IBL international), cFGF23 (Immutopics, Gen 2), iFGF23 (Immutopics, Gen 2), OPG (Biomedica), SOST (Biomedica), DKK1 (Biomedica), soluble RANKL (sRANKL Biomedica), $24,25(OH)_2D$ (LC-MS/MS)[346], $1,25(OH)_2D$ (Diasorin, Liaison XL assay) and Cystatin C (Tina-quant Cystatin C Gen.2). All assays were performed in duplicate except for $1,25(OH)_2D$, Cystatin C and phosphate. The inter and intra-assay coefficient of variation (CV) of all assays were <10% except for $24,25(OH)_2D$ and $1,25(OH)_2D$, which were <15%. Assay performance was monitored using kit and in-house controls and following Good Laboratory Practice.

All the biochemical analysis was conducted prior the start of this PhD study except markers of cystatin C and phosphate were conducted by M. Christodoulou during this PhD study.

Derived variables

eGFR was calculated using the MDRD-4 algorithm.

Calculated ratios included 25(OH)D:$24,25(OH)_2D$, $1,25(OH)_2D$:$24,25(OH)_2D$, PINP:CTX, sRANKL:OPG and cFGF23:iFGF23 and were expressed as on molar:molar ratio, except for PINP:CTX and cFGF23:iFGF23.

Free 25(OH)D was calculated using the equation[347]:

$$\text{Free 25(OH)D} = \text{total 25(OH)D} / [1+(6*10^3 \times \text{Albumin}) + (7*10^8 \times \text{DBP})]$$

Statistical analysis

The findings presented in this paper are the results of explorative secondary analyses. The primary outcome of the VDOP study was the change in BMD at the hip. A formal power calculation for secondary outcomes were not conducted but instead an estimation of the detectable effect size is provided for SOST data. In addition, correction for repeated testing was not deemed appropriate for this explorative analysis as any finding will require confirmation in RCTs specifically designed and powered for respective outcomes.

The sample size calculation was based on a detectable effect size (a 15% reduction in plasma SOST in any arm of study). This is within the observed % reduction of SOST after treatment

with pharmaceutical agents to reduce bone resorption. Data from a study in older men and women (>65 years of age; n=95) provided an estimate of the biological variability of SOST[348]. The mean (SD) plasma SOST concentration was 27.8 (14.1)ng/ml. The between subject CV % [(CV); SD*100/mean %)] was 51%. Other data suggest a CV of 30-42%[349–351]. It is assumed that the within subject variation is approximately half the size of the between subject estimate, i.e. 25-30 %. The sample size calculation was based on a conservative CV% of 30%. To detect an effect size of 15%, with a 30% CV, 5% significance level and 90% power the required sample size is 84 subjects per arm. Samples available from the VDOP study were n= 113, 114 and 116/arm.

Prior to t-tests and analysis of covariance (ANCOVA) analyses, all outcomes were assessed for normality (defined as a posterior distribution skewness <2 or >-2) and visual inspection of histograms. Non-normally distributed variables were converted to natural logarithm values (LN) and checked again for normality. The distribution of Klotho and cFGF23 at both time points (baseline and 12 months) were extremely skewed. Outliers were identified on basis of z-scores (based on interquartile range; IQR) and excluded if <-2.68 or >2.68. After excluding the extreme outliers, the LN values of both variables were normally distributed. Analyses were conducted with and without these outliers and there were no material differences between outcomes and interpretation of the data.

Differences between pre- and post- supplementation values were tested with paired sample t-tests for each supplementation group. Between group differences post-supplementation were tested by ANCOVA, with the baseline value as co-variate. Additional models included eGFR and gender as co-variates. These models did not provide substantially different results and/or these co-variates were non-significant and therefore only the result of the ANCOVAs with the baseline value as co-variate were reported, unless stated otherwise. Data were presented as mean and SD or median and IQR for normally distributed and skewed data, respectively.

To assess whether vitamin D deficiency at baseline influenced the effect of supplementation, analyses as described above were conducted separately for participants with a plasma 25(OH)D ≤25nmol/L at baseline.

Regression analyses was used to test associations with total and free 25(OH)D concentration before and after 12 months of supplementation. For post supplementation data, the dose was entered as a co-variate, but was non-significant. Therefore, results of univariate models are presented. Regression analyses for variables derived from the independent variable were not conducted (i.e. for free 25(OH)D these were: DBP, albumin and total 25(OH)D) and for any of the ratios with total 25(OH)D). Linearity of associations was visually inspected. Two outliers for free 25(OH)D were excluded from the 12 months data. Results are presented as the β-coefficient and associated p value. These regression analyses were conducted using the natural values of the data (not LN converted).

For the statistical analysis of the data IBM® SPSS® Statistics Version 25 software was used.

Results

Baseline characteristics are presented in **Table 10**. Baseline characteristics were well balanced between treatment groups and no significant differences were found.

Plasma calcium and renal function markers

Adjusted calcium remained unaltered. There was a significant decrease in plasma phosphate in all treatment groups compared to baseline ($p<0.01$), but there was no dose effect. There were significant changes in plasma creatinine in all treatment groups ($p<0.001$), with no between groups differences but eGFR was unaltered. Cystatin C significantly increased from baseline in the 24,000 IU and 48,000 IU group ($p<0.001$; $p<0.05$ respectively) with no between groups differences. Plasma cFGF23 and iFGF23 significantly increased in all treatment groups with supplementation ($p<0.05$) without a significant dose effect. The c:iFGF23 ratio remained unaltered (**Table 10**). Although Klotho remained unaltered compared to baseline, there were significant between group differences ($p<0.001$) after supplementation.

In ANCOVA models for albumin and cFGF23, eGFR was a significant covariate. Inclusion of eGFR in these models did not alter the interpretation of findings.

Table 10. Participants' characteristics and response to vitamin D supplementation[a]

	Baseline	12 months [12 000 IU]	12 months [24 000 IU]	12 months [48 000 IU]	ANCOVA[b] analysis
N	379	122	124	126	
Age (years)	74.1 [71.5-77.0]	75.6 [72.5-77.3]	76.0 [72.5-77.9]	76.4 (4.4)	n/a
Plasma calcium and renal function markers					
Albumin (g/L)	45.7 (2.2)	44.6 (2.1)*	44.5 (2.6)*	44.3 (2.0)*	0.70
Adjusted calcium (mmol/L)	2.2 (0.1)	2.3 (0.1)	2.2 (0.1)	2.2 (0.1)	0.07
Phosphate (mmol/L)	0.88 (0.18)	0.79 (0.19)*	0.81 (0.17)*	0.81 (0.19)*	0.68
Cystatin C (mg/L)	0.87 (0.22)	0.88 (0.27)	0.92 (0.25)*	0.95 (0.29)*	0.41
Creatinine (µmol/L)	82.1 (19.1)	82.5 [67-94]*	84.6 [71-96]*	78.1 (66-88]*	0.25
eGFR (mL/min per 1.73 m^2)	72 (15)	73 (14)	74 (16)	70 (15)	0.54
Klotho (pg/mL)	493.7 [392.6-627.7]	502.0 [403.1-639.6]	502.7 [395.5-611.2]	477.6 [399.6-589.2]	<0.001
cFGF23 (RU/mL)	66.7 [54.9-84.2]	90.8 [65.3-104.9]*	85.7 [59.7-94.9]*	77.8 [58.4-87.3]*	0.11
iFGF23 (pg/mL)	55.1 [44.5-72.7]	66.9 [42.5-88.4]*	71.7 [54.5-79.4]*	73.2 [52.2-84.7]*	0.80
i:cFGF23	1.9 [1.5-2.5]	2.1 [1.4-2.2]	2.2 [1.4-2.3]	2.3 [1.4-2.3]	0.75
Vitamin D metabolism markers					
Total 25(OH)D (nmol/L)	40.0 (20.1)	55.9 (15.6)*	64.6 (15.3)*	79.0 (15.1)*	<0.001
Free 25(OH)D (pmol/L)	8.4 (4.3)	11.7 (3.3)*	13.8 (3.4)*	16.9 (4.3)*	<0.001
24,25$(OH)_2$D (nmol/L)	3.2 [2.0-5.5]	6.1 (2.7)*	7.4 (2.8)*	9.4 (3.0)*	<0.001
25(OH)D:24,25$(OH)_2$D	14.5 [11.3-18.8]	12.1 [9.4-12.9]*	11.9 [10.0-13.4]*	12.9 (4.7)*	<0.001
1,25$(OH)_2$D (pmol/L)	94.5 (29.0)	100.6 (29.8)	101.0 (29.4)*	101.9 (30.8)*	0.099
1,25$(OH)_2$D:24,25$(OH)_2$D	25.7 [16.8-44.4]	17.7 [11.9-21.8]	14.6 [8.9-18.7]	15.3 [9.0-18.2]	<0.001
DBP (mg/L)	367.8 (63.4)	362.5 (74.1)	356.9 (46.1)	384.4 (57.8)*	<0.01
iPTH (pg/ml)	43.4 [33.2-57.4]	39.8 [28.8-53.5]*	40.9 [26.3-55.5]*	37.3 [27.8-47.5]*	<0.01
Wnt-signalling pathway markers					
SOST (pmol/L)	44.3 [32.4-60.0]	46.9 [32.6-63.5]	45.4 [32.1-57.8]	46.5 [33.2-61.2]	0.20

DKK1 (pmol/L)	31.2 (16.5)	40.6 (17.9)*	33.2 (19.0)	38.9 (18.1)*	0.87
OPG (pmol/L)	5.67 (2.08)	5.69 (2.04)	5.12 [4.25-6.47]	5.89 (2.17)	0.20
sRANKL (pmol/L)	0.12 [0.08-0.18]	0.14 [0.08-0.18]	0.13 [0.07-0.18]	0.14 (0.07)	0.75
sRANKL:OPG	0.02 [0.01-0.04]	0.03 [0.01-0.03]	0.04 [0.01-0.04]	0.03 [0.01-0.04]	0.85
Bone mineral density and metabolism					
Hip BMD (g/m^2)	0.98 (0.17)	0.96 (0.15)	0.98 (0.16)	0.99 (0.18)	0.19
Hip BMC (g)	35.44 (8.30)	34.08 (7.56)	35.42 (7.92)	35.73 (8.63)	0.14
FN BMD (g/m^2)	0.902 (0.152)	0.88 (0.13)	0.90 (0.14)	0.92 (0.15)	0.13
FN BMC (g)	4.90 (1.09)	4.78 (0.96)	4.82 (1.04)	4.70 (1.16)	0.72
BAP (µg/L)	9.5 [7.9-12.3]	11.4 [8.4-13.8]*	10.7 [7.7-12.7]	11.4 [8.0-14.1]	0.87
CTX (ng/mL)	0.40 [0.30-0.50]	0.36 (0.16)	0.37 (0.15)	0.35 (0.14)	0.48
PINP (µg/L)	36.2 [28.8-46.2]	40.1 [31.7-52.6]	39.1 [31.0-46.6]*	38.4 [28.9-47.1]*	0.53
PINP:CTX	101.4 [85.9-116.9]	120.5 [103.6-155.2]*	124.0 [107.6-158.5]*	118.8 [100.8-157.1]*	0.99

Participants' characteristics and response to vitamin D supplementation[a]

[a]For normally distributed data, results are expressed as mean (SD); for skewed results are expressed as median [interquartile range].

*Paired T-tests were used to analyse pre- and post- supplementation values for each supplementation group; * denotes significantly different from baseline p <0.05

[b]ANCOVA was used to test between group differences after 12 months of supplementation, with the baseline value as a co-variate.

Vitamin D metabolism

Post-supplementation all vitamin D metabolites were significantly higher in all treatment groups ($p<0.001$) compared to baseline, except $1,25(OH)_2D$ which only significantly increased in the 24,000 IU and 48,000 IU group (both $p<0.01$). Supplementation had a significant dose-dependent effect on total, free 25(OH)D and $24,25(OH)_2D$ (all $p<0.001$). The vitamin D metabolites ratios were significantly different between the groups ($p<0.001$). DBP was unchanged, except for a significant increase in the 48,000 IU group ($p<0.05$) after supplementation and there were significant differences between treatment groups ($p<0.001$). iPTH decreased in all treatment groups after supplementation ($p<0.05$) with a significant dose-dependent effect ($p<0.001$) (**Table 10**).

In ANCOVA models for total and free 25(OH)D, eGFR was a significant covariate. Both models with and without this covariate were significant (both $p<0.001$).

Wnt-signalling pathway markers

There were no changes with supplementation in plasma concentrations of SOST, OPG, sRANKL and sRANKL:OPG ratio. Differences between groups were non-significant. DKK1 significantly increased in the 12,000 IU and 48,000 IU groups ($p<0.05$) and there were significantly differences between the treatment groups ($p<0.05$) (**Table 10**).

Bone parameters and markers of bone metabolism

BMD and BMC at the hip, FN and CTX were not significantly different compared to baseline and there were no group differences as reported before [343,345]. Femoral neck area was significantly lower in the 48,000IU group and there were significant differences between the groups after supplementation ($p<0.001$). BAP significantly increased only in the 12,000 IU group ($p<0.05$). PINP:CTX ratio significantly increased, respectively with supplementation in all treatment groups (all $p<0.001$). PINP significantly increased compared to baseline only in the 24,000 IU and 48,000 IU groups ($p<0.001$) with no differences between groups (**Table 10**).

Subgroup analyses by vitamin D deficiency at baseline

Deficient study population characteristics and comparisons

At baseline, 28% of participants had a 25(OH)D concentration ≤25nmol/L (mean 25(OH)D: 18.8 ± 4.1 nmol/L and the numbers were equally distributed between supplementation groups[343]. This group had a significantly lower hip and FN BMD and BMC. Plasma concentrations of $1,25(OH)_2D$, $24,25(OH)_2D$ and PINP were lower and iPTH and cFGF23 were higher compared to the group with baseline 25(OH)D >25nmol/L (all $p<0.05$). After supplementation, no significant changes in hip and FN BMD and BMC were found. In line with the findings in the full cohort, supplementation significantly increased concentrations of total and free 25(OH)D, $24,25(OH)_2D$ and cFGF23 concentrations and decreased PTH in all supplementation groups. Plasma iFGF23 significantly increased with the 2 highest dosages. Plasma $1,25(OH)_2D$ was significantly higher in all treatment groups. Klotho, SOST, OPG, RANKL, BAP and CTX remained unchanged. The observed increase in PINP in the full cohort was not found but instead a PINP significantly decreased in the 24,000 IU group but the PINP:CTX ratio significantly increased in all groups. The observed decrease in plasma phosphate was not found in this subgroup.

Associations with total and free 25(OH)D plasma concentrations

Plasma calcium and renal function markers

At baseline, total and free 25(OH)D were significantly negatively associated with cFGF23 ($p<0.001$) (**Figure 1**) and the c:iFGF23 ratio ($p<0.05$) (**Table 11**).

After supplementation, total and free 25(OH)D were negatively associated with adjusted calcium ($p<0.05$) and eGFR ($p<0.01$ and $p<0.001$ respectively) (**Table 11**). No associations were found with the rest of the biomarkers after supplementation (**Table 11**).

Vitamin D metabolism

Pre-supplementation both total and free 25(OH)D were positively associated with $24,25(OH)_2D$ and $1,25(OH)_2D$ and negatively associated with $1,25(OH)2D$:$24,25(OH)_2D$ and

iPTH (all p<0.001) (**Table 11**; **Figure 17**). DBP was positively associated with total 25(OH)D (p<0.01) (**Table 11**).

Post- supplementation, DBP and 24,25$(OH)_2$D were positively associated with total 25(OH)D (both p<0.001) (**Table 11**)**.** Plasma PTH was negatively associated with free 25(OH)D (p<0.05) and there was tendency of significance for total 25(OH)D (p=0.09) (**Table 11**; **Figure 17**).

Wnt-signalling pathway markers

The Wnt-signalling markers DKK1, OPG and sRANKL were not significantly associated with either total or free 25(OH)D both pre- and post-supplementation. SOST was positively associated only with free 25(OH)D after supplementation (**Table 11**; **Figure 18**).

Bone density and metabolism

At baseline both total and free 25(OH)D were positively associated with hip BMD (p<0.01 and p<0.05, respectively), hip BMC (p<0.05 and p<0.01, respectively), FN BMC (p<0.05 and p<0.01, respectively) and FN BMD (both p<0.05) (**Table 11; Figure 16**). Of the bone metabolism markers, only BAP was significant (positively) associated with total and free 25(OH)D (p<0.05) (**Table 11**).

After supplementation, no significant associations were found (**Table 11**).

Table 11. Associations of total and free 25(OH)D with biomarkers at baseline and 12 months

	Baseline				12 months			
	Total 25(OH)D (nmol/L)		Free 25(OH)D (pmol/L)		Total 25(OH)D (nmol/L)		Free 25(OH)D (pmol/L)	
	β coefficient	p-value	β coefficient	p-value	β coefficient	p-value	β coefficient	p-value
Plasma calcium and renal function markers								
Albumin (g/L)	0.004	0.44	n/a	n/a	-0.003	0.72	n/a	n/a
Adjusted calcium (mmol/L)	0.000	0.34	0.001	0.46	**-0.001**	**<0.05**	**-0.002**	**<0.05**
Serum Phosphate (mmol/L)	0.001	0.08	0.003	0.18	0.000	0.84	0.003	0.18
Cystatin C (mg/L)	0.000	0.50	-0.002	0.425	0.000	0.88	0.004	0.19
Serum creatinine (µmol/L)	0.027	0.58	0.169	0.47	-0.074	0.23	-0.424	0.10
eGFR (mL/min per 1.73 m^2)	0.026	0.50	0.144	0.43	**-0.141**	**<0.01**	**-0.710**	**<0.001**
Klotho (pg/mL)	-1.398	0.40	-7.066	0.37	-1.606	0.10	-1.771	0.66
cFGF23 (RU/mL)	**-0.433**	**<0.001**	**-1.741**	**<0.01**	-0.203	0.10	-0.396	0.45
iFGF23 (pg/mL)	0.107	0.22	0.227	0.50	0.047	0.68	0.485	0.31
i:cFGF23	**-0.023**	**<0.05**	**-0.104**	**<0.05**	-0.005	0.21	-0.006	0.74
***Vitamin D metabolism* markers**								
$24,25(OH)_2D$ (nmol/L)	**0.126**	**<0.001**	**0.559**	**<0.001**	**0.124**	**<0.001**	**0.452**	**<0.001**
$1,25(OH)_2D$ (pmol/L)	**0.425**	**<0.001**	**1.964**	**<0.001**	0.130	0.15	0.268	0.49
$1,25(OH)_2D$:$24,25(OH)_2D$	**-0.916**	**<0.001**	**-0.114**	**<0.001**	-0.010	0.73	-0.015	0.91
DBP (mg/L)	**0.439**	**<0.01**	n/a	n/a	**0.803**	**<0.001**	n/a	n/a
iPTH (pg/ml)	**-0.360**	**<0.001**	**-1.612**	**<0.001**	-0.121	0.09	**-0.752**	**<0.05**
Wnt-signalling pathway markers								
SOST (pmol/L)	0.020	0.76	0.237	0.45	0.014	0.86	**0.634**	**<0.05**
DKK1 (pmol/L)	0.000	0.99	-0.03	0.89	0.035	0.55	0.194	0.46
OPG (pmol/L)	0.002	0.76	-0.016	0.53	0.005	0.51	0.024	0.45
sRANKL (pmol/L)	0.000	0.90	0.000	0.79	0.000	0.78	0.000	0.66
sRANKL:OPG	0.000	0.88	0.000	0.82	0.000	0.85	0.000	0.94
Bone density and metabolism								

Hip BMD (g/m^2)	**0.001**	**<0.01**	**0.006**	**<0.01**	0.001	0.10	0.003	0.14
Hip BMC (g)	**0.053**	**<0.05**	**0.298**	**<0.01**	0.036	0.16	0.121	0.27
FN BMD (g/m^2)	**0.001**	**<0.05**	**0.005**	**<0.01**	0.001	0.14	0.003	0.11
FN BMC (g)	**0.007**	**<0.05**	**0.039**	**<0.01**	0.000	0.99	0.000	0.99
BAP (µg/L)	**-0.019**	**<0.05**	**-0.096**	**<0.05**	0.006	0.72	0.038	0.58
CTX (ng/mL)	0.000	0.61	-0.002	0.43	0.000	0.51	-0.001	0.73
PINP (µg/L)	-0.034	0.42	-0.202	0.32	-0.031	0.63	-0.014	0.96
PINP:CTX	0.007	0.93	-0.005	0.99	-0.137	0.26	-0.315	0.54

Univariate linear regression analysis; the table displays the β coefficients and the ANOVA p-value for the β coefficient.
For abbreviations see **Table 10**.
Regression analyses for variables derived from the independent variable were not conducted (i.e. for free 25(OH)D these were: DBP, albumin and total 25(OH)D) and for any of the ratios with total 25(OH)D).

Figure 16. Correlations of total and free 25(OH)D with cFGF23 at baseline and 12 months

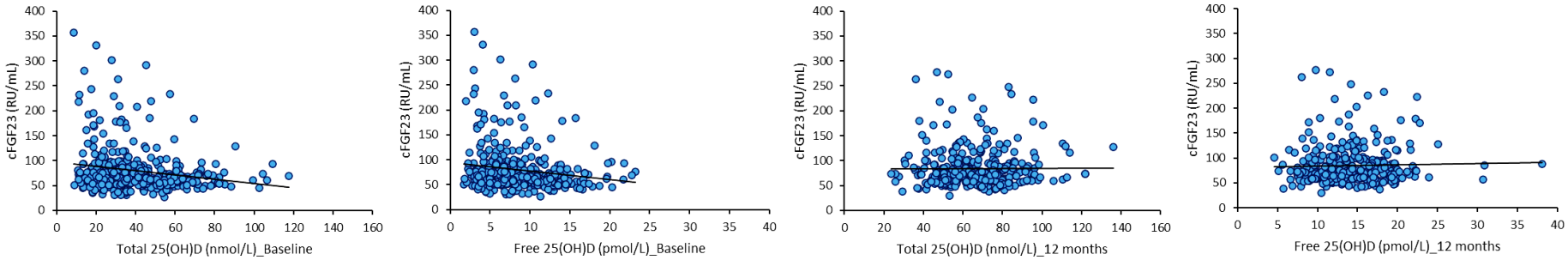
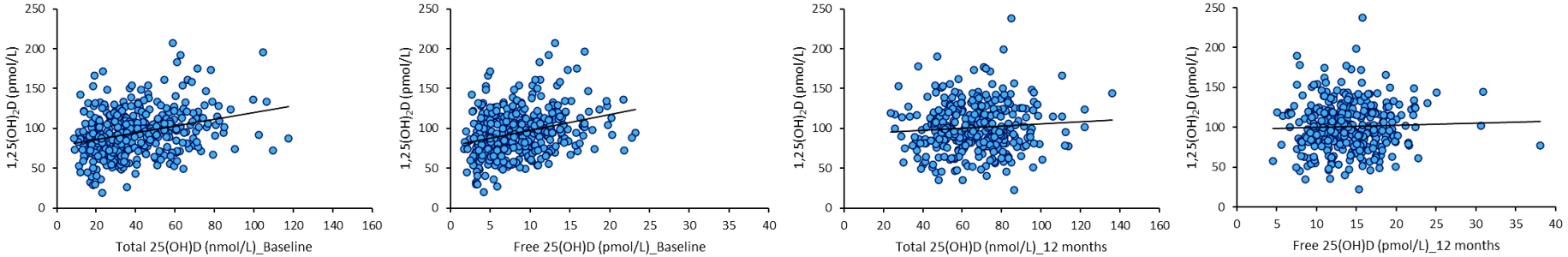
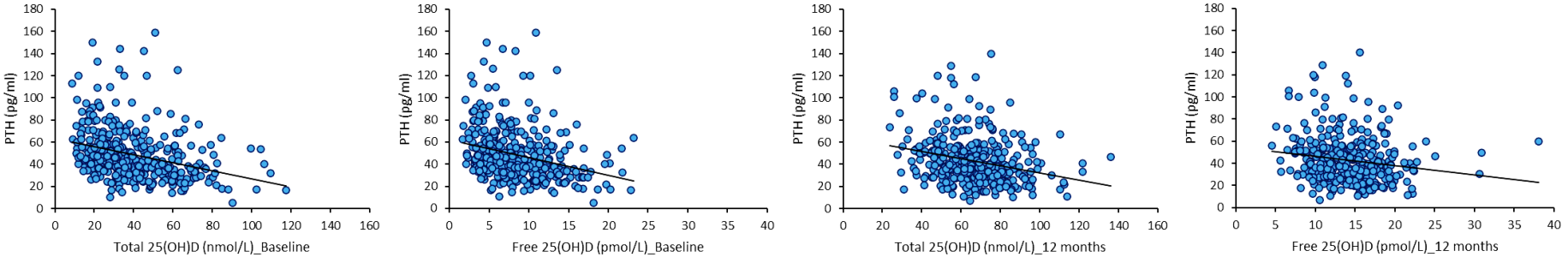


Figure 17. Correlations of 25(OH)D with 1,25 dihydroxy vitamin D and PTH at baseline and 12 months

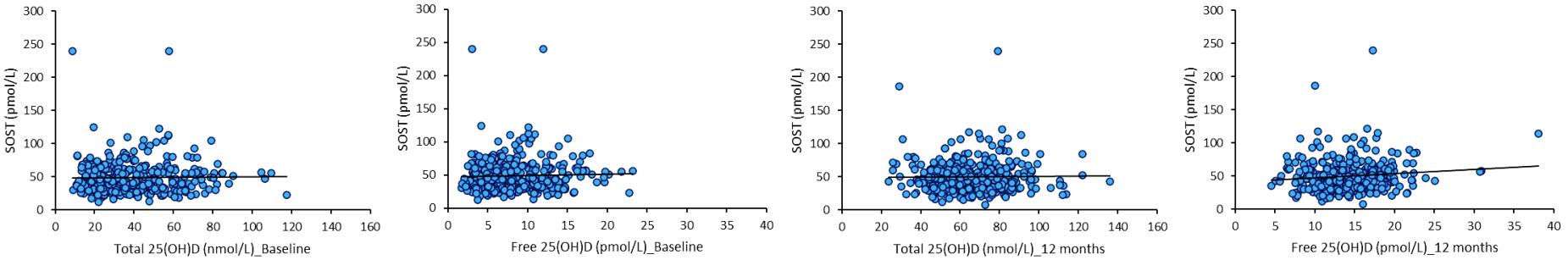


A. Correlations of total and free 25(OH)D and $1,25(OH)_2D$

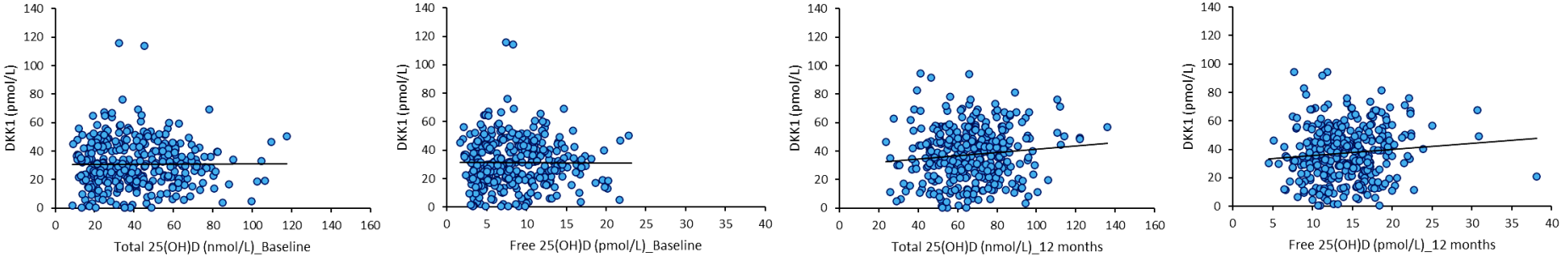


B. Correlations of total and free 25(OH)D and PTH

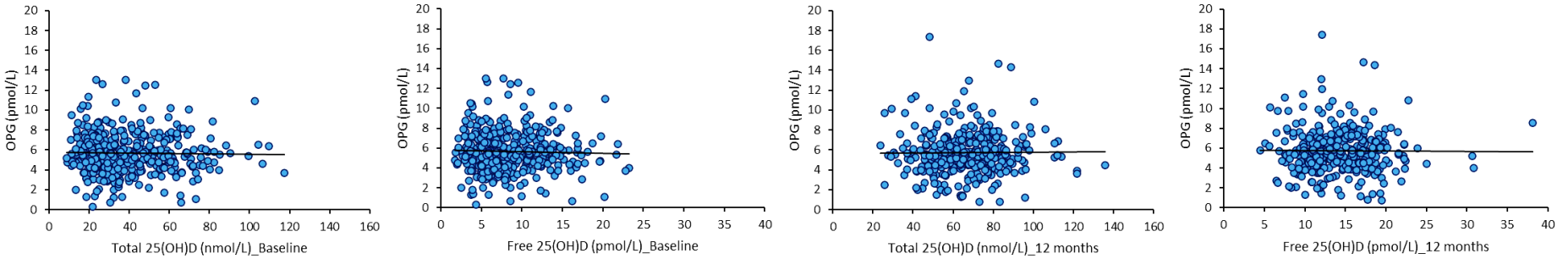
Figure 18. Correlations of 25(OH)D with Wnt-signalling pathway markers at baseline and 12 months



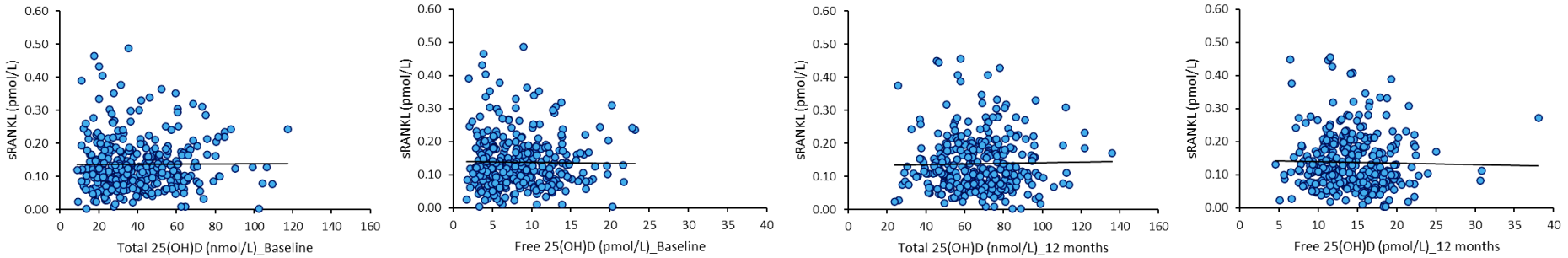
A. Correlations of total and free 25(OH)D and SOST



B. Correlations of total and free 25(OH)D and DKK1

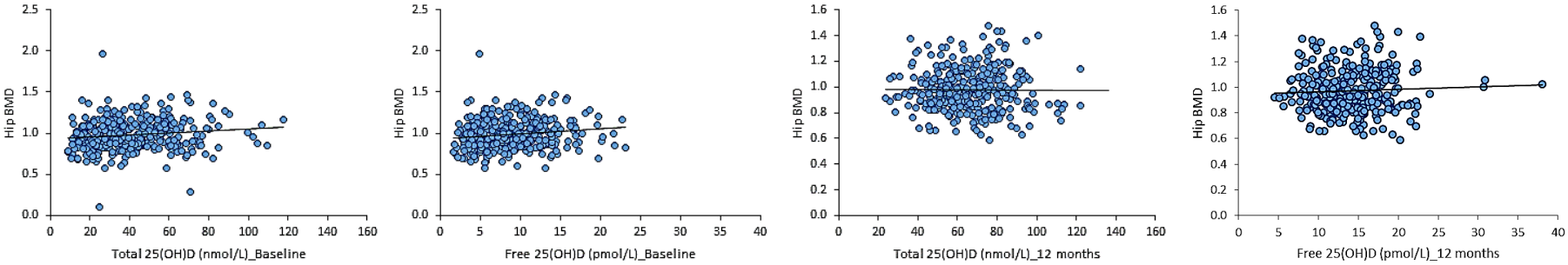


C. Correlations of total and free 25(OH)D and OPG



D. Correlations of total and free 25(OH)D and sRANKL

Figure 19. Correlations of total and free 25(OH)D with hip BMD at baseline and 12 months



Discussion

Supplementation dose-dependently increased total and free 25(OH)D concentrations and decreased plasma phosphate and iPTH in all groups (all $p<0.05$). The PINP:CTX ratio, cFGF23 and iFGF23 significantly increased with no between-group differences. Klotho was unchanged. $1,25(OH)_2D$ and PINP significantly increased in the 24 and 48,000IU groups. SOST, OPG, RANKL, BMD, BMC and CTX remained unchanged. In subgroup analyses restricted to participants deficient (25(OH)D <25 nmol/L) at baseline, findings were similar. There were no significant changes in BMD, BMC and CTX. Although an increase in PINP was not seen in this subgroup, PINP:CTX ratio increased.

Before supplementation, plasma concentrations of both total and free 25(OH)D were associated with cFGF23 and iPTH but not any of the markers of Wnt-signalling or bone metabolism, except for BAP. Both free and total 25(OH)D were positively associated with BMD and BMC of both sites at baseline. After supplementation, total and free 25(OH)D was positively associated with DBP ($p<0.001$) and negatively with adjusted calcium and eGFR ($p<0.01$). The negative association with iPTH and positive association with SOST ($p<0.05$) were only significant for free 25(OH)D after supplementation. There were no significant associations with other markers of Wnt-signalling and bone metabolism. The relationships with BMD and BMC were no longer found after supplementation.

The expected dose-dependent increase in total and free 25(OH)D and $24,25(OH)_2D$ with vitamin D supplementation was observed in this study. This was accompanied by a dose-dependent decrease in iPTH, as observed before in generally heathy people[352–356]. We also found an increase in $1,25(OH)_2D$ concentrations in the 24 and 48,000IU/month groups, despite that fact that few of the study participants had baseline values of 25(OH)D below the concentration usually considered as rate limiting for $1,25(OH)_2D$ production. This was also observed in other studies[357].

We found a significant increase in cFGF23 and iFGF23 with vitamin D supplementation, some individuals exceeding the normal ranges of cFGF23 and iFGF23 (laboratory-specific normal range: cFGF23 <100 RU/ml[358]; iFGF23 28-121 pg/mL as established in n=50 healthy individuals; personal communication Professor William Fraser). An increase in iFGF23 with vitamin D supplementation was also reported in a recent meta-analysis [359]. This may be partly

mediated by the increase in $1,25(OH)_2D$ observed in our study. There is a reciprocal regulation of FGF23 and $1,25(OH)_2D$[357]; $1,25(OH)_2D$ stimulates the expression of FGF23 and Klotho[357,360,361]. An increase Klotho concentrations was found after vitamin D supplementation[362] and in another study, Klotho has been shown to stimulate 25(OH)D activation in the kidney[31]. FGF23 however stimulates the expression of CYP24A1, thereby increasing catabolism of $1,25(OH)_2D$ and the conversion of 25(OH)D into $24,25(OH)_2D$[30,35], while at the same time inhibiting CYP27B1 expression and thus $1,25(OH)_2D$ production[357]. FGF23 has also been reported to inhibit iPTH synthesis production, a mechanism modulated by $1,25(OH)_2D$[357]. The effects of supplementation on FGF23 appear to be contradictory to the finding that at baseline, but not after supplementation, plasma 25(OH)D was negatively associated with cFGF23. This is likely linked to the effect of kidney function on both measurements.

The increase in FGF23 may be also secondary an increase in intestinal calcium and phosphate absorption[363] as mediated by the increase in $1,25(OH)_2D$. It may thus reflect a compensatory response to maintain phosphate homeostasis by increasing urinary phosphate excretion. Accordingly, we found a decrease in plasma phosphate after supplementation. FGF23, in conjunction with its co-factor Klotho is a phosphaturic hormone[30,357]. FGF23 downregulates the expression of phosphate cotransporters in renal proximal tubule membrane by phosphorylation of the NHERF-1 through a Klotho dependent mechanism[107]. Also, iPTH has a phosphaturic effect thought NHERF-1; therefore, the increase in FGF23 may be a response to the observed decrease in iPTH in this study.

Elevated plasma concentrations of cFGF23 and iFGF23 are found from early stages of renal impairment followed by an increase in plasma phosphate and PTH as CKD progresses[357,364]. This is associated with soft tissue calcification and increased risk of CVD[181]. It is also associated with low plasma concentrations of $1,25(OH)_2D$ due to FGF23 mediated inhibition of production and increased catabolism. It may therefore contribute to the risk of osteomalacia. Consistent with this hypothesis, negative associations have been reported between FGF23 and other regulators of the Wnt-signalling and markers of bone integrity and fracture risks, particularly in trabecular bone. These associations remained significant after correction for eGFR[212,365]. Several of these studies showed no relationships with BMD or BMC[212,366]. Whether an increase in FGF23 in response to vitamin D supplementation, without

a concomitant increase in plasma iPTH and phosphate and decrease in $1,25(OH)_2D$ is also associated with negative bone and CVD health outcomes needs further investigation.

Renal function is an important determinant of the response to vitamin D supplementation[356]. In post-supplementation ANCOVA models that included eGFR as a covariate, eGFR was significant for total and free 25(OH)D and cFGF23. The interaction of eGFR with post-supplementation 25(OH)D may reflect increased catabolism and impaired dose-response associated with a decline in renal function[13]. The interaction with cFGF23 may also be explained by the importance renal function in the catabolism and urinary excretion of FGF23 fragments[97]. In models for markers of Wnt-signalling, bone metabolism and BMD and BMC, eGFR was not significant. It is possible that the effect of renal function on bone markers may predominantly be observed at a lower eGFR than observed in this cohort. In reverse, supplementation did not increase or decrease eGFR, although cystatin C significantly increased in two highest dose groups.

Our study did not confirm an anabolic effect of vitamin D supplementation on components of the Wnt-signalling pathway[367,368]. The negative regulators SOST, OPG and sRANKL remained unchanged, while DKK1 significantly increased in 2 groups. sRANKL:OPG ratio remained unchanged. Data on the effect of vitamin D metabolites and vitamin D supplementation on the sRANKL:OPG ratio are conflicting [369]. Some studies reported that $1,25(OH)_2D$ can decrease the expression of RANKL and upregulate OPG/RANKL. This is partly mediated through the inhibitory effect of $1,25(OH)_2D$ on inflammatory factors[285,286]. However another study suggests that $1,25(OH)_2D$ increases the expression of RANKL and decreased OPG and enhanced osteoclast formation[287]. Although no pronounced effects on these regulators of bone metabolism were found, there was an increase in the formation marker PINP in the two highest dose groups while the PINP: CTX ratio increased in all groups. This may indicate that the balance of bone formation and resorption may have changed with supplementation. Other studies also found an anabolic effect of vitamin D supplementation on bone turnover markers[370,371].

Vitamin D supplementation may increase bone mineralization[22] and therefore BMD and BMC by increasing the bio-availability of calcium and phosphate[79]. This may be independent of potential effects of increased vitamin D status on alterations of bone cell differentiation and

function. This is most pronounced when substantial amounts of unmineralized bone matrix are present before supplementation commences, such as with osteomalacia, associated with vitamin D deficiency[8,344]. Therefore, similar to other vitamin D intervention studies, the effects of supplementation may have depended on vitamin D deficiency at baseline. In the VDOP study, 28% of participants had a baseline 25(OH)D below 25nmol/L, the threshold typically associated with impaired mineralisation[8]. This study and earlier analyses of the VDOP trial showed no interaction between the presence or absence of vitamin D deficiency (<25nmol/L) at baseline and change in BMD of the hip and femoral neck[343], markers of Wnt-signalling and bone metabolism, except for PINP. However, our study was not powered for this subgroup analyses.

In view of earlier reports of the importance of vitamin D status, rather than vitamin D supply, for musculo-skeletal health outcomes (BMD, fractures, falls, muscle function, prevention and treatment of secondary hyperparathyroidism) in both supplemented and unsupplemented individuals, we conducted regression analyses with total 25(OH)D and its free fraction before and after supplementation. We found that at baseline, BMD and BMC were positively associated with both and total and free 25(OH)D. This is consistent with other cross-sectional studies[344]. The lack of an effect of supplementation appears to be contra-dictionary to these findings. However, at baseline, 25(OH)D likely reflects a wider range of factors influencing both vitamin D status and BMD and BMC, such as time spent outdoors, physical activity or body composition[372,373]. The associations between 25(OH)D and BMD and BMC were no longer significant after supplementation. This might indicate that after supplementation, 25(OH)D concentrations ranges were achieved within which a further increase does not result in an increase in mineralization. In addition, after supplementation, vitamin D status will predominantly have been determined by oral intake and as such may override the effect of before mentioned life-style factors on 25(OH)D. Surprisingly, both at baseline and post-supplementation no significant associations were found with any of the measured markers of the Wnt-signalling pathway or bone metabolism, except for BAP.

It has been suggested that serum free 25(OH)D may be a better measure of tissue availability and utilisation and may be a better predictor of functionality of vitamin D than total plasma 25(OH)D[374,375]. The majority of vitamin D metabolites circulate tightly bound to DBP (85-90%), or with a lower affinity to albumin (10-15%) and only a small fraction circulates in its free

form[374,375]. According to the free hormone theory, only the free fraction can enter cells, unless the megalin/cubilin-mediated endocytotic uptake allows for internalisation of DBP-bound metabolites. This has so far only been demonstrated in the kidney, breast and muscle tissue[374]. Bone cells may therefore depend on the free 25(OH)D concentration in their micro-environment. Both osteocytes and osteoblast express CYP27B1[376,377]. It is therefore possible that vitamin D status regulates the auto and paracrine functions of osteocytes and osteoblast, including the production of FGF23. In healthy individuals, total and free 25(OH)D are highly correlated[374,375,378] and generally have the same relationships with health outcomes, despite the fact that DBP may have an independent association with indices of bone health[369,374,375]. This was also found in this study, in which we used calculated, not directly measured free 25(OH)D concentrations.

The plasma concentration of DBP is not considered to respond to vitamin D supply or plasma 25(OH)D. In this study, DBP significantly increased in the highest dose group. This finding therefore remains unexplained. In reverse, the concentration of DBP may be a determinant of the concentration of vitamin D metabolites since it protects against catabolism, thereby prolonging half-life[11.]. The significant association between DBP and plasma 25(OH)D in this study appears to confirm this.

This study has several limitations. The absence of placebo group did not allow to account for changes unrelated to the intervention (e.g. effect of ageing or secular trends). Our study was not powered for subgroup analyses by baseline vitamin D status. The length of supplementation may have been too short to detect significant changes in BMD and BMC as measured by DXA. We however did also not observe the anticipated 0.6% decrease in BMD (the average annual change in BMD in this age group[344,345]) the study was powered to detect[343]. We did not conduct peripheral quantitative computed tomography (pQCT) to obtain measures of bone integrity and strength and changes in these parameters may have gone undetected. Markers of bone metabolism and osteocyte signalling may however be expected to respond to interventions more rapidly and within the length of a bone remodelling cycle (~3-4 months)[379]. It is possible that markers measured after 12 months reflect a newly achieved steady state that is seemingly no different from baseline and that changes occurred within the first few months after commencement of the intervention, such as observed in pharmaceutical trials[378,379]. We did not directly measure free 25(OH)D but

instead calculated the free fraction. Although directly measured and calculated free 25(OH)D concentrations correlate well in healthy populations[369,374,375], it cannot be excluded that directly measured concentrations would have provided different findings.

In conclusion, the decrease in iPTH and increase in PINP:CTX ratio suggest a protective effect of supplementation on bone metabolism although no significant effect on BMD or pronounced changes in regulators of the Wnt-signalling pathway were found. Also, no changes in BMD were found in subgroup analyses restricted to participants that were vitamin D deficient at baseline. The increase in FGF23 warrants caution due to its negative associations with bone and cardiovascular health. Relationships between total and free 25(OH)D concentrations with biomarkers were similar and confirmed positive associations of higher vitamin D status and BMD. The change in associations after supplementation might suggest a threshold effect.

CHAPTER 4: Early renal impairment affects hormonal regulators of calcium and bone metabolism and Wnt-signalling and the response to vitamin D supplementation in healthy older adults

Chapter 4 is based on the following publication:

Christodoulou M, Aspray TJ, Piec I, Washbourne C, Tang JCY, Fraser WD, Schoenmakers I; VDOP Trial group, Terry J Aspray, Roger M Francis, Elaine McColl, Thomas Chadwick, Ann Prentice, Inez Schoenmakers. Early renal impairment affects hormonal regulators of calcium and bone metabolism and Wnt signalling and the response to vitamin D supplementation in healthy older adults. J Steroid Biochem Mol Biol. 2023 Feb 3;229:106267. doi: 10.1016/j.jsbmb.2023.106267.

Summary

Bone and renal metabolism are regulated by common factors and there is extensive cross-talk between these organs (the 'renal-bone-axis'). Ageing is associated with physiological changes including reduced bone mass, renal function and tissue sensitivity to regulatory hormones, impacting the renal-bone axis.

We aimed to investigate the influence of eGFR on plasma concentrations of vitamin D metabolites, Wnt-signalling and bone metabolism in a dose ranging RCT (12,000IU, 24,000IU, 48,000IU/month for 1 year; n=379, >70y) with a baseline eGFR >30 ml/min/1.73m^2. Participants were categorised on basis of eGFR (≥60 or <60 ml/min/1.73m^2) based on 4 commonly used algorithms for eGFR. Differences between eGFR categories were tested by ANCOVA and t-tests at baseline and after 12 months of supplementation.

Before supplementation commenced, a lower eGFR was associated with significantly higher concentrations of cFGF23, iFGF23, iPTH and SOST and lower Klotho, 1,25(OH)$_2$D and DKK1 concentrations. Differences between eGFR groups in 25(OH)D, 24,25(OH)$_2$D and iPTH were only detected with eGFR based on MDRD-4 and CKD-EPI algorithms. Differences in BMD and BMC were detected only with CG.

Pre- and post- supplementation comparisons showed differences in the response to supplementation between eGFR groups. Plasma 25(OH)D, 24,25(OH)$_2$D, DKK1 increased and

iPTH and CTX decreased in groups. Klotho only significantly increased in the group with lower eGFR. Plasma iFGF23 and $1,25(OH)_2D$, BAP, PINP increased only in the group with eGFR ≥60 ml/min/1.73m^2. Findings were largely consistent across all eGFR algorithms.

Post-supplementation, cFGF23, iFGF23, iPTH and SOST remained significantly higher and $1,25(OH)_2D$ lower in the lower eGFR group. Differences between eGFR groups in Klotho were no longer found. This was found for all eGFR algorithms, with the exception of iPTH and iFGF23, which were not significantly different with eGFR based on CG.

This study showed that even a moderate decline in eGFR has a negative impact on vitamin D metabolism, Wnt-signalling and bone turnover markers. Vitamin D supplementation has beneficial effects on markers of the renal-bone axis in older people with both normal and impaired renal function. The response depended on renal function. Supplementation improved Vitamin D status and Klotho in the group with moderate renal impairment to concentrations comparable to those found in the group with normal renal function. However, although CTX decreased, an increase in bone formation markers was not found in the group with eGFR <60 ml/min/1.73m^2.

Methods

Study design

This study is a secondary analyses utilising plasma samples collected as part of the vitamin D supplementation in older people (VDOP) randomized controlled trial[343] (ISRCTN35648481). In brief, this RCT included relatively healthy 379 adults aged ≥70 y (48% women; mean age: 75 y) from the northeast of England. The study excluded those with an eGFR <30 ml/min/1.73m^2 (CKD stage 4 and 5) at screening. Participants were randomly allocated into 3 supplementation groups of vitamin D_3 [12,000 international units (IU), 24,000 IU, or 48,000 IU] given once a month for 1 year. More details of the study design, methods, exclusion criteria and primary outcomes were previously described[343,345]. Additional methods used for this secondary analysis are provided below. The results of this explorative secondary analyses were not pre-specified in the original trial design and analyses plan.

Results for BMD and bone area (at hip and neck), plasma concentrations of 25(OH)D, iPTH, albumin, calcium and creatinine were earlier reported by Aspray *et al*.[343] but are also included here as part of secondary analyses and data interpretation.

The study was conducted in accordance with guidelines laid down in the Declaration of Helsinki. A favourable opinion was obtained from the Tyne & Wear South Research Ethics Committee (REC, 12/NE/0050) with Research and Development approval from the sponsor, Newcastle upon Tyne Hospitals NHS Foundation Trust. All participants provided written informed consent.

Measurements

Methods for measurements BMD, height and weight, collection of early morning fasting blood samples at baseline and after 12 months of supplementation[343], as well as details of blood processing, storage and biochemical analyses were provided elsewhere[343].

In brief, analyses were conducted at 2 sites (MRC Human Nutrition Research, Cambridge, UK (HNR-UK) and of University of East Anglia (UEA), UK. HNR-UK biochemical methods were: plasma 25(OH)D (LC-MS/MS), DBP (ImmunDiagnostik AG ELISA), iPTH (Immulite 2000, SIEMENS), PINP (UniQ, RIA), β-CTX (Immunodiagnostic Systems), BAP (DiaSorin, Liaison). All assays were performed in duplicate except for iPTH. Assay performance was monitored using kit and in-house controls and under strict standardisation according to ISO 9001:2000. Assay performance details were provided in Aspray T.J. *et al.* 2019[343]. Quality assurance of 25(OH)D and iPTH assays were performed as part of the Vitamin D External Quality Assessment Scheme (www.deqas.org), and the National External Quality Assessment Scheme (www.ukneqas.org.uk). Measurements of 25(OH)D were harmonised against NIST standards as part of the Vitamin D harmonisation program[343].

Measurements conducted at UEA included serum phosphate (Cobas, Roche Diagnostics), αKlotho (IBL international), plasma cFGF23 and iFGF23 (Immutopics), OPG (Biomedica), SOST, DKK1 and sRANKL (Biomedica), plasma 24,25-dihydroxy vitamin D ($24,25(OH)_2D$) (LC-MS/MS)[346], plasma $1,25(OH)_2D$ (DiaSorin, Liaison XL) and Cystatin C (Cobas, Roche Diagnostics). All assays were performed in duplicate except for $1,25(OH)_2D$, Cystatin C and phosphate on basis of consistent performance with intra and inter-assay CV < 4%. The inter and intra-assay CV of all assays were <10% except for $24,25(OH)_2D$ and $1,25(OH)_2D$, which

were <15%. Assay performance was monitored using kit and in-house controls and following Good Laboratory Practice.

All the biochemical analysis was conducted prior the start of this PhD study except markers of cystatin C and phosphate were conducted by M. Christodoulou during this PhD study.

Derived variables

Different equations to calculate eGFR are used for population health and clinical practice; they are based on plasma measurements of creatinine or cystatin C or a combination of both and include age, race and gender (Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) and Modification of Diet in Renal Disease 4 variable (MDRD-4) or age, gender and body weight (Cockcroft Gault (CG)). These equations were validated in different populations or patient groups and provide somewhat different results[232–234]. In clinical practice CKD-EPI and MDRD-4 and are most commonly used for the diagnosis and categorisation of CKD, but there are differences between guidelines. The UK National Institute of Health and Care Excellence (NICE) recommends CKD-EPI with creatinine for the majority of patients[231] but cystatin C based equations are recommended for the assessment of eGFR in those with CKD stage 3a (eGFR 45–59 mL/min/1.73m^2) and no proteinuria[317]. CKD-EPI is also recommended in guidelines specific for renal patients (the National Kidney foundation (NKF), Kidney Disease Improving Global Outcomes (KDIGO) and the Caring for Australians and New Zealanders with Renal Impairment (CARI) guidelines). For the US, the latest guidelines recommend the removal of race from the CKD-EPI algorithm[380]. In some countries, including the UK, the CG algorithm may be used for the older population, particularly in the context of decision making in the treatment of metabolic bone disease[214,318,381–383].

In the VDOP study population there were no non-white participants therefore race was removed.

The eGFR was calculated using different algorithms:

MDRD-4= 175 x $(S_{Cr})^{-1.154}$ x $(age)^{-0.203}$ x 0.742 [if female] x 1.212 [if Black]

With serum creatinine (S_{cr}) in mg/dL and age in years

CKD-EPI Creatinine = 142 x min(Scr/κ, 1)α x max(Scr/κ, 1)-1.200 x 0.9938Age x 1.012 [if female]

With serum creatinine (Scr) in mg/dL and age in years;

κ = 0.7 for females or 0.9 for males;

α = -0.241 for females or -0.302 for males;

min(Scr/κ, 1) is the minimum of Scr/κ or 1.0

max(Scr/κ, 1) is the maximum of Scr/κ or 1.0

CG= {((140–age) x weight)/(72xS_{Cr})}x 0.85 (if female)

With serum creatinine (S_{cr}) in mg/dL and age in years and weight in kg

CKD-EPI cystatin C= 133 x min(S_{cys}/0.8, 1)$^{-0.499}$ x max (S_{cys}/0.8, 1)$^{-1.328}$ x 0.996^{Age} x 0.932 [if female]

With serum cystatin C (Scys) in mg/L and age in years;

min = indicates the minimum of Scys/0.8 or 1

max = indicates the maximum of Scys/0.8 or 1

CKD-EPI Creatinine-Cystatin C= 135 × min(S_{Cr}/κ, 1)$^{\alpha}$ × max(S_{Cr}/κ, 1)$^{-0.601}$ × min(S_{cys}/0.8, 1)$^{-0.375}$ × max(S_{cys}/0.8, 1)$^{-0.711}$ × 0.995^{Age} × 0.969 [if female] × 1.08 [if black]

With serum creatinine (S_{cr}) in mg/dL, serum cystatin C (Scys) in mg/L and age in years;

κ = 0.7 for females or 0.9 for males;

α = -0.248 for females or -0.207 for males;

min(SCr/κ or 1) = indicates the minimum of SCr/κ or 1

max(SCr/κ or 1) = indicates the maximum of SCr/κ or 1

min(Scys/0.8, 1) = indicates the minimum of Scys/0.8, 1

max(Scys/0.8, 1) = indicates the maximum of Scys/0.8, 1

Free 25(OH)D was calculated as follows[347]:

Free 25(OH)D= total 25(OH)D/ [1+(6*10^{3}x Albumin) + (7*10^{8}xDBP)

Statistical analysis

The response to the intervention by supplementation group without considering eGFR was reported in detail before[342]. In brief, to test the response to supplementation, differences between pre- and post- supplementation values were analysed with paired sample t-tests for each supplementation group. For the purpose of providing descriptive data included in the analyses reported in this paper, post-supplementation data are presented pooled for all 3 groups and significant changes from baseline by group are denoted in superscripts.

Participant data were categorised based on their eGFR score (≥60 and <60 ml/min/1.73m^2) according to all 5 algorithms. Some individuals were in a different eGFR category at baseline and 12 months (6.5% (with MDRD-4) and 9.5% (with CG), respectively), but there was no difference in the numbers that changed to or from the higher category. There was no difference in eGFR between baseline and 12 months and no effect of dose of vitamin D supplementation on eGFR with any of the algorithms.

The following comparisons were conducted: (1) Pre- and post-supplementation comparisons within each eGFR category were tested with paired sample t-test. (2) Differences between eGFR categories were tested by ANCOVA pre- and post-supplementation, with supplementation group as a co-variate for 12 months data. ANCOVAs with eGFR, supplementation group and an interaction term for eGFR *supplementation group were also conducted but are not presented since these models partly replicate the comparisons addressed by (1).

Body Mass Index (BMI) was considered as a covariate (except for models with CG) but did not materially change the findings and therefore was removed from final models. Since age and gender are incorporated in all eGFR algorithms, these were not used as covariates.

Results for MDRD-4 and CG algorithms with creatinine are presented in **Table 13**. Results for CKD-EPI with creatinine were very similar to those for MDRD-4 and are only briefly summarised in the results section. Results for CKD-EPI cystatin C and CKD-EPI creatinine-cystatin C based algorithms are provided in the supplementary materials. Data were presented as mean (SD) or median (IQR) for normally distributed and skewed data, respectively.

Correction for repeated testing was not deemed appropriate as any findings will require confirmation in RCTs specifically designed and powered for respective outcomes.

All outcomes and/or the difference between pre- and post-supplementation were assessed for normality (defined as a posterior distribution skewness <2 or >-2) and visual inspection of histograms. Non-normally distributed variables were converted to LN and checked again for normality. The distribution of Klotho and cFGF23 at both time points (baseline and 12 months) were extremely skewed. Outliers were identified on basis of z-scores (based on interquartile range; IQR) and excluded if <-2.68 or >2.68. After excluding the extreme outliers, the LN values of both variables were normally distributed. Analyses were conducted with and without these outliers and there were no material differences between outcomes and interpretation of the data.

Linear regression analysis with eGFR as a continuous variable (all 5 algorithms) was conducted to evaluate the linearity of relationships and to eliminate bias due to the differences in group size. Regression analyses with variables included in the eGFR algorithms were not conducted nor were they entered as co-variates (i.e., serum creatinine, cystatin C, gender, age, weight, BMI (CG only)). For 12-month data, no effect of vitamin D supplementation dose was found on the slope of relationships and therefore the dose was removed as co-variate, resulting in univariate models. Linearity of associations was visually inspected prior to analysis. Two outliers for free 25(OH)D were excluded from the 12 months data. Results of regression analyses with CKD-EPI creatinine were very similar to MDRD-4. Therefore, these findings are not presented. Results are presented as β-coefficient and associated p-value. These regression analyses were conducted using the natural values of the data (not LN converted).

For the statistical analysis of the data IBM® SPSS® Statistics Version 28 software was used.

Results

Baseline characteristics without consideration of eGFR are presented in (**Table 12**). Baseline characteristics were well balanced between treatment groups and no significant differences were found. There were very few non-Caucasians (<1%) participants. Characteristics by eGFR categories are presented in **Table 13** and **Table 14**. Dependent on the algorithm used, 18%

(MDRD-4) to 28% (CG) of participants had an eGFR <60 mL/min/1.73 m^2, of which 3% and 5% had an eGFR <45 mL/min/1.73 m^2. The overall range was 32.5-138.3 (MDRD-4) and 33.5-145.2 (CG), respectively. There were differences between eGFR categories in age and BMI, dependent on the algorithm used.

Table 12. Participant's characteristics at baseline and after a 12-months of vitamin D supplementation[a,343]

	Baseline	12 months
Male/Female (N)[b]	195/182	176/165
Age (years)	74.1 [71.5-77.0]	76.0 [72.5-78.0]
Renal function markers		
Albumin (g/L)	45.7 (2.2)	44.5 (2.2)
Adjusted calcium (mmol/L)	2.2 (0.1)	2.2 (0.1)
Phosphate (mmol/L)	0.88 (0.18)	0.80 (0.19)$^{\nabla}$*
MDRD-4 eGFR (mL/min per 1.73 m^2)	72 (15)	73 (15)
CG eGFR (mL/min per 1.73 m^2)	69 (17)	70 (18)
Klotho (pg/mL)	493.7 [392.6-627.7]	494.5 [398.5-613.1]
cFGF23 (RU/mL)	66.7 [54.9-84.2]	73.6 [60.7-97.0] $^{\Delta}$*
iFGF23 (pg/mL)	55.1 [44.5-72.7]	63.3 [50.48-80.0] $^{\Delta}$*
Vitamin D metabolism markers		
Total 25(OH)D (nmol/L)	40.0 (20.1)	66.5 (18.0)$^{\Delta}$*
1,25$(OH)_2$D (pmol/L)	94.5 (29.0)	101.2 (29.9)$^{\Delta 24,48}$
iPTH (pg/ml)	43.4 [33.2-57.4]	38.5 [28.0-52.2]$^{\nabla}$*
Wnt-signalling pathway markers		
SOST (pmol/L)	44.3 [32.4-60.0]	46.3 [32.9-60.6]
DKK1 (pmol/L)	31.2 (16.5)	37.6 (18.5) $^{\Delta 12,48}$
OPG (pmol/L)	5.7 (2.1)	5.5 [4.5-6.9]
sRANKL (pmol/L)	0.12 [0.08-0.16]	0.12 [0.08-0.18]
Bone mineral density and metabolism		
Hip BMD (g/m^2)	0.98 (0.17)	0.98 (0.17)
Hip BMC (g)	35.44 (8.30)	35.08 (8.06)
BAP (µg/L)	9.5 [7.9-12.3]	9.9 [7.9-13.5]$^{\Delta 12}$
CTX (ng/mL)	0.40 [0.30-0.50]	0.36 (0.15)
PINP (µg/L)	36.2 [28.8-46.2]	39.1 [30.7-49.0]$^{\Delta 24,48}$

[a]For normally distributed data, results are expressed as mean (SD); for skewed data, results are expressed as median [interquartile range (IQR)]. Post supplementation data (12 months) are presented pooled for the 3 supplementation groups. [b]Number of participants for which data were available for individual variables was >85 % of the total N.

Paired T-tests were used to analyse pre- and post- supplementation values for each supplementation group; *Significant p<0.05; $^{\Delta}$increase or $^{\nabla}$ decrease from baseline in all 12,000IU/m; 24,000 IU/m and 48,000IU/m vitamin D treated groups; $^{\Delta 12,24,48}$ increase or $^{\nabla 12,24,48}$ decrease in respective supplementation group.

Supplementation effect

The effect of supplementation on markers of bone, calcium and phosphate metabolism were reported previously[342]. Post supplementation data are presented pooled for all supplementation groups (**Table 12**). In summary, when eGFR was not considered there was a dose dependent increase of 25(OH), 24,25$(OH)_2$D and decrease of iPTH (all p<0.05). Plasma 1,25$(OH)_2$D significantly increased in the 2 highest treatment groups. Both cFGF23 and iFGF23 significantly increased (all p<0.05). Vitamin D supplementation had no effect on eGFR, Klotho, SOST, OPG, sRANKL, hip and FN BMD, BMC and CTX. The remainder of markers did not follow a dose-dependent pattern (**Table 12**).

Response to supplementation by eGFR category

There were differences in the response to vitamin D supplementation by eGFR category. Findings were largely the same for all eGFR algorithms (**Table 13; Table 14**). Plasma cFGF23, total and free 25(OH)D, 24,25$(OH)_2$D and DKK1 increased and plasma phosphate, iPTH and CTX decreased in both eGFR groups (all p<0.05). The decrease in CTX as non-significant when eGFR ≥60 ml/min/1.73m^2 as calculated according with GC (**Figure 20**). Klotho significantly increased only in the group with the lower eGFR (**Figure 20**). Plasma iFGF23 and 1,25$(OH)_2$D, BAP and PINP only increased in the group with eGFR ≥60 ml/min/1.73m^2 (**Figure 21**) (all p<0.05). OPG significantly decreased in both eGFR groups but this was only found when eGFR calculated with CG. SOST and sRANKL remained unchanged in both eGFR groups. There were no changes in hip and FN BMD, BMC in either eGFR group.

Table 13. Differences between eGFR groups at baseline and after 12 months of supplementation with eGFR calculated according to MDRD-4 and Cockcroft-Gault algorithms

Characteristics	eGFR category[a]	MDRD-4		CKD-EPI creatinine		Cockcroft-Gault	
		Baseline	12 months	Baseline	12 months	Baseline	12 months
		N (%) or Mean (SD)	N (%) or Mean (SD)	N (%) or Mean (SD)	N (%) or Mean (SD)	N (%) or Mean (SD)	N (%) or Mean (SD)
Male/Female	≥60	160/149	141/133	156/151	135/136	144/127	124/118
	<60	35/33	35/32	38/31	37/29	51/55	52/47
N	≥60	309 (82%)	274 (80%)	307 (82%)	271 (80%)	271 (72%)	242 (71%)
	<60	68 (18%)	67 (20%)	69 (18%)	66 (20%)	106 (28%)	99 (29%)
eGFR (mL/min per 1.73 m^2)	≥60	76.9 (12.5)*	77.4 (12.2)*	76.4 (9.3)*	76.5 (8.8)*	78.1 (14.6) *	78.1 (15.2)*
	<60	51.7 (6.6)	52.2 (7.0)	51.5 (6.8)†	51.6 (7.4)	51.8 (6.6)	50.7 (7.4)
Age (years)	≥60	74.5 (4.0)*	-	74.4 (3.9)*	-	74.2 (3.6) *	-
	<60	77.1 (4.1)	-	77.4 (4.3)	-	76.9 (4.7)	-
BMI (kg/m^2)	≥60	26.7 (3.6)*	-	26.7 (3.7)*	-	27.7 (3.8) *	-
	<60	28.5 (4.7)	-	28.5 (4.7)	-	25.2 (3.6)	-
Markers of renal function and calcium and phosphate metabolism							
Biomarkers	**eGFR category[a]**	**Mean (SD) or median [IQR]**	**Mean (SD) or median [IQR]**	**Mean (SD) or median [IQR]**	**Mean (SD) or median [IQR]**	**Mean (SD) or median [IQR]**	**Mean (SD) or median [IQR]**
Albumin (g/L)	≥60	45.8 (2.2)†	44.6 (2.3)	45.8 (2.2)†	44.6 (2.3)†	45.8 (2.2)	44.6 (2.1)
	<60	45.8 (2.2)†	44.1 (2.1)	45.6 (2.2)†	44.2 (2.0)†	45.6 (2.2)	44.4 (2.5)
Adjusted calcium (mmol/L)	≥60	2.3 (0.1)*	2.3 (0.1)	2.2 (0.1)	2.2 (0.1)	2.2 (0.1)	2.2 (0.1)
	<60	2.2 (0.1)†	2.3 (0.1)	2.2 (0.1)†	2.3 (0.1)†	2.2 (0.1)	2.2 (0.1)
Phosphate (mmol/L)	≥60	0.89 (0.17)*†	0.80 (0.19)	0.89 (0.17)*†	0.80 (0.19)	0.87 (0.17)†	0.80 (0.19)
	<60	0.83 (0.20)†	0.82 (0.18)	0.83 (0.20)	0.82 (0.17)	0.89 (0.18)†	0.82 (0.18)
Klotho (pg/mL)	≥60	513.6 [398.7-642.4]*	492.5 [399.0-606.6]	514.3 [402.3-643.0]*	490.0 [393.8-609.8]	509.2 [399.5-644.6]*	497.1 [400.7-618.3]
	<60	440.0 [367.2-553.8]†	502.3 (1.4)	436.2 [364.9-552.3]†	497.6 (1.4)	467.0 [376.2-590.9]	493.1 [398.2-592.2]

cFGF23 (RU/mL)	**≥60**	64.5 [53.1-76.6]*†	71.1 [58.9-87.6]*	64.4 [53.1-76.8]*†	73.54 [60.66-97.09]*	64.9 [52.9-80.3]*†	71.4 [59.5-88.3]*
	<60	85.0 [68.1-121.6]†	99.2 [78.5-136.0]	84.0 [68.6-119.3]†	71.97 [60.98-96.58]	72.1 [60.0-95.6]†	84.8 [65.4-116.8]
iFGF23 (pg/mL)	**≥60**	53.2 [42.8-65.2]*†	62.6 [50.0-79.2]*	53.0 [42.7-65.1]*†	62.98 [50.41-79.83]*	53.0 [42.9-67.4]*†	63.3 [50.0-81.2]
	<60	69.4 [51.6-84.9]	66.5 [51.2-87.2]	70.4 [51.9-86.0]	65.38 [50.92-84.20]	59.1 [48.0-79.0]†	59.8 [50.2-78.8]
Markers of Vitamin D metabolism							
Total 25(OH)D (nmol/L)	**≥60**	41.3 (20.6)*†	66.6 (18.1)	41.2 (20.6)*†	66.7 (18.2)	39.6 (19.1)†	64.9 (17.2)*
	<60	34.3 (16.9)†	66.4 (17.6)	34.4 (16.8)†	66.1 (17.4)	41.1 (22.6)†	70.4 (19.2)
Free 25(OH)D (pmol/L)	**≥60**	8.7 (4.4)*†	14.0 (4.0)	8.7 (4.4)*†	14.1 (4.0)	8.4 (4.1)†	13.7 (4.0)*
	<60	7.2 (3.7)†	14.5 (5.2)	7.3 (3.1)†	14.4 (5.18)	8.6 (4.7)†	15.0 (4.8)
$24,25(OH)_2D$ (nmol/L)	**≥60**	4.4 [2.1-6.1]*†	11.3 [9.7-13.6]	4.4 [2.1-6.1]*†	7.8 (3.3)	3.2 [2.1-5.6]†	7.5 (3.1)
	<60	2.9 (1.7)†	12.1 [10.1-14.7]	3.0 (1.7)†	7.0 (2.7)	2.9 [1.9-5.3] †	7.8 (3.0)
$1,25(OH)_2D$ (pmol/L)	**≥60**	98.0 (28.1)*†	105.6 (29.4)*	98.0 (28.2)*†	105.6 (29.4)*	97.0 (28.7)*†	104.0 (30.3)*
	<60	79.7 (28.1)	83.5 (25.8)	80.4 (28.0)	83.8 (26.2)	88.9 (28.7)†	94.3 (28.1)
DBP (mg/L)	**≥60**	367.3 (63.6)	368.3 (61.9)	368.0 (63.8)	369.4 (62.4)	366.2 (63.5)	368.41 (64.7)
	<60	369.5 (64.1)	373.7 (61.0)	366.7 (63.8)	369.9 (59.1)	371.5 (64.0)	371.7 (54.0)
iPTH (pg/ml)	**≥60**	42.5 [32.3-55.3]*†	37.0 [27.7-50.4]*	41.5 [32.3-55.3]*†	38.0 [27.6-51.9]*	43.7 [33.1-57.4]†	37.7 [27.8-51.6]
	<60	52.1 [37.9-76.0]†	48.2 [32.1-64.9]	51.7 [38.1-75.9]†	39.5[29.5-55.1]	42.0 [33.1-57.0]†	41.2 [29.5-55.4]
Wnt-signalling pathway markers							
SOST (pmol/L)	**≥60**	43.0 [31.1-56.3]*	43.3 [32.0-57.8]*	42.7 [31.2-55.9]*	46.7 [34.1-61.8]*	43.2 [31.0-56.6]*	43.4 [31.1-57.8]*
	<60	54.6 (1.5)	56.3 [41.3-72.6]	54.6 (1.5)	55.5 (1.5)	49.1 (1.5)	52.0 [36.5-68.3]
DKK1 (pmol/L)	**≥60**	32.4 (16.6)*†	38.0 (18.3)*	32.2 (16.7)*†	37.6 (18.4)	32.4 (16.2)*†	37.0 (18.8)
	<60	26.3 (15.0)†	32.3 (17.3)	27.0 (15.1)†	33.6 (17.0)	28.5 (15.1)†	36.4 (16.6)
OPG (pmol/L)	**≥60**	5.6 (2.0)	5.7 [4.5-6.9]	5.6 (2.0)†	5.6 [4.5-7.]	5.5 (2.0)*†	5.2 (1.5)

	<60	5.9 (2.3)	5.4 (2.3)	5.9 (2.3)†	5.63 (1.50)	6.1 (2.1)†	5.3 [4.2-7.2]
sRANKL (pmol/L)	**≥60**	0.12 [0.08-0.18]	0.12 [0.08-0.19]	0.12 [0.08-0.18]	0.12 [0.08-0.19]	0.13 [0.08-0.18]	0.14 (0.08)*
	<60	0.13 [0.09-0.18]	0.12 [0.07-0.18]	0.12 (2.00)	0.12 [0.07-0.18]	0.11 [0.08-0.17]	0.12 [0.08-0.18]
Bone mineral density and metabolism							
Hip BMD (g/m^2)	**≥60**	0.98 (0.18)	0.99 (0.17)*	0.98 (0.18)	0.99 (0.17)*	0.99 (0.18)*	0.98 (0.17)
	<60	0.97 (0.17)	0.92 (0.13)	0.97 (0.17)	0.93 (0.14)	0.94 (0.17)	0.95 (0.16)
Hip BMC (g)	**≥60**	35.44 (8.24)	35.35 (8.35)	35.35 (8.27)	35.15 (8.30)	36.28 (8.26)*	35.19 (8.14)
	<60	35.48 (8.67)	33.01 (6.66)	35.74 (8.51)	33.69 (7.10)	33.30 (8.09)	34.18 (7.95)
FN BMD (g/m^2)	**≥60**	0.90 (0.15)	0.91 (0.15)	0.90 (0.15)	0.91 (0.15)	0.91 (0.15)*	0.91 (0.15)
	<60	0.89 (0.15)	0.87 (0.11)	0.89 (0.15)	0.87 (0.12)	0.88 (0.15)	0.88 (0.14)
FN BMC (g)	**≥60**	4.90 (1.09)	4.80 (1.07)	4.89 (1.08)†	4.77 (1.06)	5.00 (1.11)*	4.78 (1.06)
	<60	4.87 (1.11)	4.58 (1.02)	4.90 (1.09)	4.67 (1.06)	4.64 (1.01)	4.69 (1.05)
BAP (µg/L)	**≥60**	9.5 [7.8-12.3]†	9.9 [7.9-13.6]	9.5 [7.8-12.3]†	9.8 [7.9-13.6]	9.4 [7.9-12.3]†	10.1 [7.9-13.8]
	<60	10.6 (3.5)	10.2 [7.7-14.0]	10.6 (3.5)	10.3 [7.8-14.1]	10.5 (3.7)†	10.0 [7.7-13.3]
CTX (ng/ml)	**≥60**	0.40 [0.3-0.5]†	0.37 (0.16)	0.40 [0.3-0.5]†	0.36 (0.16)	0.40 [0.30-0.50]	0.37 (0.16)
	<60	0.40 [0.3-0.6]†	0.36 (0.15)	0.40 [0.30-0.55]†	0.36 (0.15)	0.40 [0.30-0.60]†	0.35 (0.15)
PINP (µg/L)	**≥60**	35.7 [28.8-46.0]†	38.7 [30.7-49.2]	35.5 [28.8-46.0]†	38.4 [30.7-49.4]	35.1 [28.1-44.6]*†	39.4 [31.2-48.8]
	<60	38.7 [28.5-51.7]	39.6 [31.1-51.1]	39.6 [28.7-50.9]	42.1 [31.6-51.4]	40.6 [30.4-50.8]	38.1 [29.7-51.9]

For normally distributed data, results are expressed as mean (SD); for skewed data, results are expressed as median [interquartile range (IQR)].

[a]Range of number of participants for which data were available by eGFR group: MDRD-4 at baseline eGFR ≥60 n= 309-277; <60 n=68-62 and at 12 months eGFR ≥60 n= 269-235; <60 n= 67-57; GC at baseline eGFR≥60 n= 271-242; <60 n=106-99 and at 12 months eGFR ≥60 n= 238-206; <60 n=99-86.

* Significant difference p<0.05 between eGFR groups tested with independent t-tests (baseline) or ANCOVA analysis (post-supplementation; 12 months)

†Significant difference p<0.05 between pre- and post-supplementation values.

Table 14. Differences between eGFR categories at baseline and after 12 months of supplementation with eGFR calculated according to CKD-EPI with cystatin C and creatinine-cystatin C algorithms

Biomarkers	eGFR category[a]	CKD-EPI cystatin C		CKD-EPI creatinine-cystatin C	
		Baseline	12 months	Baseline	12 months
		N (%), Mean (SD) or median [IQR]	N (%), Mean (SD) or median [IQR]	N (%), Mean (SD) or median [IQR]	N (%), Mean (SD) or median [IQR]
Male/Female	≥60	141/157	137/137	173/162	147/142
	<60	21/22	36/27	22/19	27/22
N	≥60	328 (88)	274 (81)	335 (89)	289 (86)
	<60	43 (12)	63 (19)	41 (11)	49 (14)
Age (years)	≥60	73.7 [71.4-76.7]*	-	73.7 [71.4-76.5]*	-
	<60	77.6 [73.1-81.1]	-	77.7 (4.7)	-
eGFR (mL/min per 1.73 m^2)	≥60	91 (17)‡	89 (20)‡	90 (18)‡	88 (21)‡
	<60	50 (7)	50 (8)	53 (11)	49 (9)
BMI (kg/m^2)	≥60	26.8 (3.8)‡	-	26.8 (3.8)‡	-
	<60	28.5 (4.8)	-	28.8 (4.6)	-
Renal function markers					
Biomarker	**eGFR category[a]**	**Mean (SD) or median [IQR]**	**Mean (SD) or median [IQR]**	**Mean (SD) or median [IQR]**	**Mean (SD) or median [IQR]**
Albumin (g/L)	≥60	45.8 (2.2)†	44.5 (2.3)	45.8 (2.2)†	44.6 (2.3)
	<60	45.5 (2.2)†	44.3 (2.1)	45.5 (2.2)†	44.1 (2.3)
Adjusted calcium (mmol/L)	≥60	2.2 (0.1)*	2.2 (0.1)	2.2 (0.1)	2.2 (0.1)
	<60	2.3 (0.1)	2.2 (0.1)	2.3 (0.1)	2.3 (0.1)
Phosphate (mmol/L)	≥60	0.87 (0.17)*†	0.79 (0.19)*	0.87 (0.17)†	0.79 (0.19)*
	<60	0.94 (0.17)†	0.89 (0.15)	0.90 (0.20)	0.86 (0.14)
Klotho (pg/mL)	≥60	488.3 [392.4-623.5]	504.2 [403.7-619.9]	507.4 [397.8-631.5]*	494.9 [403.6-609.8]
	<60	498.4 [375.7-625.5]	496.0 [390.0-599.8]	435.9 [364.1-557.0]†	498.8 [383.3-569.9]
cFGF23 (RU/mL)	≥60	65.3 [54.0-81.0]*†	71.1 [59.1-86.0]*	65.2 [54.2-80.2]*†	72.7 [59.9-97.8]*
	<60	94.1 [68.6-135.7]	105.9 [81.3-143.5]	98.6 [68.3-143.0]	83.8 [67.7-99.0]

iFGF23 (pg/mL)	**≥60**	53.8 [43.5-66.2]*†	63.7 [49.8-80.4]*	53.8 [43.0-69.8]*†	63.1 [49.7-80.0]*
	<60	73.1 [52.5-84.2]	62.2 [52.3-80.4]	68.4 [52.2-83.5]	63.3 [53.3-84.3]
Vitamin D metabolism markers					
Total 25(OH)D (nmol/L)	**≥60**	40.2 (20.2)†	66.3 (17.9)	40.6 (20.3)†	66.1 (17.5)
	<60	37.8 (19.5)†	67.9 (18.9)	35.0 (17.8)†	68.5 (20.7)
Free 25(OH)D (pmol/L)	**≥60**	8.5 (4.3)†	14.0 (4.1)	8.6 (4.3)†	13.9 (3.9)
	<60	7.9 (4.2)†	14.5 [11.5-17.0]	5.9 [4.7-9.7]†	14.4 [11.5-17.9]
$24,25(OH)_2D$ (nmol/L)	**≥60**	3.2 [2.0-5.8]*†	7.7 (3.2)	3.3 [2.1-5.8]*†	7.7 (3.2)
	<60	3.2 (2.0)†	7.2 (2.9)	3.0 (1.8)†	6.9 (2.6)
$1,25(OH)_2D$ (pmol/L)	**≥60**	96.2 (28.8)*†	103.6 (28.4)*	96.5 (28.6)*†	104.0 (29.8)*
	<60	83.2 (27.2)	85.0 [70.0-103.5]	79.3 (26.8)	84.9 (25.7)
DBP (mg/L)	**≥60**	367.1 (63.7)	369.1 (62.5)	367.8 (65.0)	368.3 (61.6)
	<60	368.9 (55.7)	372.6 (54.6)	366.8 (51.5)	378.4 (58.9)
iPTH (pg/ml)	**≥60**	42.9 [32.8-56.7]*†	37.7 [27.5-51.6]*	42.9 [32.9-56.6]*†	37.7 [27.6-51.6]*
	<60	53.4 [37.9-71.0]	43.1 [32.4-57.8]	52.4 [35.5-74.8]	46.7 [32.3-65.0]
Wnt-signalling pathway markers					
SOST (pmol/L)	**≥60**	43.4 [31.5-58.4]*	43.5 [32.1-58.7]*	43.3 [31.5-57.6]*	44.1 [32.1-58.7]*
	<60	52.5 [38.3-65.9]	56.2 (20.0)	57.7 (20.5)	58.5 (21.0)
DKK1 (pmol/L)	**≥60**	31.8 (16.6)†	37.3 (18.1)	32.2 (16.5)*†	37.1 (18.1)
	<60	27.0 (15.8)†	35.8 (18.7)	23.9 (15.0)†	36.3 (19.0)
OPG (pmol/L)	**≥60**	5.6 (2.1)	5.6 [4.5-6.9] *	5.6 (2.1)	5.6 [4.5-9.6]*
	<60	6.1 (2.3)	5.4 (2.2)	5.8 [4.8-7.0]	5.0 (2.3)
sRANKL (pmol/L)	**≥60**	0.12 [0.08-0.18]	0.12 [0.08-0.19]	0.12 [0.08-0.18]	0.12 [0.08-0.19]
	<60	0.13 [0.11-0.18]	0.12 [0.07-0.19]	0.12 [0.09-0.18]	0.11 [0.06-0.17]
Bone mineral density and metabolism					
Hip BMD (g/m^2)	**≥60**	0.98 (0.17)	0.98 (0.17)	0.98 (0.17)	0.98 (0.17)
	<60	0.94 (0.18)	0.94 (0.14)	0.94 (0.18)	0.94 (0.14)
Hip BMC (g)	**≥60**	35.64 (8.33)	35.08 (8.18)	35.51 (8.27)	35.13 (8.30)
	<60	33.52 (8.04)	33.30 (7.11)	34.72 (8.67)	33.18 (6.36)

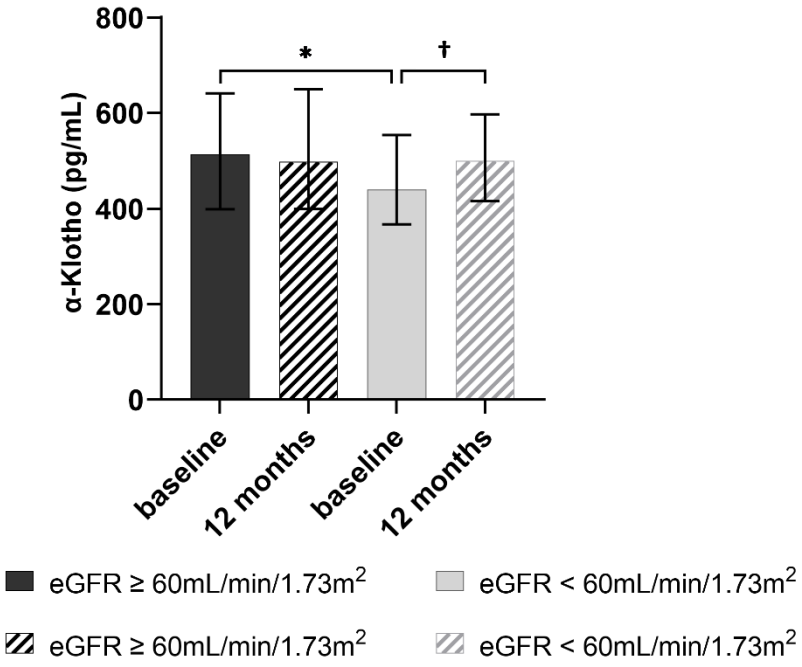
FN BMD (g/m^2)	**≥60**	0.90 (0.15)	0.90 (0.14)	0.90 (0.15)	0.91 (0.15)
	<60	0.87 (0.16)	0.88 (0.13)	0.88 (0.16)	0.88 (0.13)
FN BMC (g)	**≥60**	4.92 (1.10)	4.77 (1.05)	4.91 (1.10)	4.77 (1.06)
	<60	4.67 (1.01)	4.64 (1.07)	4.78 (1.07)	4.65 (1.05)
BAP (µg/L)	**≥60**	9.5 [7.9-12.2]†	9.9 [7.9-13.3]	9.5 [7.8-12.3]†	9.9 [7.9-13.5]
	<60	10.8 (3.6)	11.3 [8.1-15.2]	10.5 (3.2)	11.3 [7.8-15.4]
CTX (ng/ml)	**≥60**	0.40 [0.30-0.50]†	0.36 (0.16)	0.40 [0.30-0.50]†	0.36 (0.16)
	<60	0.40 [0.30-0.60]†	0.38 (0.15)	0.40 [0.30-0.60]†	0.38 (0.15)
PINP (µg/L)	**≥60**	35.7 [28.4-45.8]*†	38.1 [30.6-48.7]	35.7 [28.4-46.0]*†	38.2 [30.6-48.8]
	<60	43.8 (13.7)	45.0 [34.9-52.7]	41.4 [33.6-52.8]	44.0 [33.6-51.9]

For normally distributed data, results are expressed as mean (SD); for skewed results are expressed as median [interquartile range].

* Significant difference $p<0.05$ between eGFR categories tested with independent t-tests (baseline) or ANCOVA analysis (post-supplementation; 12 months)

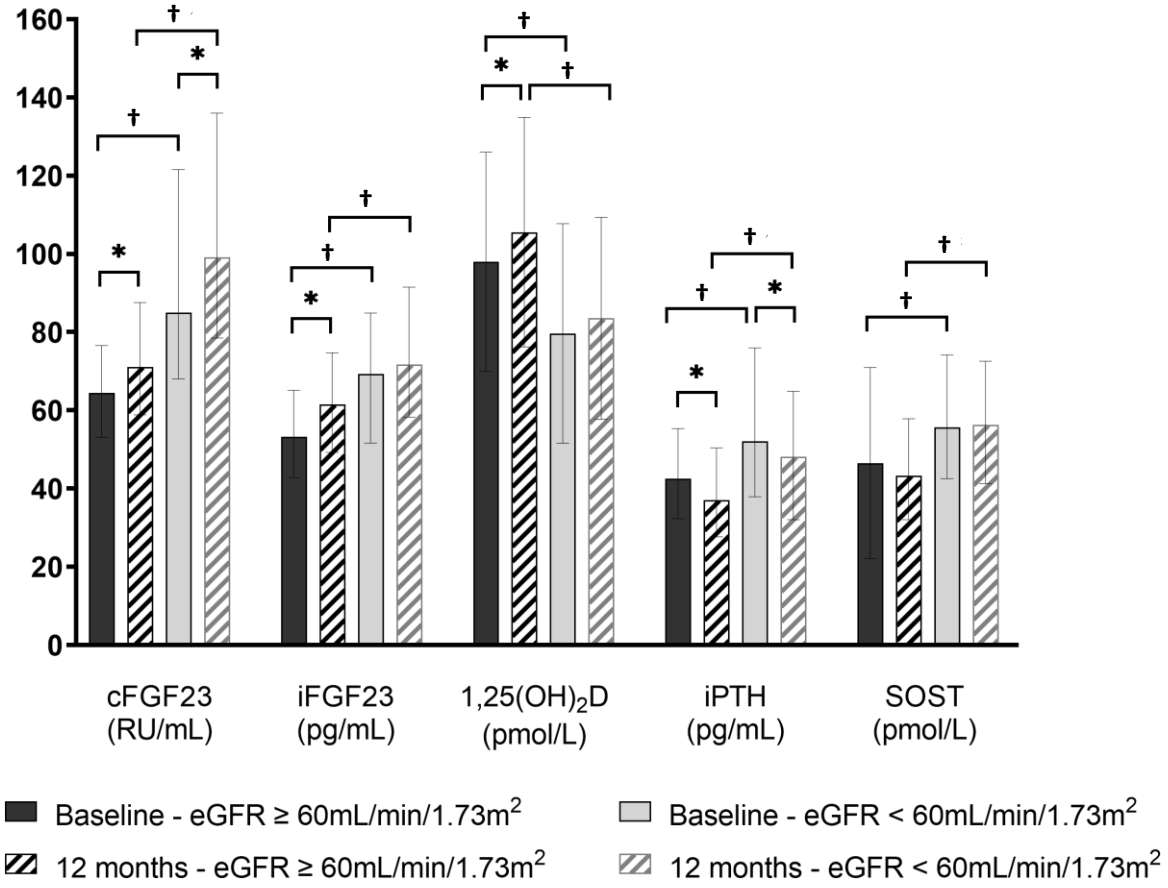
†Significant difference $p<0.05$ between pre- and post-supplementation values.

Figure 20. Serum concentrations of α-Klotho pre- and post- vitamin D supplementation in groups categorized on basis of eGFR 60< or ≥ 60 mL/min/1.73m^2 calculated according MDRD-4 algorithm



Data are presented as median (IQR).
The effect of supplementation was analysed by t-test (significance †p<0.05). Difference between eGFR categories was analysed by ANCOVA (significance *p<0.05).

Figure 21. Plasma or serum concentrations of cFGF23, iFGF23, 1,25(OH)^{2}D, PTH and SOST pre- and post- vitamin D supplementation in groups categorized on basis of eGFR as <60 or ≥ 60 mL/min/1.73m^2 according MDRD-4 algorithm



Data are presented as mean (SD) or median (IQR) values for normal and skewed data respectively.
The effect of supplementation was analysed by t-test (significance †p<0.05). Difference between eGFR categories was analysed by ANCOVA (significance *p<0.05).

Differences in biomarkers between eGFR categories

Findings with MDRD-4 and CKD-EPI equations were comparable, with some exceptions and are presented in **Table 13** and **Table 14**, respectively. Findings with MDRD-4 and CG are described in more detail below.

Markers of renal function and calcium and phosphate metabolism

At baseline, with eGFR categorised on basis of MDRD-4, adjusted calcium, serum phosphate and Klotho were significantly lower and cFGF23 and iFGF23 were significantly higher when eGFR <60 mL/min/1.73 m^2 (all p<0.05) (**Table 13; Figure 20; Figure 21**). When categorised on basis of CG, similar results were found for Klotho, cFGF23 and iFGF23 (all p<0.05) (**Table 13**). After 12 months of supplementation, when MDRD-4 was used, only cFGF23 and iFGF23 remained significantly higher in patients with eGFR <60 mL/min/1.73 m^2 (p<0.05) (**Table 13; Figure 21**). Based on CG, this was only found for cFGF23 (**Table 13**). No between groups differences in Klotho were found, regardless of eGFR equation used.

Markers of vitamin D metabolism

Before supplementation, with eGFR calculated and categorised on basis of MDRD-4, all vitamin D metabolites were significantly lower and iPTH significantly higher with eGFR <60 mL/min/1.73m^2 (all p<0.05) (**Table 13; Figure 21**). When categorised on basis of CG only $1,25(OH)_2D$ differed significantly between eGFR groups (**Table 13**). Similar to CG, no differences were found in total and free 25(OH)D between groups with CKD-EPI algorithms (**Table 14**).

Post-supplementation, when MDRD-4 was used, $1,25(OH)_2D$ remained significantly lower and iPTH higher with eGFR <60 mL/min/1.73m^2 while other metabolites were no longer significantly different between eGFR groups (all p<0.05) (**Table 13; Figure 21**). Based on CG, total and free 25(OH)D were significantly higher and $1,25(OH)_2D$ significantly lower with eGFR <60 mL/min/1.73 m^2 (all p<0.05) (**Table 13**). DBP remained not significant regardless the eGFR equation used.

Wnt-signalling pathway markers

At baseline, with eGFR categorised on basis of both MDRD-4 and CG, SOST was significantly higher and DKK1 significantly lower with eGFR <60 mL/min/1.73 m^2 (all p<0.05) (**Table 13; Figure 21**). In addition, when using CG, OPG was significantly higher with eGFR <60 mL/min/1.73 m^2 (p<0.05) (**Table 13**). There were no between-group differences for sRANKL regardless of equation used.

After supplementation, when MDRD-4 was used, SOST remained higher and DKK1 significantly lower in the group with eGFR <60 mL/min/1.73 m^2 similar to findings at baseline (all p<0.05) (**Table 13; Figure 21**). Based on CG, SOST was higher and sRANKL lower with eGFR <60 mL/min/1.73 m^2 (all p<0.05) (**Table 13**).

Bone parameters and markers of bone metabolism

At baseline, with eGFR categorised on basis of MDRD-4, no significant differences in any of the bone markers were found (**Table 13**). Categorised on the basis of CG, however, BMD and BMC at both the hip and femoral neck were lower in the eGFR category <60 mL/min/1.73 m^2 and PINP significantly higher compared to the group with ≥60 mL/min/1.73 m^2 (all p<0.05) but trends were in a similar direction (**Table 13**).

After 12 months of supplementation, when MDRD-4 was used, hip BMD was significantly lower with eGFR <60 mL/min/1.73 m^2 (p<0.05) (**Table 13**). Based on CG, there were no significant differences between the eGFR groups after supplementation (**Table 13**). There were no differences by eGFR category in markers of bone metabolism with either algorithm.

<u>Associations with eGFR (by four equations for eGFR)</u>

Regression analyses showed that all relationships were linear and there were no apparent thresholds of eGFR at which slopes changed.

Regression analyses with eGFR as the independent, continuous variable mostly confirmed findings of t-tests comparing differences between eGFR categories. Significant negative associations were found for cFGF23, iFGF23, iPTH and SOST and positive associations with $24,25(OH)_2D$, $1,25(OH)_2D$ for both MDRD-4 and CG data (**Table 15**). A significant association with Klotho was only found with MDRD-4 data and OPG with CG data. The differences

between eGFR groups in total and free 25(OH)D found with MDRD-4 and in DKK1 with both MDRD-4 and CG, were however not reflected in significant associations between eGFR and these variables (**Table 15**). Also, in accordance with findings with t-tests, no significant associations of eGFR by MDRD-4 or CG with BMD, BMC and the bone metabolism markers (BAP, CTX, PINP) were found.

Similar to analyses with MDRD-4, regression analyses with CKD-EPI algorithms also confirmed findings with t-tests; significant association with cFGF23, iFGF23, iPTH, SOST, 24,25$(OH)_2$D and 1,25$(OH)_2$D were found (**Table 16**). In addition, significant negative associations were found for serum phosphate and OPG (**Table 14**).

After supplementation, only the association with 1,25$(OH)_2$D and cFG23 remained significant by both MDRD-4 and CG. Associations of iFGF23, Klotho and iPTH with eGFR by both MDRD-4 and CG were no longer significant. Total and free 25(OH)D and SOST remained significant only with MDRD-4 and 24,25$(OH)_2$D remained significant only with CG. Regression analyses thus did not confirm findings of t-tests for iFGF23, iPTH, total and free 25(OH)D (***Table 15***). With MDRD-4, there were significant positive associations with BMD and BMC, in accordance with findings of t-tests. No significant associations of eGFR by CG with BMD and BMC were found. No associations with the bone metabolism markers were found with either algorithm. Post-supplementation, regression analyses with CKD-EPI algorithms confirmed findings of t-tests. Significant associations were found with serum phosphate and 1,25$(OH)_2$D. Negative associations with free 25(OH)D and iPTH were only found with CKD-EPI creatinine-cystatin C. Similar to analyses with MDRD-4, associations with cFGF23, iFGF23, and SOST were no longer significant after supplementation (**Table 14; Table 16**).

Table 15. Association between biomarkers and eGFR calculated according to MDRD-4 and Cockcroft-Gault algorithms at baseline and 12 months[a]

Biomarkers	MDRD-4				CKD-EPI Creatinine				Cockcroft-Gault			
	Baseline		12-months		Baseline		12-months		Baseline		12-months	
	β-coefficient	p-value	β-coefficient	p-value	β-coefficient	p-value	β-coefficient	p-value	β-coefficient	p-value	β-coefficient	p-value
Renal function markers												
Albumin (g/L)	-0.003	0.69	**0.020**	**0.02**	0.006	0.525	0.019	0.058	0.011	0.05	0.009	0.215
Adjusted calcium (mmol/L)	0.000	0.56	0.000	0.95	0.000	0.844	0.000	0.898	0.000	0.03	0.000	0.99
Phosphate (mmol/L)	0.001	0.10	-0.001	0.36	**0.002**	**0.014**	-0.001	0.513	**-0.002**	**<0.001**	**-0.004**	**<0.001**
Klotho (pg/mL)	**5.560**	**0.01**	0.802	0.49	**5.713**	**0.023**	0.447	0.737	1.062	0.16	1.086	0.14
cFGF23 (RU/mL)	**-0.648**	**<0.001**	**-0.985**	**<0.001**	**-0.743**	**<0.001**	-0.081	0.638	**-0.588**	**<0.001**	**-0.428**	**0.018**
iFGF23 (pg/mL)	**-0.254**	**0.03**	-0.058	0.66	-0.296	0.028	-0.107	0.487	**-0.176**	**0.04**	0.049	0.57
Vitamin D metabolism markers												
Total 25(OH)D (nmol/L)	0.046	0.50	**-0.196**	**<0.001**	0.091	0.251	**-0.162**	**0.031**	0.043	0.40	-0.272	**<0.001**
Free 25(OH)D (pmol/L)	0.011	0.43	**-0.056**	**<0.001**	0.020	0.246	**-0.059**	**0.001**	0.011	0.32	-0.057	**<0.001**
$24,25(OH)_2D$ (nmol/L)	**0.022**	**0.04**	-0.007	0.51	**0.032**	**0.007**	0.007	0.612	**0.023**	**<0.001**	**-0.029**	**0.002**
$1,25(OH)_2D$ (pmol/L)	**0.569**	**<0.001**	**0.606**	**<0.001**	**0.665**	**<0.001**	**0.779**	**<0.001**	**0.202**	**0.01**	**0.238**	**<0.001**
DBP (mg/L)	-0.067	0.76	-0.380	0.10	-0.010	0.968	-0.377	0.167	0.060	0.71	-0.137	0.36
iPTH (pg/ml)	**-0.162**	**0.04**	-0.152	0.05	**-0.281**	**0.002**	-0.127	0.183	0.012	0.861	-0.013	0.84
Wnt-signalling pathway markers												
SOST (pmol/L)	**-0.282**	**<0.001**	**-0.356**	**<0.001**	**-0.376**	**<0.001**	0.154	0.169	**-0.226**	**<0.001**	-0.096	0.19
DKK1 (pmol/L)	0.099	0.10	0.092	0.19	**0.143**	**0.035**	0.089	0.277	0.060	0.17	0.016	0.72
OPG (pmol/L)	-0.008	0.28	0.007	0.41	-0.012	0.153	0.007	0.465	**-0.015**	**<0.001**	0.008	0.15
sRANKL (pmol/L)	0.000	0.71	0.000	0.85	0.000	0.089	0.000	0.737	0.000	0.08	0.000	0.55
Bone mineral density and metabolism												
Hip BMD (g/m^2)	-0.001	0.17	**0.002**	**0.002**	-0.001	0.214	**0.002**	**0.002**	**0.002**	**<0.001**	0.001	0.062
Hip BMC (g)	-0.043	0.14	**0.076**	**0.01**	-0.063	0.056	**0.095**	**0.008**	**0.135**	**<0.001**	0.021	0.42

FN BMC (g)	-0.002	0.61	0.008	0.06	-0.004	0.313	**0.010**	**0.033**	**0.018**	**<0.001**	0.001	0.69
FN BMD (g/m^2)	-0.001	0.29	**0.001**	**0.02**	-0.002	0.373	0.001	0.646	**0.002**	**<0.001**	0.001	0.22
BAP (µg/L)	0.011	0.36	-0.001	0.96	0.010	0.462	-0.010	0.653	0.003	0.79	-0.002	0.92
CTX (ng/mL)	0.000	0.92	0.000	0.95	0.000	0.697	0.000	0.688	**-0.002**	**0.005**	0.000	0.46
PINP (µg/L)	-0.002	0.98	-0.028	0.71	-0.017	0.798	-0.010	0.909	-0.087	0.08	0.007	0.92

[a]β-coefficients and associated p-values from linear regression analysis with eGFR as a continuous variable. Significant associations p<0.05 are indicted in bold.

Table 16. Association between biomarkers and eGFR calculated according to CKD-EPI cystatin C and CKD-EPI creatinine-cystatin C algorithms at baseline and 12 months

Biomarkers	CKD-EPI cystatin C				CKD-EPI creatinine-cystatin C			
	Baseline		12-months		Baseline		12-months	
	β-coefficient	p-value	β-coefficient	p-value	β-coefficient	p-value	β-coefficient	p-value
Renal function markers								
Albumin (g/L)	0.011	0.05	>0.000	1.00	>0.010	0.15	0.006	0.42
Adjusted calcium (mmol/L)	**>0.000**	**0.03**	>0.000	0.99	>0.000	0.10	>0.000	0.95
Phosphate (mmol/L)	**-0.002**	**<0.001**	**-0.004**	**<0.001**	**-0.002**	**<0.001**	**-0.004**	**<0.001**
Klotho (pg/mL)	1.062	0.160	1.086	0.139	2.248	0.257	1.320	0.158
cFGF23 (RU/mL)	**-0.588**	**<0.001**	-0.094	0.33	**-0.774**	**0.00**	-0.112	0.35
iFGF23 (pg/mL)	**-0.176**	**0.04**	0.049	0.57	**-0.246**	**0.02**	0.005	0.96
Vitamin D metabolism markers								
Total 25(OH)D (nmol/L)	0.043	0.40	-0.005	0.90	0.076	0.22	-0.054	0.31
Free 25(OH)D (pmol/L)	0.011	0.32	-0.015	0.12	0.017	0.19	**-0.032**	**0.01**
$24,25(OH)_2D$ (nmol/L)	**0.023**	**<0.001**	0.010	0.19	**0.032**	**<0.001**	0.009	0.34
$1,25(OH)_2D$ (pmol/L)	**0.202**	**0.01**	**0.238**	**<0.001**	**0.040**	**<0.001**	**0.417**	**<0.001**
DBP (mg/L)	0.060	0.71	-0.137	0.36	0.063	0.75	-0.219	0.25
iPTH (pg/ml)	**-0.185**	**0.00**	-0.079	0.114	**-0.250**	**<0.001**	**-0.147**	**0.020**
Wnt-signalling pathway markers								
SOST (pmol/L)	**-0.226**	**<0.001**	0.036	0.56	**-0.320**	**<0.001**	0.072	0.36
DKK1 (pmol/L)	0.060	0.17	0.016	0.72	0.097	0.07	0.037	0.52
OPG (pmol/L)	**-0.015**	**<0.001**	0.008	0.15	**-0.015**	**0.02**	0.010	0.15
sRANKL (pmol/L)	>0.000	0.08	>0.000	0.55	>0.000	0.16	>0.000	0.65
Bone mineral density and metabolism								
Hip BMD (g/m^2)	>0.000	0.54	>0.000	0.99	>0.000	0.98	0.001	0.26
Hip BMC (g)	0.008	0.70	-0.009	0.64	-0.010	0.69	0.015	0.56

FN BMD (g/m^2)	>0.000	0.86	>0.000	0.71	>0.000	0.87	>0.000	0.59
FN BMC (g)	0.002	0.43	-0.002	0.46	0.001	0.77	0.001	0.84
BAP (µg/L)	-0.006	0.52	-0.013	0.31	-0.001	0.93	-0.013	0.42
CTX (ng/mL)	-0.001	0.07	0.000	0.40	-0.001	0.10	0.000	0.51
PINP (µg/L)	-0.073	0.08	-0.007	0.88	-0.080	0.13	-0.006	0.92

[a]β-coefficients and associated p-values from linear regression analysis with eGFR as a continuous variable. Significant associations p<0.05 are indicted in bold.

Discussion

In a cohort of apparently healthy older adults, 18-28% had an eGFR below <60 ml/min/1.73m^2 (CKD G3a) and 3-5% <45 ml/min/1.73m^2 (CKD G3b). Before supplementation, significantly higher concentrations cFGF23, iFGF23, iPTH and SOST and lower Klotho, 1,25(OH)$_2$D and DKK1 concentrations were found in the group with CKD G3a/b compared to the group with eGFR ≥60 ml/min/1.73m^2. Differences in 25(OH)D, 24,25(OH)$_2$D and iPTH by eGFR category were only detected with MDRD-4 and CKD-EPI equations. Differences in BMD and BMC were detected only with CG.

Pre- and post- supplementation comparisons showed differences in the response to supplementation by eGFR category. Supplementation resulted in an increase in 25(OH)D, 24,25(OH)$_2$D, DKK1 and a decrease in plasma phosphate, iPTH and CTX in both eGFR groups. Plasma Klotho only significantly increased in the group with a lower eGFR. Plasma iFGF23 and 1,25(OH)$_2$D, BAP, PINP increased only in the group with eGFR ≥60 ml/min/1.73m^2. Findings were largely consistent across all eGFR algorithms. After vitamin D supplementation, cFGF23, iFGF23, iPTH and SOST remained significantly higher in the lower eGFR group and 1,25(OH)$_2$D lower. Klotho did no longer differ between eGFR groups. Findings were consistent for all eGFR algorithms, with the exception of iPTH and iFGF23, for which no significant differences were found when eGFR was categorised on basis of CG.

Regression analyses mostly confirmed comparisons between eGFR categories, although these did not all reach statistical significance. Regression analyses further showed that relationships between eGFR and markers of calcium, phosphate and bone metabolism were continuous without an obvious threshold effect.

Consistent with earlier reports we found that, even with moderate renal impairment, Klotho was lower and both iFGF23 and cFGF23 were higher[153,154,384]. We also found differences in markers of vitamin D status and metabolism, iPTH and in markers of Wnt-signalling. Secondary hyperparathyroidism is a known complication of advanced stages of CKD and an increase in iPTH is usually reported from stage 3 or 4[214,385]. However we found significantly higher iPTH concentrations already with moderate renal impairment, with iPTH concentrations above the assay-specific references range (4.7-114 pg/ml[386]) in a considerable proportion of participants, similar to other reports[98]. A reduction in plasma 1,25(OH)$_2$D is

reported from early renal impairment and continues to decrease with declining renal function[98,387]. The changes in plasma 1,25(OH)$_2$D concentrations with CKD are thought to be the result of the combined effect of a reduced renal hydroxylation capacity and increased catabolism induced by FGF23.

FGF23 and SOST are both produced by osteocytes and regulate renal mineral and vitamin D metabolism and influence bone formation and resorption[63]. The Wnt/β-catenin signalling pathway plays a key role bone homeostasis by regulating osteocyte function and osteoblast and osteoclast differentiation and function[192] and its effect is mainly anabolic[193]. Inhibitors of Wnt-signalling include SOST, DKK1 and, indirectly, RANKL. Increases in plasma concentrations of these factors is associated with loss of bone mass and integrity[60,134,192,388–391]. OPG antagonises the actions of RANKL by binding and preventing interaction with its receptor, RANK[54,55,60]. We showed that SOST concentrations were higher and DKK1 lower with a moderate decline in kidney function. Similar findings were reported by Sabbagh *et al.* 2012[392]. Reference ranges of plasma concentrations of these regulators of Wnt-signalling in healthy and CKD patients are not well defined. However, comparing to the reported normal ranges in a limited number of healthy subjects for the kits used for these analyses, plasma concentrations of SOST and OPG were above and RANKL below these ranges in the group with CKD G3a/b (normal range SOST: 13.31-41.77pmol/L; OPG median: 1.8pmol/L; sRANKL: 0.37-0.46 pmol/L). The clinical relevance of these findings needs be established. However, together with the changes in FGF23, intact iPTH and 1,25(OH)$_2$D it may be anticipated that these changes with early CKD are associated with negative effects on mineral and bone metabolism[134,388,390,391]. Such early changes in renal function are usually not detected or give rise to clinical monitoring but may signify a stage during which early intervention may offer health benefits.

There were differences in the response to vitamin D supplementation by eGFR category. As expected, plasma total and free 25(OH)D and 24,25(OH)$_2$D increased with supplementation. We earlier reported an increase in 1,25(OH)$_2$D in the full cohort[343], similar to findings in other RCTs[270,296]. Here we show that this increase was only observed in the group with an eGFR ≥60 ml/min/1.73m^2. This illustrates the reduced 1 α-hydroxylation capacity and/or increased catabolism with lower renal function[167], which resulted in lower plasma 1,25(OH)$_2$D concentration, despite increased availability of 25(OH)D for hydroxylation.

Plasma iPTH decreased with supplementation in both eGFR groups. In healthy individuals, iPTH and 25(OH)D are inversely correlated and vitamin D supplementation is associated with a decrease in iPTH[13], although this may only be observed at lower baseline 25(OH)D concentrations. However, reports on the effect of vitamin D supplementation on iPTH in patients with CKD and secondary hyperparathyroidism (SHPT) are conflicting and the effect depends on the stage of CKD. This is partly explained by the fact that the aetiology of SHPT with impaired renal function is complex and multifactorial. It is unclear at what stage of CKD and/or SHPT, vitamin D supplementation is no longer effective in reducing iPTH[98,214].

The response of iFGF23 and Klotho differed by eGFR category, while plasma cFGF23 increased and plasma phosphate decreased in both eGFR groups. An increase in cFGF23 and iFGF23 with vitamin D supplementation was reported before[342,343] and may be a response to increased intestinal phosphate absorption and the decline in iPTH. In this study, iFGF23 only significantly increased in the group with the higher eGFR, while Klotho remained unaltered. In the group with eGFR <60 ml/min/1.73m^2 however, Klotho increased and iFGF23 remained unchanged with supplementation. This might indicate an increase in FGF23 sensitivity as result of an increase in its co-factor Klotho and thus a potential benefit of supplementation in people with compromised kidney function. There is limited evidence that vitamin D supplementation may increase the expression of Klotho, but data are conflicting[31,362]. Our findings suggest that the effect may depend on renal function.

Vitamin D may modulate SOST expression in osteocytes[368,393]. Recent findings show an inverse correlation between vitamin D status and SOST concentrations in healthy postmenopausal women and adults[343,367,394]. However, conflicting results have been reported regarding the effect of vitamin D supplementation on SOST and DKK1[393,395,396]. Several studies showed that vitamin D supplementation can lead to decline in SOST[396,397]. Other studies showed the opposite, reporting an increase in SOST following supplementation with native[395,398] or activated forms of vitamin D in non-CKD and CKD subjects[393]. Another RCT with CKD patients (G3-4) showed that vitamin D supplementation did not significantly affect SOST[264]. In our study, SOST concentrations remained unchanged with supplementation, irrespective of category of eGFR. We also observed no significant effect of supplementation on OPG and RANKL, while DKK1 increased. The bone turnover markers however suggest that supplementation altered the rate bone remodelling. We found a decrease in plasma CTX in

both eGFR groups after supplementation, while BAP and PINP increased only in the group with higher renal function. No effects were found on bone mineral density and content in either eGFR group. Recent studies have shown associations between FGF23, SOST and other regulators of the Wnt-signalling and micro-architectural changes and fracture risk, particularly in trabecular bone, in the absence of marked changes in (DXA measured) BMD or BMC[212,366]. Future research with pQCT measures of bone integrity is required to investigate such effects of vitamin D.

The use of different eGFR algorithms provided, as expected somewhat different results. Although the MDRD-4 and CKD-EPI algorithms, as based on either creatinine or cystatin C provided similar results, those with CG differed. CG is the only algorithm tested that includes weight. This might explain the differences in bone density detected between the eGFR categories when using the CG algorithm. In addition, the higher number of participants categorised to the eGFR <60 ml/min/1.73m^2 group (baseline n=106; 12 months n=99) may have influenced the statistical power to detect these differences.

In medical practice, kidney function is routinely assessed in older adults[399] and mostly based on the calculation of eGFR based on serum creatinine or more recently, cystatin C[380,400]. Urinary creatinine to estimate clearance or tracer clearance are seldomly measured. The use of serum creatinine in older adults may lead to an overestimation of the GFR[232,401]. Plasma creatinine is also influenced by muscle mass and dietary protein intake. Both of these are known to decrease with age[402] and this can lead to misclassification of patients[400]. Cystatin C is produced at a constant rate by all nucleated cells and filtered in proximal tubules[230]. Recent studies have shown that cystatin C may be a better marker for progression of CKD[403–405], since it is less affected by exogenous factors[402]. In this study, relationships between cystatin C and creatinine based eGFR with the investigated markers were comparable. That might be due to nature of the study population used for this analysis.

This study has several limitations. The VDOP study included relatively healthy older adults and excluded those with an eGFR <30 ml/min/1.73m^2 at screening. The eGFR categorisation was solely based on eGFR, without data regarding albuminuria. In addition, since participants had no prior diagnosis of renal disease, the underlying causes of impaired renal function are unknown and may be expected to be heterogenous. As a result, the bone phenotype may also

be of a heterogenous nature. Participants of the VDOP study were predominantly of Caucasian origin and therefore results may not be applicable to other populations.

Since the design of this study, evidence has been published that a monthly vitamin D dosing regimen may have different, less beneficial effects on musculoskeletal function and metabolism compared to more frequent dosing.

Changes in bone metabolism may not be reflected in DXA measured BMD and BMC. Although the study was powered to detect a change in hip BMD from baseline in each supplementation group as based on an earlier, similar study in the North of the UK[343,344], this was found to be non-significant in this study. This may be explained by differences in the study protocol or population The response to supplementation may have depended on both renal function and baseline 25(OH)D. There was however insufficient statistical power to test this hypothesis and studies to address this question are required. The large number of comparisons conducted in this study will have increased the chance of type 2 errors; research specifically designed and powered to confirm our findings are required. Group sizes were unequal; the group with the lower eGFR had limited numbers compared to the group with the higher eGFR.

The follow-up time was 12 months, and this may have been too short to detect a change in BMD. Markers of bone metabolism and osteocyte signalling may be expected to respond to interventions within the length of a bone remodelling cycle (~3-4 months)[379]. Therefore, changes in bone metabolism may be identified with these biochemical markers before they are reflected in BMD. However, it is possible that markers measured after 12 months reflect a newly achieved steady state that is seemingly no different from baseline and that changes occurred within the first few months after commencement of the intervention, such as observed in pharmaceutical trials[378,379].

Further, we did not directly measure free 25(OH)D but instead calculated the free fraction and thus considers the potential influence of binding proteins. Although directly measured and calculated free 25(OH)D concentrations correlate well in healthy populations[369,374,375], it cannot be excluded that directly measured concentrations would have provided different findings.

In conclusion, this study showed that a moderate decline in eGFR has a negative impact on vitamin D metabolism, Wnt-signalling and bone turnover markers. Vitamin D supplementation has beneficial effects on markers of the renal-bone axis in older people with

both normal and impaired renal function. The response depended on renal function. Supplementation improved Vitamin D status and Klotho in the group with moderate renal impairment to concentrations comparable to those found in the group with normal renal function. However, although CTX decreased, no effect on bone formation markers was found in the group with eGFR <60 ml/min/1.73m^2.

CHAPTER 5: Vitamin D supplementation improves iron status and inflammation markers in older people with renal impairment

Summary

CKD leads to alterations in FGF23 and the renal-bone axis, may partly be driven by altered inflammation and iron status. Vitamin D supplementation was reported to reduce inflammation.

Older adults with normal renal function (eGFR >90mL/min/1.73m^2; CKDG1; n=35) or early CKD (eGFR 30-60mL/min/1.73m^2; CKDG3a/b; n=35) received 12,000, 24,000 or 48,000IU D_3/month for 1 year. Markers of inflammation, iron and renal-bone axis were investigated pre- and post-supplementation. Predictors of cFGF23 and iFGF23 were identified by univariate and multivariate regression.

Pre-supplementation, plasma cFGF23, iFGF23, iPTH, sclerostin and TNFα were significantly higher and Klotho, 1,25-dihydroxyvitamin D and iron lower with CKDG3a/b compared to CKDG1. Post-supplementation, only 25(OH)D, cFGF23 and IL6 differed between groups.

The effect of supplementation was eGFR dependent. In the CKDG3a/b group TNFα significantly decreased and iron increased. In the CKDG1 group, phosphate decreased, cFGF23, iFGF23, PINP, IL10 and 25(OH)D increased and CTX decreased in both groups. No significant differences were found between vitamin D doses.

In univariate models cFGF23 and iFGF23 were predicted by eGFR and regulators of calcium/phosphate metabolism at both time points; IL6 predicted cFGF23 (post-supplementation) and iFGF23 (pre-supplementation) but was not significant in multivariate models. Hepcidin predicted cFGF23 in a multivariate model with eGFR and iFGF23 post-supplementation.

Alterations in regulators of the renal-bone axis, inflammation and iron status were observed in early CKD. The response to vitamin D supplementation differed between eGFR groups. IL6 predicted cFGF23 and iFGF23, hepcidin cFGF23.

Methods

Study design

This study utilised plasma samples and data collected as part of the vitamin D supplementation in older people (VDOP) randomized controlled trial[343] (ISRCTN35648481). In brief, this RCT included 379 ambulatory, community dwelling adults aged ≥70y (48% women; mean age: 75y) from the northeast of England. Participants were recruited through general practices and those with known CKD or MDRD-based eGFR <30 ml/min/1.73m^2 at pre-screening were excluded. Participants were randomly allocated into 3 groups supplemented with vitamin D_3 [12,000 international units (IU), 24,000 IU, or 48,000 IU] given once a month for 1 year. More details of the study design, methods and primary outcomes were previously described[343,345]. From this cohort, participants were selected if the mean eGFR (baseline and 12 months) based on MDRD-4 was <30-60 mL/min/1.73 m^2 or >90 mL/min/1.73 m^2, and additionally, if samples were available at both time points. Haemolytic samples were not used. This resulted in n=70 sets of data, with n=35 participants in each eGFR group.

The analyses were explorative and secondary and were not pre-specified in the original trial design and analyses plan. Results for plasma concentrations of 25(OH)D, iPTH, BMD and Wnt-signalling markers in the full cohort were earlier reported by Aspray *et al*.[343] and *Christodoulou et al.*[227] but are also included here to support data interpretation.

The study was conducted in accordance with guidelines laid down in the Declaration of Helsinki. A favourable opinion was obtained from the Tyne & Wear South Research Ethics Committee (REC, 12/NE/0050) with Research and Development approval from the sponsor, Newcastle upon Tyne Hospitals NHS Foundation Trust. All participants provided written informed consent.

Measurements

Methods for measurements BMD, height and weight, collection of early morning fasting blood samples at baseline and after 12 months of supplementation, as well as details of blood processing, storage and biochemical analyses were provided elsewhere [343]. Sample collection

and processing methods were used suitable for the measurement or iron status and other markers included in this study.

In brief, analyses were conducted at 3 sites (Newcastle upon Tyne hospitals NHS Foundation Trust; MRC Human Nutrition Research, Cambridge, UK (HNR-UK) and of University of East Anglia (UEA), UK. HNR-UK biochemical methods were: 25(OH)D (LC-MS/MS), iPTH (Immulite 2000, SIEMENS), PINP (UniQ, RIA), β-CTX (Immunodiagnostic Systems), BAP (DiaSorin, Liaison). All assays were performed in duplicate except for iPTH. Assay performance was monitored using kit and in-house controls and under strict standardisation according to ISO 9001:2000. Assay performance details were provided in Aspray T.J. *et al.* 2019[343] and Christodoulou M. *et al*. 2022[342]. Quality assurance of 25(OH)D and iPTH assays are performed as part of the Vitamin D External Quality Assessment Scheme (www.deqas.org) and the National External Quality Assessment Scheme (www.uknegas.org.uk). Measurements of 25(OH)D were harmonised against NIST standards as part of the Vitamin D harmonisation program[343].

Measurements conducted at UEA included plasma phosphate (Cobas, Roche Diagnostics), αKlotho (IBL international), cFGF23 and iFGF23 (Immutopics), OPG (Biomedica), SOST, DKK1 and soluble RANKL (sRANKL) (Biomedica), 1,25$(OH)_2$D (DiaSorin, Liaison XL), iron (Cobas, Roche Diagnostics), hepcidin (R&D systems Bio-Techne), CRP (High sensitivity; Cobas, Roche Diagnostics), TNFα (R&D systems Bio-Techne), IL-6 (Cobas, Roche Diagnostics) and IL-10 (High sensitivity; R&D systems Bio-Techne). All assays were performed in duplicate except for phosphate, 1,25$(OH)_2$D, CRP, iron and IL-6 on basis of consistent performance with intra and inter-assay CV <4%. The inter- and intra-assay CVs of all other assays were <10%. Assay performance was monitored using kit and in-house controls and following Good Laboratory Practice. The measurement ranges of TNFα and IL-10 were expanded by diluting the lowest standards of the calibration curve.

All the biochemical analysis was conducted prior the start of this PhD study except markers of cystatin C, phosphate, iron, hepcidin, CRP, TNFα, IL-6 and IL-10 were conducted by M. Christodoulou during this PhD study.

MDRD-4 eGFR was calculated as follows[406,407]: MDRD-4= 175 x $(S_{Cr})^{-1.154}$ x $(age)^{-0.203}$ x 0.742 [if female] x 1.212 [if Black], with serum creatinine (S_{cr}) in mg/dL and age in years. Since there

were no non-white participants therefore race was removed. Analyses were also conducted with eGFR calculated according to the CKD-EPI algorithm with creatinine and without race[380]. CKD-EPI data provided very similar results and are therefore not presented.

Statistical analysis

The findings presented in this paper aimed to test the following:

(1) Differences in markers of iron status and inflammation and regulators of Wnt-signalling and bone metabolism between eGFR categories before and after 12 months of vitamin D supplementation.
(2) The effect of vitamin D supplementation by eGFR group.
(3) Predictors of cFGF23 and iFGF23.

A power calculation was not conducted due to a lack of relevant data. Correction for repeated testing was not deemed appropriate for this explorative analysis as any finding will require confirmation in RCTs specifically designed and powered for respective outcomes.

The response to the intervention by supplementation group without considering eGFR was reported in detail before[226,227]. Differences in the response to the dosage of vitamin D was considered by the inclusion of the dose as a co-variate in statistical modelling, where appropriate. Since there were no significant differences between dosage groups post-supplementation, descriptive data at 12 months are presented pooled for all 3 supplementation groups. Pre- and post-supplementation comparisons within each eGFR group were tested with paired t-tests. Between eGFR group differences pre- and post-supplementation were tested by ANCOVA, with baseline values and supplementation group as co-variates for 12 months data.

All outcomes were assessed for normality (defined as a posterior distribution skewness <2 or >-2) and visual inspection of histograms. Non-normally distributed variables were converted to natural LN and checked again for normality. One extreme outlier was identified for TNFα and was excluded. For normally distributed data, results are expressed as mean (SD); for skewed data, results are expressed as median [IQR].

Univariate linear regression analysis was conducted to identify predictors of cFGF23 and iFGF23. Variables included were selected a prior on the basis of a theoretical biological

mechanism and included those related to renal function, calcium and phosphate, iron status and inflammation. Multivariate linear regression was subsequently performed including all variables with a p-value <0.2 in univariate analyses (**Table 18; Table 19**), followed by hierarchical elimination of non-significant variables. Co-linearity of independent variables (R>0.6) was checked prior to inclusion in multivariate models. Linearity of associations were checked visually. These regression analyses were conducted using the natural values of the data (not LN converted).

IBM® SPSS® Statistics Version 28 software was used.

Results

Differences in biomarkers between eGFR categories

Median eGFR was 51.0 [IQR: 45.8-53.8] mL/min/1.73 m^2 (CKDG3a: n=23; CKDG3b: n=7) and 95.7 [92.1-102.8] for the CKDG3a/b and G1, respectively. Age was significantly higher in the group with CKD3a/b; gender and BMI did not differ (**Table 17**).

At baseline, compared to the group with normal renal function, plasma PTH, cFGF23, iFGF23 were higher, klotho and $1,25(OH)_2D$ were lower in the group CKDG3a/b. Also, plasma iron was lower, but there was no difference in the hepcidin concentration between groups. Of the inflammation markers, the pro-inflammatory cytokine TNFα was significantly higher and IL6 near significantly (p=0.075) higher in the group with CKDG3a/b, but plasma CRP and IL10 did not differ.

SOST was higher in the group with eGFR <60 mL/min/1.73m^2 but no differences were found in other markers of the Wnt-signalling pathway and bone density and remodelling (**Table 17**).

Post vitamin D supplementation, cFGF23 concentrations remained significantly different between eGFR groups. Post-supplementation 25(OH)D was higher in the group with CKDG3a/b. Plasma iron and hepcidin and the markers of inflammation, CRP, TNFα and IL10 were not different between groups, but the pro-inflammatory cytokine IL6 was significantly higher in the group with CKDG3a/b. There were no between eGFR group differences in markers of Wnt-signalling and bone density and remodelling (**Table 17**).

Table 17. Between group comparisons at baseline and 12 months[a]

Characteristics[a]	eGFR<60 mL/min/1.73 m² (n=35)[c]		eGFR>90 mL/min/1.73 m² (n=35)[d]	
	BASELINE	12 MONTHS	BASELINE	12 MONTHS
Men/ Women	18/17	-	20/15	-
Age (years)	76.9 (4.1)	-	72.3 (4.1)‡	-
BMI (kg/m²)	27.9 (4.5)	-	27.6 (4.5)	-
MDRD eGFR (mL/min/1.73 m²)[e]	51.0 [45.8-53.8]		95.7 [92.1-102.8]‡	
MDRD eGFR (mL/min/1.73 m²)	50.0 [44.8-53.1]	51.0 [44.6-54.4]	97.5 [93.3-103.0]	94.5 [89.6-105.0]
Markers of calcium, phosphate and vitamin D metabolism				
Acalcium (mmol/L)	2.2 (0.1)	2.2 (0.1)	2.2 [2.2-2.3]	2.2 (0.1)
Phosphate (mmol/L)	0.81 (0.2)	0.83 (0.2)	0.88 (0.17)†	0.78 (0.16)
iPTH (pg/mL)	59.9 (27.1)	52.7 (23.9)	42.9 [27.9-67.8]*	42.3 (22.6)
iFGF23 (pg/mL)	69.4 [49.7-78.4]	78.4 [54.9-91.5]	53.4 (19.0)*†	57.1 [48.1-68.2]
cFGF23 (RU/mL)	81.9 [71.0-154.6]	112.0 [84.8-139.8]	58.2 (18.5)**†	61.1 [53.4-74.6]*
Klotho (pg/mL)	436 (102)	471.1 (109)	509 [392-643]*	507.4 [403-730]
25(OH)D (nmol/L)	30.0 [21.8-39.0]†	73.5 (20.3)	39.9 (21.2)†	61.5 (18.7)*
$1,25(OH)_2D$ (pmol/L)	71.2 [56.1-102.0]	82.4 (29.6)	111.5 (30.9)**	112.0 (31.6)
Iron status and inflammation markers				
Iron (µmol/L)	12.6 (6.4)†	16.2 (6.4)	16.1 (6.0)*	18.0 (5.8)
Hepcidin (ng/mL)	24.7 (16.0)	20.2 (14.1)	19.7 [11.9-31.6]	19.6 11.2
CRP (nmol/L)	18.8 [8.1-35.5]	18.3 [8.2-39.2]	15.5 [7.5-27.7]	12.8 [4.9-27.9]
TNFα (pg/mL)	9.4 [7.8-11.1]†	6.5 [5.1-12.0]	6.8 [5.8-9.8]*	7.0 [4.2-8.8]
IL6 (pg/mL)	2.54 [0.75-4.61]	2.93 [1.78-5.85]	0.75 [0.75-2.90]	0.75 [0.75-2.80]*
IL10 (pg/mL)	0.33 [0.02-0.61]†	0.52 [0.38-1.00]	0.18 [0.02-0.60]†	0.69 [0.52-0.81]
Bone turnover and Wnt-signalling markers				
Hip BMD (g/ m²)	1.01 (0.19)	1.00 (0.19)	0.95 (0.14)†	0.94 (0.15)
BAP (µg/L)	9.5 [7.4-11.3]	9.8 (3.1)	11.4 (3.6)	11.6 [8.3-14.2]
CTX (ng/mL)	0.41 [0.29-0.58]†	0.36 (0.15)	0.37 [0.28-0.53]†	0.34 [0.26-0.42]

PINP (µg/L)	41.4	[29.1-53.5]	40.3	[31.8-50.7]	38.3	[26.8-46.0]†	40.0	[32.7-47.5]
SOST (pmol/L)	61.39	(22.6)	62.3	(24.0)	41.5	(16.7)**	41.9	(18.6)
DKK1 (pmol/L)	25.0	(12.1)	32.9	(15.7)	30.6	(13.3)	40.9	(19.2)
OPG (pmol/L)	5.40	[4.39-6.29]	5.45	[4.45-6.61]	5.25	(1.86)	5.26	(1.61)

[a]For normally distributed data, results are expressed as mean (SD); for skewed data, results are expressed as median [interquartile range (IQR)].

[c] Arm: 12000 IU n=4; 24000 IU n=13; 48000 IU n=18

[d] Arm: 12000 IU n=8; 24000 IU n=16; 48000 IU n=11

[e]Average of pre- and post-supplementation values

‡ Independent t-test significant difference $p<0.05$ between eGFR groups

* ANCOVA significant difference $p<0.05$ between eGFR groups

** ANCOVA significant difference $p<0.001$ between eGFR groups

† Paired t-test significant difference $p<0.05$ between pre- and post-supplementation values within eGFR groups; the vitamin D dose was non-significant and therefore removed from models.

Supplementation effect within each eGFR group

Estimated GFR did not change. There were differences in the response to supplementation between eGFR groups. In the group with CKDG3a/b, plasma phosphate, iFGF23, cFGF23 was unchanged, whereas in the group with normal renal function plasma phosphate decreased and both cFGF23, iFGF23 increased (both $p<0.01$). Plasma 25(OH)D increased in both groups and the remainder markers of calcium and phosphate metabolism did not significantly change in either group.

In the group with CKDG3a/b, plasma iron increased, while hepcidin was unchanged. The pro-inflammatory cytokine TNFα decreased and the anti-inflammatory cytokine IL10 increased. Plasma CRP and IL6 did not change. The bone resorption marker CTX decreased, but BMD, BAP, P1NP and markers of Wnt-signalling were unchanged. In the group with normal renal function, no changes were observed in markers of iron status and inflammation, except for an increase in IL10. CTX and BMD decreased and PINP increased. BAP and markers of Wnt-signalling were unchanged (**Table 17**). As indicated in the methods section, there were no significant differences between vitamin D dosages in the response.

Predictors of cFGF23 and iFGF23

At baseline, predictors of cFGF23 were eGFR, iFGF23, PTH and $1,25(OH)_2D$, but no significant associations were found with markers of inflammation and iron status in univariate analyses, although a tendency of significance was detected for plasma iron ($p=0.076$) (**Table 18**). In a multivariate model, only eGFR remained significant (R^2 =19%) (**Table 19**).

Significant predictors of iFGF23 were eGFR, cFGF23, $1,25(OH)_2D$, albumin adjusted calcium and IL6 (all $p<0.05$) and a tendency for hepcidin ($p=0.078$). No significant associations were found with other markers of inflammation and iron status (**Table 18**). In a multivariate model, cFGF23, $1,25(OH)_2D$ and adjusted calcium remained significant (total R^2 =31%) (**Table 19**).

At 12 months, predictors of cFGF23 were the same as at baseline, i.e., eGFR, iFGF23, PTH, $1,25(OH)_2D$ and also albumin adjusted calcium and IL6 and there was a tendency of significance for hepcidin ($p=0.077$) and CRP ($p=0.070$) (**Table 18**). In a multivariate model, eGFR, iFGF23 and hepcidin were significant (total R^2 =37%) (**Table 19**).

Predictors of iFGF23 were eGFR, cFGF23, Klotho and 1,25(OH)$_2$D, and a tendency for iron (p=0.08), of which cFGF23 and 1,25(OH)$_2$D were significant in a multivariate model (Total R^2=30%) (**Table 18; Table 19**).

Table 18. Predictors of c-terminal and intact FGF23 at baseline and 12 months[a] in univariate regression models

	Baseline		12-months	
	β-coefficient (SE)	**p-value**	**β-coefficient (SE)**	**p-value**
cFGF23 (RU/mL)				
eGFR (mL/min/1.73 m^2)	**-1.70 (0.266)**	**<0.001**	**-1.13 (0.269)**	**<0.001**
Acalcium (mmol/L)	24.0 (131.775)	0.856	**293.6 (113.670)**	**0.012**
Phosphate (mmol/L)	-74.6 (41.539)	0.077	53.1 (46.710)	0.26
iFGF23 (pg/mL)	**0.72 (0.261)**	**0.007**	**0.92 (0.226)**	**<0.001**
Klotho (pg/mL)	-0.037 (0.035)	0.294	-0.051 (0.037)	0.167
iPTH (pg/mL)	**0.66 (0.304)**	**0.033**	**0.67 (0.327)**	**0.045**
25(OH)D (nmol/L)	-0.56 (0.413)	0.178	0.15 (0.392)	0.711
1,25(OH)$_2$D (pmol/L)	**-0.56 (0.220)**	**0.013**	**-0.50 (0.228)**	**0.03**
Iron (µmol/L)	-2.26 (1.253)	0.076	-0.67 (1.295)	0.599
Hepcidin (ng/mL)	<-0.001 (0.001)	0.513	-0.001 (0.001)	0.077
CRP (nmol/L)	0.143 (0.128)	0.269	0.309 (0.168)	0.07
TNFa (pg/mL)	1.23 (2.102)	0.561	1.26 (1.520)	0.41
IL6 (pg/mL)	1.32 (1.690)	0.437	**3.54 (1.764)**	**0.049**
IL10 (pg/mL)	-9.33 (18.806)	0.622	2.99 (4.434)	0.502
iFGF23 (pg/mL)				
eGFR (mL/min/1.73 m^2)	**-0.38 (0.122)**	**0.003**	**-0.45 (0.135)**	**0.002**
Acalcium (mmol/L)	**187.4 (53.316)**	**<0.001**	100.5 (55.969)	0.077
Phosphate (mmol/L)	9.1 (18.660)	0.627	15.9 (22.591)	0.484
cFGF23 (pg/mL)	**0.14 (0.051)**	**0.007**	**0.21 (0.052)**	**<0.001**
Klotho (pg/mL)	-0.012 (0.015)	0.445	**-0.037 (0.017)**	**0.036**
iPTH (pg/mL)	-0.07 (0.138)	0.614	0.06 (0.162)	0.711
25(OH)D (nmol/L)	0.29 (0.181)	0.111	0.03 (0.186)	0.153
1,25(OH)$_2$D (pmol/L)	**-0.34 (0.092)**	**<0.001**	**-0.40 (0.102)**	**<0.001**
Iron (µmol/L)	-0.28 (0.563)	0.619	-1.08 (0.610)	0.08
Hepcidin (ng/mL)	<0.001(0.0002)	0.078	<0.001 (0.0003)	0.258
CRP (nmol/L)	0.042 (0.057)	0.464	0.064 (0.082)	0.437
TNFa (pg/mL)	0.04 (0.913)	0.965	1.07 (0.723)	0.143
IL6 (pg/mL)	**1.57 (0.721)**	**0.031**	0.73 (0.686)	0.404
IL10 (pg/mL)	-10.24 (8.029)	0.207	-0.34 (2.139)	0.874

[a]β-coefficients and associated p-values from univariate linear regression analysis; Dependent variables are indicted in bold in grey bars; Significant (p<0.05) associations are indicted in bold.

Table 19. Predictors of c-terminal and intact FGF23 at baseline and 12 months[a] in multivariate regression models

	Baseline		12-months	
	β-coefficient (SE)	**p-value**	**β-coefficient (SE)**	**p-value**
cFGF23 (RU/mL)				
eGFR (mL/min/1.73 m^2)	**-1.07 (0.27)**	**<0.001**	**-0.83 (0.26)**	**0.002**
iFGF23 (pg/mL)	-	-	**0.74 (0.22)**	**0.001**
Hepcidin (ng/mL)	-	-	**-0.001 (0.001)**	**0.007**
iFGF23 (pg/mL)				
Acalcium (mmol/L)	**157.5 (50.0)**	**0.002**	-	-
cFGF23 (pg/mL)	**0.10 (0.05)**	**0.034**	**0.17 (0.05)**	**0.001**
1,25$(OH)_2$D (pmol/L)	**-0.223 (0.091)**	**0.017**	**-0.319 (0.099)**	**0.002**

[a]β-coefficients and associated p-values from multivariate regression analysis; Dependent variables are indicted in bold in grey bars; Significant ($p<0.05$) predictors are indicted in bold. Supplementation effect is significant within each eGFR group.

Discussion

In this post-hoc analyses of a 12-month double-blind RCT with vitamin D in older people, we showed that at baseline plasma concentration of cFGF23, iFGF23, PTH, SOST and TNFα were higher and Klotho, 1,25$(OH)_2$D and iron lower in the group with CKD G3a/b compared to the group with normal renal function. After supplementation, only 25(OH)D, cFGF23 and IL6 differed between groups. The response to supplementation differed by eGFR category; a significant decrease in TNFα and increase in iron was only found in the group with CKD G3a/b. In the group with normal renal function, plasma phosphate decreased and cFGF23, iFGF23 and PINP increased. A significant increase in 25(OH)D and IL10 and a decrease in CTX was found in both groups. In univariate regression analyses, both cFGF23 and iFGF23 were predicted by renal function and regulators of calcium and phosphate metabolism. IL6 significantly predicted iFGF23 at baseline and cFGF23 after supplementation, which did not remain significant in multivariate models. Post-supplementation hepcidin predicted cFGF23 in a multivariate model.

The alterations in regulators of calcium, phosphate and vitamin D metabolism found at baseline in the group with predominantly CKDG3a are consistent with changes reported in more advanced stages of CKD[153,154,384]. The pro-inflammatory cytokines TNFα and IL6 were

significantly or tended to be higher in the group with renal impairment, indicating a state of higher chronic inflammation. The majority of participants (86%), regardless renal function, had CRP values within the International Federation of Clinical Chemistry reference range (<47.6 nmol/L)[408], indicating absence of acute infection and inflammation. Our findings also suggest that a decline in iron status occurs from early renal impairment, although this was not accompanied by higher hepcidin concentrations. This may potentially only occur when iron falls below the threshold of deficiency (5.8 µmol/L as per Cobas, Roche Diagnostics kit insert), observed in very few study participants. Recent studies suggest that iron deficiency and systemic inflammation upregulate FGF23 transcription[409–411] and cleavage into C-terminal FGF23, leading to a mild increase in iFGF23 but a significant increase in cFGF23[205–207,412]. Also in our study, cFGF23 was proportionally higher in the CKDG3a compared to the CKDG1 groups (iFGF3 to cFGF23 ratio 0.72 and 0.98, respectively; p< 0.01). One of the suggested mechanisms involves a reduction of N-acetylgalactosaminyltransferase 3 (GALNT3) which inhibits proteolysis of FGF23 through glycosylating its cleavage site. This potentially modulates sensitivity to plasma phosphate[413]. In return, iFGF23 stimulates renal and hepatic inflammation[411,414] , possibly resulting a self-reinforcing loop, since an increase in pro-inflammatory cytokines, such as IL-6 and TNFα, upregulate hepcidin and suppress erythropoiesis[415,416]. Together with a hepcidin mediated increase in iron retention in enterocytes and macrophages this leads to a reduction in circulating iron concentrations[416–418].

Our data indicate that the effect of vitamin D supplementation on iron and inflammation status may be influenced by renal function. Supplementation significantly increased plasma iron and decreased TNFα concentrations only in the group with renal impairment, to values comparable to those in the group with normal renal function. IL10 increased in both groups. These results are similar to the reported effects of vitamin D supplementation and/or VDR activation in *in-vitro* models on pro-inflammatory (including TNFα and IL6) and anti-inflammatory cytokines (IL10), mostly in conditions with increased inflammation[419–425]. It has been suggested that an increase in iron status after vitamin D supplementation is mediated through a decline in IL6 and TNFα and an increase in the anti-inflammatory cytokine IL10[209,222,225,268,426,427], possibly explaining that an increase in iron status was only seen in the group with renal impairment. Although a reduction in hepcidin may be expected to be

observed simultaneously and has been observed after a bolus of vitamin D[426,428–431] in our study hepcidin did not significantly change.

Only in the group with normal renal function, the increase in iFGF23 and cFGF23 was significant, but the data distribution in group with renal impairment was wide, limiting the statistical power to detect a change. A corresponding decrease in plasma phosphate was only found in the group with normal renal function. It may be speculated this was the result of differences in iFGF23 sensitivity due to lower klotho expression and iron status in the group with renal impairment. The mechanisms of the increase in FGF23 with vitamin D supplementation remains to be elucidated but has been reported before by our group and others[214,227,342]. It may be a response to increased intestinal phosphate absorption and plasma 25(OH)D. In addition, it may be a compensatory mechanism to maintain phosphate homeostasis when iPTH declines in response to vitamin D supplementation[214,227,342].

Vitamin D supplementation did not substantially change the predictors of cFGF23 and iFGF23. Both cFGF23 and iFGF23 were predicted by each other, by renal function and regulators of calcium and phosphate metabolism; for cFGF23 these were PTH and $1,25(OH)_2D$ and for iFGF23, $1,25(OH)_2D$, klotho and albumin adjusted calcium. Associations with plasma IL6 were significant for cFGF23 (12 month) and iFGF23 (at baseline) and there were tendencies ($p<0.1$) of significant associations with CRP and markers of iron status. In a multivariate model, hepcidin significantly predicted cFGF23 at 12 months, independent of eGFR and iFGF23. These data provide limited evidence that in a small cohort of people with normal and early renal impairment, inflammatory factors and iron status are determinants of plasma iFGF23 and cFGF23 [206,207,208,209] although these variables explained a minor part of variance.

Whether renal function, FGF23, inflammation and iron status have independent effects on the renal-bone axis and the response to vitamin D supplementation needs further detailed investigation. Many of differences between eGFR categories in regulators of the renal bone axis (PTH, iFGF23, Klotho; $1,25(OH)_2D$, SOST), inflammation (TNFα) and plasma iron found at baseline and were no longer significant after supplementation, although eGFR did not significantly change. Together these data may indicate that vitamin D supplementation may partly abate the effects of renal impairment. However, although the bone resorption marker CTX decreased in both groups, an increase in the bone formation marker PINP was only found in the group with normal renal function. As nett bone mass depends on the balance between

bone formation and resorption, this potentially indicates a more limited response in those with impaired renal function.

This study has several limitations. The VDOP study included relatively healthy older adults and excluded those with an eGFR <30 ml/min/1.73m^2 (CKD stage 4 and 5) at screening. Albuminuria was not considered. In addition, since participants had no prior diagnosis of renal disease, the underlying causes of impaired renal function are unknown and may be expected to be heterogeneous. As a result, the bone phenotype may also be of a heterogeneous nature. Also, the creatinine analyses was conducted using the Roche kinetic-Jaffe method. The disadvantage of this method is its low specificity due to interfering substances which introduces a potential limitation to our creatinine measurements. We did not measure other markers of iron status or metabolism e.g. ferritin, transferrin or total iron-binding capacity, due to limitations in available sample types. Power for statistical analyses was limited due to the small group sizes and wide, non-normal distribution of the data. We did not collect data allowing the assessment of dietary iron intake. Therefore, it cannot excluded that group differences in iron status were explained by nutrient intake rather altered iron homeostasis or can we distinguish between absolute and functional low iron supply[201].

In this community dwelling cohort of older people considered to be generally healthy, a substantial proportion of participants had undetected renal impairment. This study showed that early renal impairment (CKDG3a/b) has a negative impact on PTH, FGF23, vitamin D and iron status, inflammation and bone metabolism markers. The effect of vitamin D supplementation depended on renal function and after supplementation few differences between the group with impaired and normal renal function remained. However, our data suggest an altered response in FGF23 and bone metabolism to vitamin D supplementation with renal impairment. Predictors of cFGF23 and iFGF23 were eGFR and regulators of calcium/phosphate metabolism.

This study identified changes in the renal bone-axis which occur before patients are generally clinically monitored. Vitamin D supplementation may partly abate the effects of renal impairment. Diagnosis in the early stages of renal impairment may provide opportunities for the prevention and progression of renal disease and CKD-MBD and other complications.

CHAPTER 6: General discussion

Methodology overview

The research described in this dissertation is composed of firstly, a systemic review and meta-analysis of recent RCTs with different forms of vitamin D in CKD patients with a focus on CKD-MBD related outcomes, accompanied by an overview of existing guidelines of vitamin D intake for CKD patients. Secondly, a series of studies designed to provide novel insights into the crosstalk of the kidney and bone -the renal and bone axis- and the effects of vitamin D supplementation. These studies utilised samples and data from a dose-ranging (12,000; 24,000 and 48,000 IU vitamin D_3 per month for 1 year) RCT study (the VDOP study) with relatively healthy older men and women (n=379; >70 years old) in UK. We investigated the response to vitamin D supplementation without (Chapter 3) and with consideration of renal function (Chapter 4 and 5). The analysis included markers of: (a) vitamin D metabolism, (b) kidney function, (c) bone turnover, (d) Wnt signalling, and in subgroups selected on basis of renal function, (e) iron and (f) inflammation (Chapter 5). Also, a series of regression analyses was performed to investigate relationship of 25(OH)D, eGFR and FGF23 with the different markers as mentioned above.

Discussion of findings

Vitamin D supplementation guidelines for CKD patients and recent RCTs

Aims of this part of the research were:

I. To provide a comprehensive review of guidelines for adult, pre-dialysis renal patients for the management of vitamin D status and SHPT.
II. To conduct a systematic review of recent RCTs with different forms of vitamin D in CKD patients with a focus on CKD-MBD related outcomes and a meta-analyses of the effectiveness of supplementation on plasma PTH concentrations.

Systematic review and meta-analysis

Major gaps remain in the evidence base for the management of vitamin D status in relation to CKD and CKD–MBD. Our systematic review and meta-analysis showed that the effect of supplementation with the parent molecule, vitamin D on PTH concentrations was inconsistent between the 22 studies included and meta-analysis showed a tendency (p=0.08) but non-significant, significant reduction in PTH. Three other studies using calcidiol, the 25 hydroxylated form of vitamin D, showed a consistent, reducing effect on PTH concentrations. Treatment with calcitriol and paricalcitol also led to a consistent and greater suppression of PTH, but increased the risk of hypercalcaemia. In addition, treatment with vitamin D analogues and calcitriol showed an increase in FGF23, in contrast with vitamin D or 25(OH)D treatments. The increase in FGF23 with analogue administration requires further attention as this hormone is already elevated in CKD patients and is a predictor of vascular calcification and CVD. This may indicate an undesirable side effect of administration of these forms of vitamin D. Limited data are available on the effect of vitamin D treatment on markers of bone metabolism and the variations in the range of reported markers prevented direct comparisons.

A summary of the latest guidelines is summarized in **Figure 15.** Guidelines depend on the stage of CKD. In the first stages (G1-G3a), general population recommendations for the prevention of vitamin D deficiency are followed. For more advanced stages, monitoring of 25(OH)D and PTH is recommended and correction of deficiency as required. For the correction of deficiency, general or CKD specific patient guidelines provide recommendations. In more advanced stages of CKD, treatment with Vitamin D analogues or $1,25(OH)_2D$ is recommended as an alternative to Vitamin D_2 and D_3 only when PTH is persistently high and progressively over the upper limit of the assay.

<u>The effect of vitamin D supplementation</u>

Aims of this part of the research were:

III. To investigate changes in regulators and markers of bone metabolism, BMD and BMC in response to different dosages of vitamin D supplementation in older people for 12

months. Further, we investigated their associations with total 25(OH)D and free 25(OH)D at baseline and after 12 months of supplementation.

Bone metabolism and Wnt-signalling pathway markers

- *Full cohort analysis (VDOP)*

The main findings of the investigation of the response of markers of bone metabolism, BMD and BMC in response to different dosages of vitamin D supplementation and their relationship with total 25(OH)D and free 25(OH)D are summarised in this section.

Vitamin D supplementation dose-dependently increased total and free 25(OH)D concentrations and decreased plasma phosphate and PTH in all supplementation groups (all $p<0.05$). The PINP:CTX ratio, cFGF23 and iFGF23 significantly increased with no between-group differences. Klotho was unchanged. Plasma $1,25(OH)_2D$ and PINP significantly increased in the highest dose groups (24,000IU; 48,000IU). SOST, OPG, RANKL, BMD, BMC and CTX remained unchanged. In subgroup analyses restricted to participants deficient (25(OH)D <25 nmol/L) at baseline, findings were similar. There were no significant changes in BMD, BMC and CTX. Although an increase in PINP was not seen in this subgroup, the PINP:CTX ratio increased.

Before supplementation, in all groups plasma concentrations of both total and free 25(OH)D were associated with cFGF23 and PTH but not any of the markers of Wnt-signalling or bone metabolism, except for BAP. Both free and total 25(OH)D were positively associated with BMD and BMC of both sites at baseline. After supplementation, total and free 25(OH)D was positively associated with DBP and negatively with adjusted calcium and eGFR. The negative association with PTH and positive association with SOST were only significant for free 25(OH)D after supplementation. There were no significant associations with other markers of Wnt signalling and bone metabolism. The relationships with BMD and BMC were no longer found after supplementation.

The expected dose-dependent increase in total and free 25(OH)D and $24,25(OH)_2D$ with vitamin D supplementation was confirmed. This was accompanied by a dose-dependent decrease in PTH, as previously reported in generally heathy people[352–356]. We also showed an

increase in 1,25(OH)$_2$D concentrations in the 24 and 48,000IU/m groups, despite that only 28% of participants had baseline values of 25(OH)D below the concentration of <25nmol/L usually considered as limiting factor for 1,25(OH)$_2$D production[357].

We found a significant increase in cFGF23 and iFGF23 with vitamin D supplementation, some individuals exceeding the normal ranges of cFGF23 and iFGF23. Increase of FGF23 after vitamin D supplementation was also reported in a recent meta-analysis[359]. This may be partly mediated by the increase in 1,25(OH)$_2$D observed in our study. There is a reciprocal regulation of FGF23 and 1,25(OH)$_2$D[357]; 1,25(OH)$_2$D stimulates the expression of FGF23 and Klotho[357,360–362] and in reverse, Klotho has been shown to stimulate 25(OH)D activation in the kidney[31]. FGF23 however stimulates the expression of CYP24A1, resulting to the catabolism of 1,25(OH)$_2$D and 25(OH)D[30,35], at the same time inhibiting CYP27B1 expression and thus 1,25(OH)$_2$D production[357]. It has been reported before that FGF23 can inhibit PTH synthesis through a 1,25(OH)$_2$D mediated mechanism[357].

The increase in FGF23 may be secondary effect of an increase in intestinal calcium and phosphate absorption[363] as mediated by the increase in 1,25(OH)$_2$D. Thus, this might reflect a compensatory response of FGF23 to maintain phosphate homeostasis by increasing urinary phosphate excretion[30,107,357]. Accordingly, we found a decrease in plasma phosphate after supplementation. Also, PTH has a phosphaturic effect; therefore, the increase in FGF23 may also be a response to the observed decrease in PTH. The increase in FGF23 after vitamin D supplementation requires further understanding due to its negative associations with bone and cardiovascular health.

Published data on the effect of vitamin D supplementation on RANKL and OPG are conflicting[369]. My study did not confirm an anabolic effect of vitamin D supplementation on components of the Wnt-signalling pathway as reported elsewhere[367,368]. In the VDOP study the regulators SOST, OPG and sRANKL remained unchanged. Although no pronounced effects Wnt-signalling markers were found, there was an increase in the formation marker PINP in the two highest dose groups and the PINP: CTX ratio increased in all groups. This may indicate that the balance of bone formation and resorption may have changed with supplementation, consistent with other published findings[370,371].

Vitamin D supplementation may increase bone mineralization[22] and therefore BMD and BMC by increasing the bio-availability of calcium and phosphate[79]. This may be independent of potential effects of increased vitamin D status on alterations of bone cell differentiation and function, particularly when unmineralised bone matrix was present at baseline. The effects of supplementation may therefore have depended on vitamin D deficiency at baseline. However, we showed no interaction between the presence or absence of baseline vitamin D deficiency and change in BMD of the hip and femoral neck (also presented in the VDOP primary paper)[343], markers of Wnt-signalling and bone metabolism, except for PINP. However, our study was not powered for this subgroup analyses.

Regression analyses showed similar patterns of associations of total and free 25(OH)D concentrations with markers of calcium and bone metabolism. After supplementation, most of the associations disappeared and PTH and SOST were significantly associated only with free 25(OH)D.

The positive associations of BMD and BMC with both and total and free 25(OH)D at baseline, are consistent with findings in other cross-sectional studies[344]. Our findings that showed a lack of an effect of supplementation seems to be contra-dictionary to these findings. However, in unsupplemented individuals, 25(OH)D likely reflects a wider range of factors influencing both vitamin D status and BMD and BMC, such as time spent outdoors, physical activity or body composition[372,373]. The associations between 25(OH)D and BMD and BMC were no longer significant after supplementation. The change in these associations with supplementation suggests a threshold effect of total and free 25(OH)D. More specifically this might indicate that there is no additional effect of vitamin D supplementation after sufficiency is achieved. In addition, after supplementation, vitamin D status will predominantly have been determined by oral intake and as such may override the effect of before mentioned life-style factors on 25(OH)D. Although BMD, BMC and BAP at baseline were associated with 25(OH)D this was not reflected on the Wnt signalling markers. No significant associations were found with any of the measured markers of the Wnt signalling pathway.

ANCOVA analyses showed that renal function was significant determinant of the response to vitamin D supplementation[356]; we showed that eGFR was a significant (positive) predictor of post-supplementation concentrations of total and free 25(OH)D (and a negative predictor

of FGF23). The interaction of eGFR with post-supplementation 25(OH)D may reflect increased catabolism and impaired dose-response associated with a decline in renal function[13]. The interaction with cFGF23 may also be explained by the importance of renal function in the catabolism and urinary excretion of FGF23 fragments[97]. Estimated GFR was not a significant covariate (ANCOVA analysis) for values of markers of Wnt signalling, bone metabolism and BMD and BMC post supplementation. However, further post-hoc analyses with data categorized on the basis of eGFR detected differences between individuals with normal and early CKD, partially at baseline. This is presented in the next section.

<u>The effect of early renal impairment</u>

Aims of this part of the research were:

IV. To identify differences in markers of vitamin D metabolism, bone turnover and Wnt-signalling between adults categorised on the basis of kidney function. Differences were investigated before after 12 months of vitamin D supplementation.

V. To investigate the differences in the response to vitamin D supplementation by category of CKD.

Bone metabolism, Wnt-signalling and the response to vitamin D supplementation

- *Full VDOP population categorized on basis of eGFR: ≥60 and <60 ml/min/1.73m^2*

To investigate influence of renal function on vitamin D metabolism, bone turnover and Wnt-signalling markers, we investigated differences between subgroups categorised on the basis of their kidney function.

In this cohort of relatively healthy older adults without a prior diagnosis of renal disease we found that dependent on the algorithm for eGFR used, 18 and 28% of the study population had an eGFR <60 ml/min/1.73m^2 (CKD G3a) and 3 and 5% <45 ml/min/1.73m^2 (CKD G3b) calculated with MDRD-4 and CG respectively. The choice of algorithms therefore introduced variation in categorization of patients and thus is expected to influence any subsequent data analyses. The percentage of participants, using MDRD-4 data as a reference is presented in **Figure *22***. These algorithms were developed based on populations with different

characteristics (i.e. generally healthy, renal patients), and the variables included in each algorithm vary **Figure *22***.

Analyses of VDOP data, comparing subgroups divided on basis of renal function (≥60 versus <60 ml/min/1.73m^2) showed that a moderate decline in eGFR has a negative impact on vitamin D metabolism, Wnt-signalling and bone turnover markers. Also the response to supplementation depended on renal function, but vitamin D supplementation had beneficial effects on markers of the renal-bone axis in older people with both normal and impaired renal function. We explored these differences with data categorised on basis of 5 different algorithms for calculation of eGFR. Differences these algorithms are discussed at the end of this section. Here data are described for MDRD-4 and CKD-EPI unless otherwise stated.

Secondary hyperparathyroidism is a known complication of advanced stages of CKD and an increase in iPTH is usually reported from stage 3 or 4[214,385]. However, we found significantly higher iPTH concentrations already with moderate renal impairment. A reduction in plasma 1,25(OH)$_2$D is reported from early renal impairment and continues to decrease with declining renal function[98,387]. The changes in plasma 1,25(OH)$_2$D concentrations with CKD are hypothesised to be the result of the combined effect of a reduced renal hydroxylation capacity and increased catabolism induced by FGF23. This study confirms that eGFR <60 ml/min/1.73m^2 is related to higher iPTH and FGF23 and lower 1,25(OH)$_2$D with compared to people with eGFR ≥60 ml/min/1.73m^2. At baseline, also plasma 25(OH)D concentrations were significantly lower with MDRD-4 eGFR <60 ml/min/1.73m^2 (41.3 vs 34.3 nmol/L) and may have contributed to the relatively high PTH values.

FGF23 and SOST are both produced by osteocytes and regulate renal mineral and vitamin D metabolism and influence bone formation and resorption[63]. We reported that SOST concentrations were higher and DKK1 lower with a moderately lower eGFR, confirming similar findings reported by Sabbagh et al. 2012[392]. Reference ranges of plasma concentrations of these regulators of Wnt-signalling in healthy and CKD patients are not well defined. However, plasma concentrations of SOST and OPG were above and RANKL below the reference ranges reported for the kits used, in the group with CKD G3a-b (normal range SOST: 13.31-41.77pmol/L; OPG median: 1.8pmol/L; sRANKL: 0.37-0.46 pmol/L). The exact clinical relevance of these findings it is not established yet. However, in combination with the changes in FGF23, iPTH and 1,25(OH)$_2$D it may be anticipated that these changes with early CKD are

associated with negative effects on mineral and bone metabolism[134,388,390,391]. Such early changes in renal function are usually not detected or give rise to clinical monitoring but may signify a stage during which early intervention may offer health benefits.

The responses in biomarkers to vitamin D supplementation were dependent on the eGFR category. As expected, plasma total and free 25(OH)D and 24,25$(OH)_2$D increased with supplementation. The analysis in the full cohort showed an increase in 1,25$(OH)_2$D[343], similar to findings in other RCTs[270,296]. However, when dividing the population based on eGFR (≥60 and <60 ml/min/1.73m^2) this increase was only observed in the group with an eGFR ≥60 ml/min/1.73m^2. This illustrates the reduced 1 α-hydroxylation capacity and/or increased catabolism with lower renal function[167], which resulted in lower plasma 1,25$(OH)_2$D concentrations, despite increased availability of 25(OH)D after supplementation for hydroxylation.

Plasma iPTH decreased with supplementation in both eGFR groups. Vitamin D supplementation is associated with a decrease in iPTH[13], although this may only be observed at lower baseline 25(OH)D concentrations. However, reports on the effect of vitamin D supplementation on iPTH in patients with CKD and SHPT are conflicting as mentioned in our systematic review[214]. This is partly explained by the fact that the aetiology of SHPT with impaired renal function is complex and multifactorial. It is unclear at what stage of CKD and/or SHPT, vitamin D supplementation is no longer effective in reducing iPTH[98,214].

Our data suggest that in early CKD the response of iFGF23 and Klotho differed by eGFR category, while plasma cFGF23 increased and plasma phosphate decreased regardless of the eGFR. An increase in cFGF23 and iFGF23 with vitamin D supplementation was reported before[342,343] and may be a response to increased intestinal phosphate absorption and a decline in iPTH. In this study, iFGF23 only significantly increased in the group with eGFR ≥60 ml/min/1.73m^2, while Klotho remained unaltered. On the other hand, in the group with eGFR <60 ml/min/1.73m^2 Klotho increased and iFGF23 remained unchanged with supplementation. This might indicate an increase in FGF23 sensitivity as result of an increase in its co-factor Klotho and thus a potential benefit of supplementation in people with compromised kidney function. There is limited evidence that vitamin D supplementation may increase the expression of Klotho, and data are conflicting[31,362]. Our findings thus suggest that

the effect may depend on renal function. Other factors, including iron status and inflammation may also play a role and were subject of investigation of Chapter 5.

Effect of early renal impairment on the response to vitamin D supplementation

- *Full VDOP population categorized on basis of eGFR: ≥60 and <60 ml/min/1.73m^2*

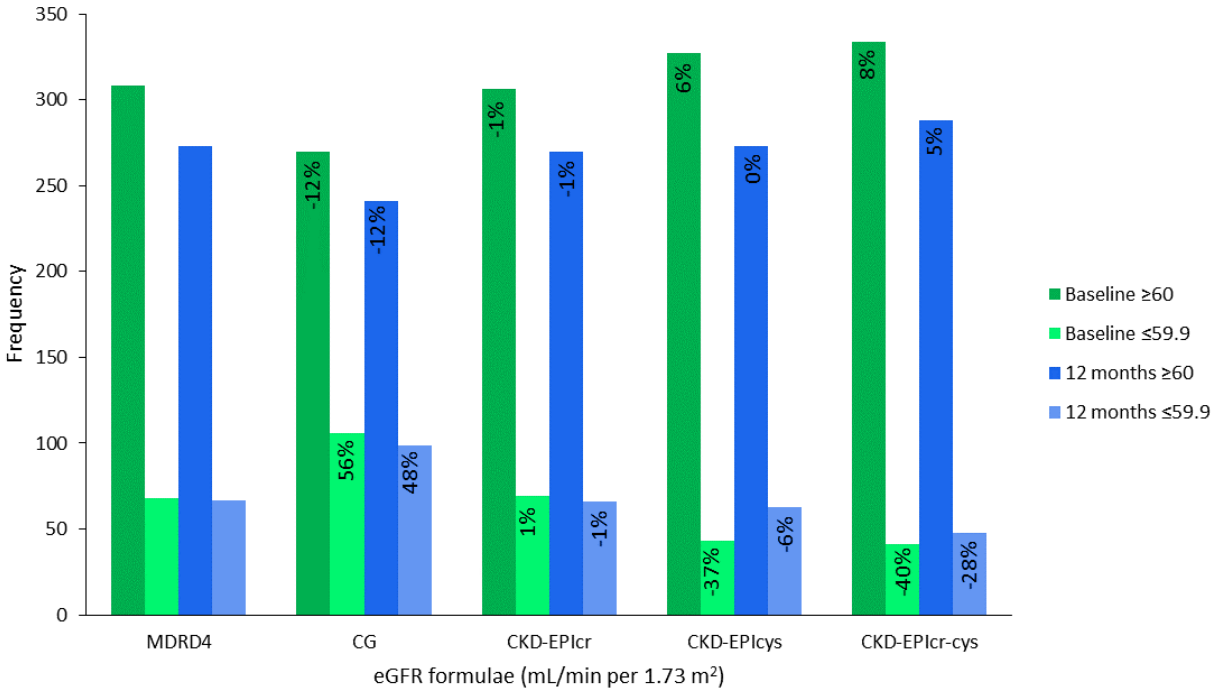
Vitamin D may modulate SOST expression in osteocytes[368,393]. Recent findings show an inverse correlation between vitamin D status and SOST concentrations in healthy postmenopausal women and adults[343,367,394]. However, conflicting results have been reported regarding the effect of vitamin D supplementation on SOST and DKK1[393,395,396]. In our study, SOST, OPG and RANKL concentrations remained unchanged with supplementation, irrespective of eGFR while DKK1 increased, suggesting limited effects on the Wnt-signalling pathway. The bone turnover markers however indicate that supplementation altered the rate bone remodelling. A decrease in plasma CTX was found in both eGFR groups after supplementation, while BAP and PINP increased only in the group with higher renal function. No effects were found on bone mineral density and content as measured by DXA in either eGFR group. Micro-architectural changes and fracture risk changes might have not been reflected in DXA measurements. Future research with pQCT measures of bone integrity is required to investigate such effects of vitamin D. The relatively short duration of the study and/or the sensitivity of DXA to detect changes and the limited numbers in each eGFR subgroups may also have limited the power to detect potential changes.

The use of different eGFR algorithms provided, as expected somewhat different results. Although the MDRD-4 and CKD-EPI algorithms, as based on either creatinine or cystatin C provided similar results, based on CG differed. CG is the only algorithm tested that includes weight. This might explain the differences in bone density detected between the eGFR categories when using the CG algorithm. In addition, the higher number of participants categorised to the eGFR <60 ml/min/1.73m^2 group (baseline n=106; 12 months n=99) with CG may have influenced the statistical power to detect these differences. In addition, the CG algorithm tends to underestimate eGFR compared to MDRD-4 and CKD-EPI influencing the characteristics of participants in respective groups (**Table *13***).

In clinical practice, kidney function is routinely assessed in older adults[399] and mostly based on the calculation of eGFR based on serum creatinine or more recently, cystatin C[380,400]. The

use of serum creatinine in older adults may lead to an overestimation of the GFR[232,401], due to the influenced of muscle mass and dietary protein intake on creatinine plasma concentrations. Both of these factors are known to decrease with age[402] and this can lead to misclassification of patients[400]. On the other hand, cystatin C is produced at a constant rate by all nucleated cells and filtered in proximal tubules[230]. Recent studies have suggested that cystatin C may be a better marker for progression of CKD[403–405], since it is less affected by exogenous factors[402]. In this study, relationships between cystatin C and creatinine based eGFR with the investigated markers were comparable. Thus, our findings do not provide evidence of pronounced differences in correlation with the biomarkers. That might be due to nature of the study population used for this analysis.

Figure 22. Re-classification of the VDOP population with the use of different algorithms at baseline and 12 months data



The numbers on the bars indicated the percentage of population re-classification compared to MDRD-4.

Iron and Inflammation markers, bone metabolism, Wnt-signalling and the response to vitamin D supplementation

- *Subgroup analysis on basis of eGFR: ≥60 and <60 ml/min/1.73m^2*

In this part of my research, I investigated markers of inflammation, iron status and regulators of Wnt-signalling and bone metabolism and their associations with FGF23 in in subgroups with impaired and normal renal function (eGFR <60 ml/min/1.73m^2 (CKD stage G3a and G3b) and eGFR >90 ml/min/1.73m^2 and CKD stage G1; normal renal function) and their response to vitamin D supplementation.

In this further subgroup analysis with study participants selected on the basis of eGFR (CKD 3a/ b and CKD G1), we found, similar to the findings in the full cohort, negative impacts of early CKD (CKD3a/b) on PTH, FGF23 and vitamin D metabolites. We also found differences in iron status, inflammation and bone metabolism markers.

The inflammation markers TNFα and IL6 were or tended to be elevated in the people with CKD G3, suggesting a state of higher chronic inflammation. Absence of acute inflammation or infection was confirmed in most of the participants since CRP concentrations were within the reference range[408]. Our findings also suggest a decline in iron starts from early renal impairment. Literature shows that iron deficiency and systemic inflammation can upregulate FGF23 transcription[409–411] leading to significant increase of cFGF23 and mild increase of iFGF23[205–207,412]. One of the suggested mechanisms involves decrease in GALNT3 which inhibits proteolysis of FGF23 through glycosylating its cleavage site[413]. In return, iFGF23 stimulates renal and hepatic inflammation[411,414], possibly resulting a self-reinforcing loop, since an increase in pro-inflammatory cytokines, such as IL-6 and TNFα, upregulate hepcidin and suppress erythropoiesis[415,416]. Together with a hepcidin mediated increase in iron retention in enterocytes and macrophages this leads to a reduction in circulating iron concentrations[416–418].

After supplementation many of the differences between the people with G1 and G3a/b disappeared. However, in this smaller data set, the response of FGF23 and bone metabolism to vitamin D supplementation was dependent on kidney function.

Supplementation significantly increased plasma iron and decreased TNFα concentrations only in the group with renal impairment, to values comparable to those in the group with normal

renal function. Plasma IL10 increased regardless of kidney function. These results confirmed findings of other human studies using different forms of vitamin D supplementation findings of *in-vitro* models on pro-inflammatory (including TNFα and IL6) and anti-inflammatory cytokines (IL10), mostly in conditions with increased inflammation[419–425]. It has been suggested that the increase in plasma iron after vitamin D supplementation may be mediated through a decline in IL6 and TNFα and increase in the anti-inflammatory cytokine IL10[209,222,225,268,426,427], possibly explaining that the observed increase in iron status in my study was only seen in the group with renal impairment. In this study we did not observe an increase of hepcidin after vitamin D supplementation as reported in other studies [426,428–431].

Also this secondary analysis suggest that changes in the renal bone-axis and regulatory factors occur before patients are generally clinically monitored. Also the findings of this sub-study suggest that vitamin D supplementation may partly abate the effects of renal impairment.

Predictors of cFGF23 and iFGF23

Subgroup analysis (pooled data)

To investigate whether iron status and inflammation were predictors of cFGF23 and iFGF23, I conducted a series of regression analyses before and after supplementation. cFGF23 was predicted by iFGF23, renal functions PTH and $1,25(OH)_2D$. iFGF23 was predicted by cFGF23, renal function, $1,25(OH)_2D$, klotho and albumin adjusted calcium.

Associations with plasma IL6 were significant for cFGF23 (12 month) and iFGF23 (at baseline) and there were tendencies ($p<0.1$) of significant associations with CRP and markers of iron status. In a multivariate model, hepcidin significantly predicted cFGF23 at 12 months, independent of eGFR and iFGF23. Vitamin D supplementation did thus not substantially change the predictors of cFGF23 and iFGF23. These data provide limited evidence that inflammatory factors and iron status are determinants of plasma iFGF23 and cFGF23[206–209] although these variables explained a minor part of variance.

Limitations

Systematic review and meta-analysis

Studies included in the systematic review varied in design and form of vitamin D supplementation. Also, there was a high degree of heterogeneity regarding duration, dose, and population characteristics. Therefore, the statistical power of the meta-analysis was limited by a high degree of heterogeneity. Our systemic review and meta-analyses demonstrated Major gaps remain in the evidence base for the management of vitamin D status in relation to characteristics of CKD–MBD, i.e. SHPT, altered bone metabolism, bone density and integrity and fracture risk.

The VDOP study design

This study has several limitations. The absence of placebo group did not allow to account for changes unrelated to the intervention (e.g. effect of ageing or secular trends). The VDOP study was not powered for subgroup analyses by baseline vitamin D status or eGFR. The length of supplementation may have been too short to detect significant changes in BMD and BMC as measured by DXA. We however, did also not observe the anticipated 0.6% decrease in BMD (the average annual change in BMD in this age group[344,345]), even though the study was powered to detect a change in hip BMD from baseline in each supplementation groups based on an earlier, similar study in the North of the UK[343,432]. This may be explained by differences in the study protocol or population (only female population, age-range, dosing frequency). pQCT measurement was not conducted to obtain measures of bone integrity and strength and changes in these parameters may have gone undetected. Markers of bone metabolism and osteocyte signalling may however be expected to respond to interventions more rapidly and within the length of a bone remodelling cycle (~ 3-4 months)[379]. It is possible that markers measured after 12 months reflect a newly achieved steady state that is seemingly no different from baseline and that changes occurred within the first few months after commencement of the intervention, such as observed in pharmaceutical trials[378,379].

Since the design of this study, evidence has been published that a monthly and other less frequent vitamin D dosing regimens may have different, less beneficial effects on musculoskeletal function and metabolism compared to more frequent dosing (daily/weekly).

The VDOP study included relatively healthy older adults and excluded those with an eGFR <30 ml/min/1.73m^2 and/or renal disease at screening. The eGFR categorisation was solely based on eGFR, without data regarding albuminuria, although this was checked at screening. The underlying cause of the impaired renal function are unknown in these participants and may be expected to be heterogenous. Even though CKD patients were excluded from the study, we found that a significant proportion of participants has an eGFR classified as CKD G3a/b (30-60 ml/min/1.73m^2). As a result, the bone phenotype may also be of a heterogenous nature. Participants of the VDOP study were predominantly of Caucasian origin and therefore results may not be generalizable to other populations.

Secondary analysis methodology

The response to supplementation may have depended on both renal function and baseline 25(OH)D. There was however insufficient statistical power to fully test this hypothesis and studies to address this question are required.

The subgroup analysis conducted in Chapter 4 includes a large number of comparisons conducted. This will have increased the chance of type 1 errors. Group sizes were unequal; the group with the lower eGFR had limited numbers compared to the group with the higher eGFR. This will have increased the chance of type 2 errors. Research specifically designed and powered to confirm our findings are required.

Also, for this secondary analysis we did not directly measure free 25(OH)D but instead calculated the free fraction. Although directly measured and calculated free 25(OH)D concentrations correlate well in healthy populations[369,374,375], it cannot be excluded that directly measured concentrations would have provided different findings.

Future research- suggested leads

The data presented in this PhD dissertation have shown that CKD stage 3 a/b (early stages of renal impairment) are associated with changes in markers of calcium and phosphate metabolism, bone metabolism, Wnt signalling, iron and inflammation compared to those with normal kidney function. Although a beneficial effect of vitamin D supplementation was

reported on some of these markers, the statistical power in the VDOP study may have limited the detection of potential differences or changes with supplementation due to the small group size of those with renal impairment. However, even with limited power we showed that supplementation improved vitamin D status and bone turnover markers, well known complications of CKD. This also applies to the small group size of the study of inflammation and iron status markers, presented in Chapter 5. Further research needs to be done in a larger population size with established kidney impairment in order to develop a better understanding of the changes of these markers and effect of supplementation. Also, a placebo group in this type of study would have enabled us to consider any changes occur during ageing for the duration of the supplementation and follow-up. The increase of FGF23 after supplementation, particularly in those with normal renal function, requires also further research to understand its potential properties when that increase occurs after vitamin D supplementation. An RCT study of CKD patients supplemented with vitamin D supplementation or placebo (for the duration of >12 months, to allow more time to observe changes on BMD and BMC markers) compared to healthy individuals will allow us to study further the changes in all the above biomarkers. Further studies are also needed in order to identify potential differences between the different types of vitamin D supplementation on the response of these biomarkers in CKD patients.

This study has shown that early CKD is associated with alterations in different vitamin D, Wnt signalling, bone turnover, inflammation and iron status markers in a stage that people are not yet clinically monitored. The series of studies have shown that considering renal function is important in the investigation of the response to vitamin D supplementation. A RCT designed specifically to investigate the effects in early CKD, with the aim to prevent CKD progression and the development of CKD-MBD.

The future application of this work applies mainly to clinical practise to promote healthy ageing. Vitamin D status and kidney function screening in older adults is really important to be included in routine screening as a preventative measure for CKD-MBD. Also, vitamin D supplementation shows to be a potential beneficial tool to partly abate the effects of renal impairment.

Conclusion

This study showed that vitamin D supplementation increases the concentrations of the vitamin D metabolites in plasma and decreases iPTH. Also, it increases intact and c-terminal FGF23 and PINP:CTX ratio. However, over 12 months no effect on bone mineral density and content and Wnt signalling markers were found. No additional benefit was found from the higher doses in calcium and renal function markers, Wnt signalling markers, bone turnover, iron status and inflammation markers of vitamin D supplementation (24,000 and 48,000 IU) compared to 12,000 IU. These indicate no further beneficial effects of higher dosages of vitamin D supplementation on most vitamin D metabolites, iPTH and bone turnover markers compared to the recommended amount of 400 IU/daily (equivalent of 12,000 IU/month) for the general population were found.

However, when eGFR is considered, we see differences in the markers of the renal-bone axis and their response to vitamin D supplementation. Firstly, the characteristics of the people with <60 ml/min/1.73m^2 are: lower vitamin D metabolites and Klotho and higher PTH and SOST compared to people with >60 ml/min/1.73m^2. In both these eGFR categories iPTH and CTX decreased in response to vitamin D supplementation and increase cFGF23and DKK1. However, only the group with eGFR >60 ml/min/1.73m^2 had increased of iFGF23, 1,25(OH)$_2$D, BAP and PINP. And only the group with eGFR <60 ml/min/1.73m^2 had and increased Klotho post-supplementation.

Moreover, early renal impairment (CKD G3a/b <60 ml/min/1.73m^2) has a negative impact on iron status and inflammation. After vitamin D supplementation plasma iron, IL10 increased and TNFα decreased in the group with early renal impairment. Also, predictors of cFGF23 and iFGF23 were eGFR and regulators of calcium and phosphate metabolism.

Overall, our data suggest an altered response in FGF23 and bone metabolism to vitamin D supplementation with renal impairment. The effect of vitamin D supplementation depended on renal function.

In conclusion, these outcomes highlight the potential benefit of vitamin D supplementation to people with compromised kidney functions even for the early stages of kidney decline. Diagnosis in the early stages of renal impairment may provide opportunities for the prevention and progression of renal disease and CKD-MBD and other complications.

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