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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

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ABSTRACT

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 estimated Glomerular Filtration Rate (eGFR) on plasma concentrations of vitamin D metabolites, Wnt signalling and bone metabolism in a dose ranging vitamin D_3 RCT (12,000 IU, 24,000 IU, 48,000 IU/month for 1 year; n = 379, >70 y) with a baseline eGFR > 30 mL/min/1.73 m^2. Participants were categorised on basis of eGFR (≥60 or mL/min/1.73 m^2) based on 5 commonly used algorithms for eGFR. Differences between eGFR categories were tested with ANCOVA. Before supplementation commenced, a lower eGFR was associated with significantly higher concentrations of c-terminal and intact Fibroblast Growth Factor-23 (cFGF23; iFGF23), intact Parathyroid Hormone (iPTH) and Sclerostin (SOST) and lower Klotho, 1,25-dihydroxy Vitamin D (1,25$(OH)_2$D) and Dickkopf-related Protein 1 (DKK1) concentrations. Differences between eGFR groups in 25-hydroxy Vitamin D (25(OH)D), 24,25-dihydroxy Vitamin D (24,25$(OH)_2$D) and iPTH were only detected with eGFR based on Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) and Modification in Diet for Renal Disease (MDRD-4) algorithms. Differences in Bone Mineral Density and Content (BMD; BMC) and bone turnover markers were detected only with Cockcroft-Gault (CG). Pre- and post- supplementation comparisons showed differences in the response to supplementation by eGFR group. Plasma 25(OH)D, 24,25$(OH)_2$D, 1,25$(OH)_2$D and DKK1 increased and iPTH and C-terminal telopeptide (CTX) decreased in both groups. Plasma iFGF23, bone specific alkaline phosphatase (BAP) and Procollagen 1 intact N-terminal Propeptide (PINP) increased and phosphate decreased only in the group with eGFR ≥ 60 mL/min/1.73 m^2. Findings were largely consistent across all eGFR algorithms. Post-supplementation, cFGF23, iFGF23, iPTH and SOST remained significantly higher in the lower eGFR group. Plasma 1,25$(OH)_2$D and Klotho did no longer differ between eGFR groups. This was found for all eGFR algorithms, with the exception of iPTH and iFGF23, which were not significantly different with eGFR based on CG. Differences in BMD and BMC were detected with CKD-EPI-creatinine and MDRD-4 but not GC. This study showed that even a moderate decline in eGFR is associated with alterations in vitamin D metabolism, Wnt signalling and bone turnover markers. Renal function influenced

Abbreviations: CKD, Chronic kidney disease; eGFR, estimated Glomerular Filtration Rate; CG, Cockroft-Gault; MDRD-4, Modification of Diet in Renal Disease- 4 variable; CKD-EPI, Chronic Kidney Disease Epidemiology Collaboration; CKD-MBD, Chronic Kidney Disease-mineral bone disorder; BMI, Body Mass Index; IU, International Unit; iFGF23, Intact-Fibroblast Growth Factor-23; cFGF23, c-terminal Fibroblast Growth Factor-23; 25OHD, 25-hydroxy Vitamin D/cholecalciferol; 1, 25$(OH)_2$D, 1,25-dihydroxy Vitamin D/calcitriol; 24, 25$(OH)_2$D, 24,25-dihydroxy Vitamin D; DBP, Vitamin D Binding Protein; iPTH, intact Parathyroid Hormone; PTHR1, PTH Receptor type 1; SHPT, Secondary Hyperparathyroidism; SOST, Sclerostin; DKK1, Dickkopf-related Protein 1; OPG, Osteoprotegerin; sRANKL, Soluble Receptor Activator of Nuclear Factor Kappa-B Ligand; BMD, Bone Mineral Density; FN BMD, Femoral Neck Bone Mineral Density; BMC, Bone Mineral Content; FN BMC, Femoral Neck Bone Mineral Content; BAP, Bone specific Alkaline Phosphatase; CTX, C-terminal Telopeptide; PINP, Procollagen 1 intact N-terminal Propeptide.

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the response to vitamin D supplementation. Supplementation increased Vitamin D metabolites 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/1.73 m^2. In conclusion, vitamin D supplementation had beneficial effects on markers of the renal-bone axis in older people with both normal and impaired renal function.

1. Introduction

Ageing is associated with a decline in renal function. This 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 glomerular filtration rate (GFR), changes in tubular reabsorption of components in the glomerular filtrate, urinary concentration and production of the kidney derived hormones [1–4]. The volume of kidneys and GFR are strongly correlated with age [5]; 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 [2,6]. Estimated GFR (eGFR) is the most commonly used measure of renal function in medical practice and values of < 60 mL/min/1.73 m^2 are defined as chronic kidney disease (CKD) [7].

CKD leads to abnormalities in bone and mineral metabolism and their regulatory hormones [8], resulting in chronic kidney disease-mineral bone disorder (CKD-MBD). Renal impairment causes a decline in calcitriol (1,25-dihydroxy vitamin D; $1,25(OH)_2D$) production by the kidney, phosphate and calcium retention and is associated with increased plasma concentrations of parathyroid hormone (PTH) and fibroblast growth factor-23 (FGF23) [9,10]. In addition, CKD is associated with progressive resistance to PTH due to the downregulation of the expression of PTH receptor type 1 (PTHR1) and downstream signals [11].

From the early stages of CKD, plasma FGF23 increases before an increase in plasma phosphate is detectable [12]. With the progression of CKD FGF23 continues to increase [13]. In parallel, the expression of Sclerostin (SOST) and receptor activator of nuclear factor kappa-B ligand (RANKL), both inhibitors of the Wnt/β-catenin signalling pathway increase, stimulating osteoclast activity [13]. This leads to a decrease of bone integrity, increased fracture risk and calcification of soft tissues [8], the hallmarks of CKD-MBD. Although the endocrine changes with advanced CKD-MBD are well-known, little is known about the effects of mild to moderate CKD. Limited evidence suggests that ensuring vitamin D sufficiency may have an anabolic effect on bone metabolism through the Wnt/β-catenin signalling pathway [14,15].

Vitamin D deficiency or insufficiency is highly prevalent in CKD patients and plays a role in the development of CKD-MBD; 70–80% of CKD patients are vitamin D insufficient (plasma 25(OH)D < 25–50 nmol/L [16,17]). Guidelines for renal patients may recommend higher target concentrations (>75 nmol/L) depending on the stage of CKD [18] and recommend vitamin D supplementation to prevent and correct secondary hyperparathyroidism and the complications of CKD-MBD [19].

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 [20–22]. 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 [16] but cystatin C based equations are recommended for the assessment of eGFR in those with CKD stage 3a (eGFR 45–59 mL/min/1.73 m^2) and no proteinuria [23]. 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 Australasians with Renal Impairment (CARI) guidelines). For the US, the latest guidelines recommend the removal of race from the CKD-EPI algorithm [24]. 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 [19,25–28].

In this study we investigated the influence of renal function on markers of calcium, phosphate, vitamin D and bone metabolism and Wnt signalling in a dose-ranging vitamin D supplementation study with apparently healthy older men and women. Differences between subgroups with an eGFR ≥ 60 mL/min/1.73 m^2 (representing CKDG1 and G2) versus eGFR 30–60 mL/min/1.73 m^2 (CKD3a and 3b; mild to moderately and moderately to severe impairment, respectively) [7] were investigated. Comparisons were made at baseline and after 1 year of vitamin D supplementation and within and between group responses to supplementation were analysed. Linearity of relationships was assessed by linear regression analysis with eGFR as a continuous variable. Results of 5 commonly used eGFR algorithms are reported.

2. Methods

2.1. Study design

This study is a secondary analyses utilising data and plasma samples collected as part of the vitamin D supplementation in older people (VDOP) randomized controlled trial [29] (ISRCTN35648481). In brief, this RCT included apparently healthy 379 adults aged ≥ 70 y (48% women) from the northeast of England. The study excluded those with an eGFR < 30 mL/min/1.73 m^2 (CKD stage >G4) 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 [29,30]. Additional methods used for this secondary analysis are provided below. The analyses and outcomes presented in this manuscript were not pre-specified in detail in VDOP analyses plan but were predefined in the study protocol for this secondary study. For the comparisons presented here, participant data were categorised based on their eGFR score (≥60 and <60 mL/min/1.73 m^2) according to 5 algorithms for eGFR.

Results for bone mineral density (BMD) and bone area (at hip and neck), plasma concentrations of cholecalciferol (25-hydroxy vitamin D; 25(OH)D), intact-parathyroid hormone (iPTH), albumin, calcium and creatinine were earlier reported by Aspray et al. [29] 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.

2.2. Measurements

Methods for measurements BMD, height and weight, collection of early morning fasting blood samples at baseline and after 12 months of

supplementation [29], as well as details of blood processing, storage and biochemical analyses were provided elsewhere [29].

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), vitamin D binding protein (DBP) (ImmunDiagnostik AG ELISA), iPTH (Immulite 2000, SIEMENS), PINP (UniQ, RIA), C-terminal telopeptide (CTX) (Immunodiagnostic Systems), bone specific alkaline phosphatase (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 [29]. 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 [29].

Measurements conducted at UEA included serum phosphate (Cobas, Roche Diagnostics), αKlotho (IBL international), plasma cFGF23 and iFGF23 (Immutopics), OPG (Biomedica), SOST, DKK1 and soluble RANKL (sRANKL) (Biomedica), plasma 24,25-dihydroxy vitamin D ($24,25(OH)_2D$) (LC-MS/MS) [31], 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 coefficient of variation (CV) $< 4\%$. The inter and intra-assay CV of all assays were $< 10\%$ except for $24,25(OH)_2D$, which was $< 15\%$. Assay performance was monitored using kit and in-house controls and following Good Laboratory Practice.

2.3. Derived variables

The estimated glomerular filtration rate (eGFR) was calculated using different algorithms [24]:

CKD-EPI Creatinine = 142 x min(Scr/κ, 1)α x max(Scr/κ, 1)− 1.200 × 0.9938^{Age} 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.

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.

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 [32]:

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

2.4. Statistical analysis

Pre- and post-supplementation data and the response to the intervention by supplementation group without considering eGFR was reported in detail before[33]. 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.

The statistical analyses included the following steps and comparisons.

The effect of supplementation on eGFR was assessed by ANCOVA analyses. No significant effect was found with any of the algorithms.

To test the influence of renal function on pre-defined markers, the following analyses were conducted:

Differences between eGFR categories at baseline and post-supplementation and the response to supplementation were tested by ANCOVA with an interaction term for eGFR category and time-point (i.e., pre- and post-intervention), the latter testing the difference in response between eGFR categories.

Supplementation dose and Body Mass Index (BMI) were considered as co-variates in, except for models with CG. Supplementation dose was removed from models if non-significant. Inclusion of BMI did not materially change the findings and were therefore removed from final models. Any variables incorporated in respective eGFR algorithms were a priori excluded as covariates (i.e., serum creatinine, cystatin C, gender, age, weight, BMI (CG only)).

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 (as above). For 12-month data, multivariate regression analyses showed no effect of vitamin D supplementation dose 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.

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 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.

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

Results of ANCOVA analyses with CKD-EPI with creatinine (CKD-EPIcr) and CG algorithms are presented in Table 2 and include p-values for between and within eGFR group differences and the eGFR x time point interaction term. Results for MDRD-4 were very similar to those for CKD-EPIcr and presented together with results for CKD-EPI cystatin C and CKD-EPI creatinine-cystatin C based algorithms in the supplementary materials. Data were presented as mean (SD) or median (IQR) for normally distributed and skewed data, respectively.

Results of regression analyses are presented as β-coefficients and associated p-value in Table 3 (CKD-EPIcr and CG) and Table 2 of supplementary materials (MDRD-4, CKD-EPI cystatin C and CKD-EPI creatinine-cystatin C).

For the statistical analysis of the data IBM® SPSS® Statistics Version

28 software was used.

3. Results

Baseline characteristics without consideration of eGFR are presented in Table 1. Baseline characteristics were well balanced between treatment groups and no significant differences were found. There were very few non-white (<1%) participants. Characteristics by eGFR categories are presented in Table 2 and Supplementary Table 1. Dependent on the algorithm used, 18% (CKD-EPIcr) 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 32.7–141.7 (CKD-EPIcr) and 33.5–145.2 (CG), respectively. There were differences between eGFR categories in age and BMI, dependent on the algorithm used.

Table 1
Participant's characteristics at baseline and after 12 months of vitamin D supplementation[a] [33].

	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)$^{\nabla}$*
CKD-EPI creatinine eGFR (mL/min per 1.73 m^2)	72 (13)	72 (13)
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]$^{\Delta 24,48}$

ANCOVA was 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.

Abbreviations: Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI creatinine); estimated Glomerular Filtration Rate (eGFR); Cockcroft-Gault (CG); Intact Fibroblast Growth Factor-23 (iFGF23); c-terminal Fibroblast Growth Factor-23 (cFGF23); 25-hydroxy Vitamin D (25(OH)D); 1,25-dihydroxy Vitamin D (1,25$(OH)_2$D); intact Parathyroid Hormone (iPTH); Sclerostin (SOST); Dickkopf-related Protein 1 (DKK1); osteoprotegerin (OPG); Soluble Receptor Activator of Nuclear Factor Kappa-B Ligand (sRANKL); Bone Mineral Density (BMD); Bone Mineral Content (BMC); Bone specific Alkaline Phosphatase (BAP); C-terminal Telopeptide (CTX); Procollagen 1 intact N-terminal Propeptide (PINP).

[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.

3.1. Post-supplementation characteristics and supplementation effect

The effect of supplementation on markers of bone, calcium and phosphate metabolism were reported previously [33]. Post supplementation data are presented pooled for all supplementation groups (Table 1). In summary, when eGFR was not considered there was a dose dependent increase of 25(OH)D, 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 ($p < 0.05$). Vitamin D supplementation had no effect on Klotho, SOST, OPG, sRANKL, hip and FN BMD, BMC and CTX (Table 1).

3.2. Differences in biomarkers between eGFR categories

Findings with CKD-EPIcr and MDRD-4 equations were comparable, with some exceptions. Results for MDRD-4, CKD-EPI cystatin C and CKD-EPI creatinine-cystatin C based algorithms are presented in Supplementary Table 1, respectively. Findings with CKD-EPIcr and CG (Table 2) are described in more detail below.

3.2.1. Markers of renal function and calcium and phosphate metabolism

At baseline, with eGFR categorised on basis of CKD-EPIcr, phosphate and Klotho were significantly lower and cFGF23 and iFGF23 were significantly higher when eGFR < 60 mL/min/1.73 m^2 (Table 2; Fig. 1). When categorised on basis of CG, similar results were found for Klotho, cFGF23 and iFGF23 (Table 2).

After 12 months of supplementation, when CKD-EPIcr was used, plasma phosphate was higher and cFGF23 and iFGF23 remained significantly higher in the group with eGFR < 60 mL/min/1.73 m^2 (Table 2; Fig. 1). Also based on CG, serum phosphate and cFGF23 were higher with eGFR < 60 mL/min/1.73 m^2 (Table 2). No between groups differences in Klotho were found, regardless of eGFR equation used.

3.2.2. Markers of vitamin D metabolism

Before supplementation, with eGFR calculated and categorised on basis of CKD-EPIcr, all vitamin D metabolites were significantly lower and iPTH significantly higher with eGFR < 60 mL/min/1.73 m^2 (Table 2; Fig. 1). When categorised on basis of CG only 1,25$(OH)_2$D differed significantly between eGFR groups (Table 2).

Post-supplementation, there were no differences between eGFR groups in vitamin D metabolites, but iPTH remained higher with eGFR < 60 mL/min/1.73 m^2 with CKD-EPIcr (Table 2; Fig. 1). Based on CG, total and free 25(OH)D were significantly higher with eGFR < 60 mL/min/1.73 m^2 (Table 2). DBP remained not significant regardless the eGFR equation used.

3.2.3. Wnt signalling pathway markers

At baseline, with eGFR categorised on basis CKD-EPIcr, SOST was significantly higher and DKK1 significantly lower with eGFR < 60 mL/min/1.73 m^2 (Table 2; Fig. 1). Group differences were numerically similar with GC, although not all reached significance (Table 2). There were no between-group differences for sRANKL regardless of equation used.

After supplementation, SOST remained higher in the group with eGFR < 60 mL/min/1.73 m^2 similar to findings at baseline (Table 2; Fig. 1). Based on CG, sRANKL was lower with eGFR < 60 mL/min/1.73 m^2 (Table 2).

3.2.4. Bone parameters and markers of bone metabolism

At baseline, no significant differences between eGFR categories in any of the bone markers and measures were found with CKD-EPIcr (Table 2). 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 CTX and PINP significantly higher compared to the group with ≥ 60 mL/min/1.73 m^2 (Table 2).

After 12 months of supplementation, with CKD-EPIcr, hip and FN

Table 2
Differences between eGFR categories (eGFR <60 mL/min/1.73 m^2 and eGFR ≥60 mL/min/1.73 m^2) at baseline and after 12 months of supplementation with eGFR calculated according to CKD-EPI creatinine and Cockcroft-Gault algorithms.

Characteristics	eGFR category[a] (mL/min per 1.73 m^2)	CKD-EPI creatinine			Cockcroft-Gault		
		Baseline	12 months		Baseline	12 months	
		N (%) or Mean (SD)	N (%) or Mean (SD)		N (%) or Mean (SD)	N (%) or Mean (SD)	
Male/Female	≥ 60	156/151	135/136		144/127	124/118	
	< 60	38/31	37/29		51/55	52/47	
N[a,b]	≥ 60	307 (82%)	-		271 (72%)	-	
	< 60	69 (18%)	-		106 (28%)	-	
eGFR (mL/min per 1.73 m^2)	≥ 60	76 (9)‡	77 (9)‡		78 (15)‡	78 (15)‡	
	< 60	52 (7)	52 (7)		52 (7)	51 (7)	
Age (years)	≥ 60	74.4 (3.9)‡	-		74.2 (3.6)‡	-	
	< 60	77.4 (4.3)	-		76.9 (4.7)	-	
BMI (kg/m^2)	≥ 60	26.7 (3.7)‡	-		27.7 (3.8)‡	-	
	< 60	28.5 (4.7)	-		25.2 (3.6)	-	
Markers of renal function and calcium and phosphate metabolism							
Biomarker	eGFR category[a]	Mean (SD) or median [IQR][c]	Mean (SD) or median [IQR][c]	p-value pre vs post supplementation[c]	Mean (SD) or median [IQR]	Mean (SD) or median [IQR]	p-value pre vs post supplementation
Albumin (g/L)	≥ 60	45.8 (2.2)	44.6 (2.3)	< 0.001#	45.8 (2.2)	44.5 (2.3)	< 0.001
	< 60	45.6 (2.2)	44.2 (2.1)	< 0.001	45.6 (2.2)	44.5 (2.1)	< 0.001
Adjusted calcium (mmol/L)	≥ 60	2.2 (0.1)	2.2 (0.1)	0.103#	2.2 (0.1)	2.2 (0.1)	0.048
	< 60	2.2 (0.1)	2.3 (0.1)	< 0.01	2.2 (0.1)	2.2 (0.1)	0.053
Phosphate (mmol/L)	≥ 60	0.89 (0.17)*	0.79 (0.19)*	< 0.001#	0.89 (0.18)	0.79 (19)*	< 0.001
	< 60	0.83 (0.20)	0.85 (0.17)	0.604	0.89 (0.18)	0.84 (0.18)	0.031
Klotho (pg/mL)	≥ 60	510 [395–554] ***	502 [406–625]	0.522	492 [392–640]*	497 [393–626]	0.676
	< 60	436 [372–552]	498 (1.4)	0.229	464 [384–591]	495 [416–583]	0.611
cFGF23 (RU/mL)	≥ 60	64.5 [53.0–78.6] ***	72.1 [59.9–95.1]***	< 0.017#	64.9 [52.8–80.8]**	72.7 [59.9–95.4]*	0.006
	< 60	87.4 [70.9–125.5]	83.9 [66.4–97.1]	< 0.001	73.2 [60.1–96.3]	73.6 [63.9–95.7]	0.043
iFGF23 (pg/mL)	≥ 60	52.6 [42.6–64.2] ***	62.3 [49.6–77.2]**	< 0.001	52.8 [42.9–65.5]*	62.6 [50.0–78.5]	< 0.001#
	< 60	69.4 [52.4–84.4]	65.0 [52.5–85.8]	0.168	59.1 [48.7–79.1]	62.1 [50.2–77.0]	0.018
Markers of Vitamin D metabolism							
Total 25(OH)D (nmol/L)	≥ 60	41.2 (20.6)*	65.9 (17.9)	0.002#	39.6 (19.1)	64.2 (17.3)***	0.004#
	< 60	34.4 (16.8)	69.1 (18.7)	< 0.001	41.1 (22.6)	72.1 (18.7)	< 0.001
Free 25(OH)D (pmol/L)	≥ 60	8.7 (4.4)*	13.7 (4.0)	0.009#	8.4 (4.1)	13.6 (4.0)*	< 0.013#
	< 60	7.3 (3.1)	15.2 (5.2)	< 0.001	8.6 (4.7)	15.3 (4.8)	< 0.001
24,25$(OH)_2$D (nmol/L)	≥ 60	3.4 [2.1–5.8]**	7.8 (3.3)	< 0.001#	3.2 [2.1–5.5]	7.5 (3.1)	< 0.001
	< 60	3.0 (1.7)	7.0 (2.7)	< 0.001	3.0 [2.0–5.1]	7.8 (3.0)	< 0.001
1,25$(OH)_2$D (pmol/L)	≥ 60	98.0 (28.2)***	99.9 (29.3)	0.001#	97.0 (28.7)*	101.0 (28.8)	< 0.001
	< 60	80.4 (28.0)	106.5 (32.5)	< 0.001	88.9 (28.7)	101.2 (31.1)	< 0.001
DBP (mg/L)	≥ 60	368.0 (63.8)	365.2 (62.4)	0.590	366.2 (63.5)	365.1 (64.5)	0.839
	< 60	366.7 (63.8)	381.5 (62.7)	0.182	371.5 (64.0)	375.8 (53.2)	0.633
iPTH (pg/mL)	≥ 60	41.5 [32.3–55.3] ***	37.7 [28.0–51.0]**	< 0.001	43.3 [33.0–57.1]	37.6 [27.9–50.4]	< 0.001
	< 60	52.0 [40.0–77.6]	38.4[28.8–55.4]	0.040	43.4 [34.8–57.3]	38.4 [28.3–58.9]	0.055
Wnt signalling pathway markers							
SOST (pmol/L)	≥ 60	42.7 [31.5–55.6] ***	47.0 [34.7–61.9]***	0.625	43.2 [31.1–56.4]**	45.5 [33.7–60.3]*	0.584
	< 60	58.9 [42.5–75.4]	41.5 [31.8–57.2]	0.754	48.7 [36.6–63.4]	46.4 [32.1–61.2]	0.915
DKK1 (pmol/L)	≥ 60	32.2 (16.7)*	38.0 (18.1)	< 0.001	32.4 (16.2)*	38.8 (18.9)	< 0.001
	< 60	27.0 (15.1)	35.3 (19.3)	0.008	28.5 (15.1)	34.5 (16.8)	0.013
OPG (pmol/L)	≥ 60	5.6 (2.0)	5.5 [4.5–6.8]	0.504	5.5 (2.0)*	5.2 (1.5)	0.090
	< 60	5.9 (2.3)	5.7 (2.4)	0.608	6.1 (2.1)	5.3 [4.2–7.2]	0.039
sRANKL (pmol/L)	≥ 60	0.12 [0.08–0.17]	0.12 [0.08–0.19]	0.878	0.12 [0.08–0.18]	0.12 (0.08–0.19)*	0.838
	< 60	0.12 (2.00)	0.12 [0.07–0.18]	0.935	0.11 [0.08–0.15]	0.11 [0.07–0.18]	0.660
Bone mineral density and metabolism							
Hip BMD (g/m^2)	≥ 60	0.98 (0.18)	0.99 (0.17)**	0.617	0.99 (0.18)*	0.99 (0.16)	0.583
	< 60	0.97 (0.17)	0.92 (0.16)	0.112	0.94 (0.17)	0.96 (0.17)	0.627
Hip BMC (g)	≥ 60	35.35 (8.27)	35.58 (8.10)*	0.747#	36.28 (8.26)**	35.39 (8.10)	0.221
	< 60	35.74 (8.51)	32.73 (7.10)	0.038	33.30 (8.09)	34.27 (7.95)	0.399
FN BMD (g/m^2)	≥ 60	0.90 (0.15)	0.91 (0.14)*	0.233	0.91 (0.15)*	0.91 (0.14)	0.795
	< 60	0.89 (0.15)	0.86 (0.14)	0.032	0.88 (0.15)	0.88 (0.15)	0.707
FN BMC (g)	≥ 60	4.89 (1.08)	4.83 (1.05)*	0.524	5.00 (1.11)**	4.81 (1.04)	0.047
	< 60	4.90 (1.09)	4.48 (1.04)	0.026	4.64 (1.01)	4.66 (1.11)	0.883
BAP (μg/L)	≥ 60	9.2 [7.7–12.1]	9.7 [7.9–13.0]	0.027	9.2 [7.8–12.0]	9.8 [8.0–13.8]	0.039

(*continued on next page*)

Table 2 (*continued*)

Characteristics	eGFR category[a] (mL/min per 1.73 m^2)	CKD-EPI creatinine			Cockcroft-Gault		
		Baseline	12 months		Baseline	12 months	
		N (%) or Mean (SD)	N (%) or Mean (SD)		N (%) or Mean (SD)	N (%) or Mean (SD)	
	< 60	10.6 (3.5)	10.6 (3.5)	0.851	10.5 (3.7)	10.2 [7.7–13.1]	0.762
CTX (ng/mL)	≥ 60	0.40 (0.18)	0.36 (0.15)	0.010	0.39 (0.17)**	0.37 (0.15)	0.136[#]
	< 60	0.44 (0.22)	0.36 (0.14)	0.006	0.45 (0.21)	0.34 (0.15)	< 0.001
PINP (μg/L)	≥ 60	35.6[28.3–46.0]	38.6 [30.6–48.9]	0.008	35.4 [27.9–44.7]*	39.2 [30.7–49.8]	0.07
	< 60	39.9 [28.8–50.9]	40.3 [31.1–50.8]	0.689	40.7 [32.3–50.2]	37.9 [29.4–48.2]	0.558

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 for individual variables by eGFR category: CKD-EPI creatinine at baseline eGFR ≥ 60 n = 307–283; < 60 n = 69–62 GC at baseline eGFR≥ 60 n = 271–242; < 60 n = 106–99. Total number of participants is given in Table 1. [b] % of total number of participants per category eGFR.
‡Significant difference $p < 0.001$ between eGFR categories (eGFR <60 mL/min/1.73 m^2 versus eGFR ≥60 mL/min/1.73 m^2) tested with independent t-test.
[c] Differences between eGFR categories (eGFR <60 mL/min/1.73 m^2 versus eGFR ≥60 mL/min/1.73 m^2) at baseline and post-supplementation and the response to supplementation within eGFR category were tested by ANCOVA with an interaction term for eGFR category and time-point (i.e., pre- and post-intervention). P-values for pre- and post-supplementation comparisons within eGFR categories are given in the table. [#]Significance ($p < 0.05$) of the for eGFR x time point interaction term representing the difference in pre- to post supplementation change between eGFR categories. Significant difference *$p < 0.05$ * *$p < 0.01$; * **$p < 0.001$ between eGFR categories tested by ANCOVA.
Abbreviations: 24,25-dihydroxy Vitamin D ($24,25(OH)_2D$); Vitamin D Binding Protein (DBP); Femoral Neck (FN).
Further abbreviations see Table 1.

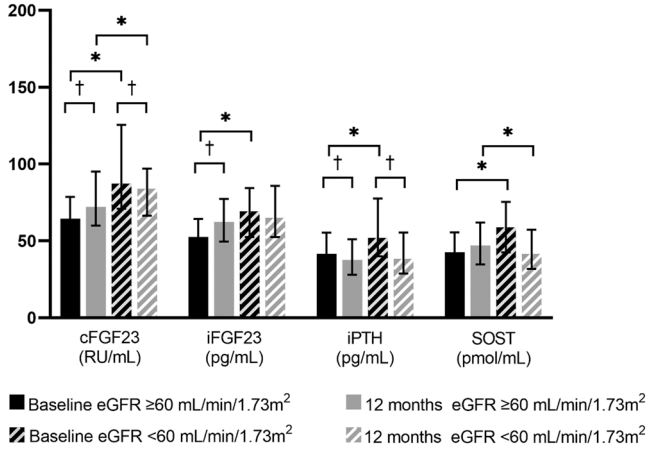

Fig. 1. Plasma or serum concentrations of cFGF23, iFGF23, PTH and SOST pre- and post- vitamin D supplementation in groups categorized on basis of eGFR as < 60 or ≥ 60 mL/min/1.73 m^2 according to the CKD-EPIcr algorithm. Data are presented as median (IQR) values. Differences between eGFR categories at baseline and post-supplementation and the response to supplementation within eGFR category were tested by ANCOVA with an interaction term for eGFR category and time-point (i.e., pre- and post-intervention). Significant differences ($p < 0.05$) between eGFR categories are indicated by * . Differences between pre- and post-supplementation values are indicated by †. Abbreviations: estimated Glomerular Filtration Rate (eGFR); Chronic Kidney Disease Epidemiology Collaboration with creatinine (CKD-EPIcr): c-terminal Fibroblast Growth Factor-23 (cFGF23); intact Fibroblast Growth Factor-23 (iFGF23); intact Parathyroid Hormone (iPTH); Sclerostin (SOST).

BMD and BMC were significantly lower with eGFR < 60 mL/min/1.73 m^2 (Table 2). Based on CG, there were no significant differences between the eGFR groups (Table 2). After supplementation, there were no differences by eGFR category in markers of bone metabolism with either algorithm.

3.3. Response to supplementation by eGFR category

There were differences in the response to vitamin D supplementation by eGFR category. (Table 2**,** Fig. 1 **and** Supplementary Table 1). Plasma cFGF23, total and free 25(OH)D, $24,25(OH)_2D$, $1,25(OH)_2D$ and DKK1 increased and plasma iPTH and CTX decreased in both eGFR groups. The decrease in CTX was non-significant when eGFR ≥ 60 mL/min/1.73 m^2 was calculated according with GC. Plasma iFGF23, BAP and PINP increased only in the group with eGFR ≥ 60 mL/min/1.73 m^2. A decrease in serum phosphate was found only in the group eGFR ≥ 60 mL/min/1.73 m^2 based on CKD-EPIcr and in both groups for CG. SOST and sRANKL remained unchanged in both eGFR groups. Hip BMC (but not BMD) and FN BMD and BMC decreased in the group with eGFR < 60 mL/min/1.73 m^2 based on CKD-EPIcr. This was not seen with data based on GC or any of the other algorithms in either eGFR group.

The change of many of these variables differed between eGFR groups, as reflected in significance of the ANCOVA interaction term for eGFR group x time point (Table 2).

3.4. Associations with eGFR (by four equations for eGFR)

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 ANCOVA analyses. At baseline, 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 CKD-EPIcr and CG data (Table 3). Significant associations with Klotho, SOST and DKK1 were only found with CKD-EPIcr data and OPG with CG data. The differences between eGFR groups in total and free 25(OH)D were not reflected in significant associations between eGFR and these variables (Table 3). In accordance with findings of ANCOVA, no significant associations of eGFR by CKD-EPIcr with BMD, BMC and the bone metabolism markers (BAP, CTX, PINP) were found, whereas these were detected when using CG.

Findings with MDRD-4 and CKD-EPI with cystatin C algorithms also mostly confirmed findings of ANCOVA and paralleled those with CKD-EPIcr and CG. These are described in Supplementary Table 2.

After supplementation, associations of iFGF23, Klotho, iPTH and SOST with eGFR by both CKD-EPI and CG were no longer significant, whereas the association with $1,25(OH)_2D$ remained significant. There were significant associations with total and free 25(OH)D, not detected in eGFR group comparisons (Table 3). With CKD-EPIcr, there were significant positive associations with BMD and BMC, in accordance with findings of ANCOVAs. No significant associations of eGFR by CG with BMD and BMC were found. No associations with the bone metabolism

Table 3
Association between biomarkers and eGFR calculated according to CKD-EPI creatinine and Cockcroft-Gault algorithms at baseline and after 12 months of supplementation[a,b].

Biomarker	CKD-EPI creatinine				Cockcroft-Gault			
	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)	0.006	0.525	0.019	0.058	0.011	0.05	0.009	0.215
Adjusted calcium (mmol/L)	0.000	0.844	0.000	0.898	> 0.000	0.03	> 0.000	0.99
Phosphate (mmol/L)	0.002	0.014	-0.001	0.513	-0.002	< 0.001	-0.004	< 0.001
Klotho (pg/mL)	5.713	0.023	0.447	0.737	1.062	0.16	1.086	0.14
cFGF23 (RU/mL)	-0.743	< 0.001	-0.081	0.638	-0.588	< 0.001	-0.428	0.018
iFGF23 (pg/mL)	-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)	0.091	0.251	-0.162	0.031	0.043	0.40	-0.272	< 0.001
Free 25(OH)D (pmol/L)	0.020	0.246	-0.059	0.001	0.011	0.32	-0.057	< 0.001
$24,25(OH)_2D$ (nmol/L)	0.032	0.007	0.007	0.612	0.023	< 0.001	-0.029	0.002
$1,25(OH)_2D$ (pmol/L)	0.665	< 0.001	0.779	< 0.001	0.202	0.01	0.238	< 0.001
DBP (mg/L)	-0.010	0.968	-0.377	0.167	0.060	0.71	-0.137	0.36
iPTH (pg/mL)	-0.281	0.002	-0.127	0.183	0.012	0.861	-0.013	0.84
Wnt signalling pathway markers								
SOST (pmol/L)	-0.376	< 0.001	0.154	0.169	-0.226	< 0.001	-0.096	0.19
DKK1 (pmol/L)	0.143	0.035	0.089	0.277	0.060	0.17	0.016	0.72
OPG (pmol/L)	-0.012	0.153	0.007	0.465	-0.015	< 0.001	0.008	0.15
sRANKL (pmol/L)	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	0.214	0.002	0.002	0.002	< 0.001	0.001	0.062
Hip BMC (g)	-0.063	0.056	0.095	0.008	0.135	< 0.001	0.021	0.42
FN BMC (g)	-0.004	0.313	0.010	0.033	0.018	< 0.001	0.001	0.69
FN BMD (g/m^2)	-0.002	0.373	0.001	0.646	0.002	< 0.001	0.001	0.22
BAP (μg/L)	0.010	0.462	-0.010	0.653	0.003	0.79	-0.002	0.92
CTX (ng/mL)	0.000	0.697	0.000	0.688	-0.002	0.005	> 0.000	0.46
PINP (μg/L)	-0.017	0.798	-0.010	0.909	-0.087	0.08	0.007	0.92

aβ-coefficients and associated p-values from linear regression analysis with eGFR as a continuous variable. Significant associations $p < 0.05$ are indicted in bold.
bNumber of participants included in regression analyses is provided in Table 1. For abbreviations see Tables 1 and 2.

markers were found with either algorithm.

Similar to CKD-EPIcr and CG, regression analyses with MDRD-4 and CKD-EPI with cystatin C algorithms mostly confirmed eGFR group comparisons. These are described in Supplementary Table 2.

4. Discussion

In a cohort of apparently healthy older adults, 18–28% had an eGFR below < 60 mL/min/1.73 m^2 (CKD G3a) and 3–5% < 45 mL/min/1.73 m^2 (CKD G3b). Before supplementation, significantly higher concentrations cFGF23, iFGF23, iPTH and SOST and lower Klotho, $1,25(OH)_2D$ and DKK1 concentrations were found in the group with CKD G3a&b compared to the group with eGFR ≥ 60 mL/min/1.73 m^2. Differences in 25(OH)D, $24,25(OH)_2D$ and iPTH by eGFR category were only detected with CKD-EPI and MDRD-4 and 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 all measured vitamin D metabolites and DKK1 and a decrease in plasma iPTH and CTX in both eGFR groups. Plasma iFGF23, BAP, PINP increased and phosphate decreased only in the group with eGFR ≥ 60 mL/min/1.73 m^2. Findings were largely consistent across all eGFR algorithms, except for BMC and BMD. After vitamin D supplementation, cFGF23, iFGF23, iPTH and SOST remained significantly higher in the lower eGFR group. Plasma $1,25(OH)_2D$ and Klotho did no longer differ between eGFR groups. Also, these findings were largely consistent for all eGFR algorithms. 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.

These findings are consistent with earlier reports in more advanced stages of CKD. This study showed that, even moderate renal impairment is associated with alterations in vitamin D metabolism, Wnt signalling and bone turnover markers [34–36]. Secondary hyperparathyroidism is a known complication of advanced stages of CKD and an increase in iPTH is usually reported from stage 3 or 4 [19,37]. We found iPTH concentrations above the assay-specific references range (4.7–114 pg/mL [38]) in a considerable proportion of participants with moderate renal impairment, similar to other reports [11]. A reduction in plasma $1,25(OH)_2D$ is reported from early renal impairment and continues to decrease with declining renal function [11,39]. The changes in plasma $1,25(OH)_2D$ 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 [40]. The Wnt/β-catenin signalling pathway plays a key role bone homeostasis by regulating osteocyte function and osteoblast and osteoclast differentiation and function [41] and its effect is mainly anabolic [42]. 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 [41,43–48]. OPG antagonises by binding and preventing interaction with its receptor, RANK [43,49,50]. 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 [51]. 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: 13.3–41.8 pmol/L; OPG median: 1.8 pmol/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 $1,25(OH)_2D$ it may be anticipated that these changes

with early CKD are associated with negative effects on mineral and bone metabolism [44,45,47,48]. 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 [29], similar to findings in other RCTs [52,53]. An increase was shown in both eGFR groups and after supplementation there were no differences between groups. This may indicate that the impact of reduced 1 α-hydroxylation capacity and/or increased catabolism with lower renal function [17], may be diminished by increased availability of 25(OH)D for hydroxylation.

Plasma iPTH decreased with supplementation in both eGFR groups and the change did not differ between groups. In healthy individuals, iPTH and 25(OH)D are inversely correlated and vitamin D supplementation is associated with a decrease in iPTH [54], 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[11,19].

The response of plasma iFGF23 and phosphate differed by eGFR category. An increase in cFGF23 and iFGF23 with vitamin D supplementation was reported before [29,33] 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/1.73 m^2, iFGF23 and phosphate remained unchanged with supplementation. Although also klotho did not change significantly, this was no longer significantly lower in the group with impaired renal function. There is limited evidence that vitamin D supplementation may increase the expression of Klotho, and thus increase FGF23 sensitivity but data are conflicting [55,56].

Vitamin D may modulate SOST expression in osteocytes [15,57]. Recent findings show an inverse correlation between vitamin D status and SOST concentrations in healthy postmenopausal women and adults [14,29,58]. However, conflicting results have been reported regarding the effect of vitamin D supplementation on SOST and DKK1 [57,59,60]. Several studies showed that vitamin D supplementation can lead to decline in SOST [60,61]. Other studies showed the opposite, reporting an increase in SOST following supplementation with native [59,62] or activated forms of vitamin D in non-CKD and CKD subjects [57]. Another RCT with CKD patients (G3–4) showed that vitamin D supplementation did not significantly affect SOST [63]. 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 of 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 consistent 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 [64,65]. 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. The CKD-EPI and MDRD-4 algorithms, calculated on basis creatinine provided similar results, while those including cystatin C or calculated by CG differed for some of the outcomes. In medical practice, kidney function is routinely assessed in older adults [66] and mostly based on the calculation of eGFR based on serum creatinine or more recently, cystatin C [24,67]. 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 [20,68], since plasma creatinine is also influenced by muscle mass and dietary protein intake. Both of these are known to decrease with age [69]. CG is the only algorithm tested that includes weight. This might explain the differences findings for bone density and content measurements between the eGFR categories. In addition, the higher number of participants categorised to the eGFR < 60 mL/min/1.73 m^2 group (baseline $n = 106$; 12 months $n = 99$) with GC may have influenced the statistical power to detect these differences. Cystatin C is thought to be less influenced by muscle mass since it is produced at a constant rate by all nucleated cells and filtered in proximal tubules [70]. Recent studies have shown that cystatin C may be a better marker for the estimation of GFR in older people and to predict the progression of CKD [69,71–73]. The recently revised and recalibrated algorithms for CKD-EPI, including the removal of race [24] may also be anticipated to provide different results.

This study has several limitations. The absence of a placebo group (as per guidelines for pharmaceutical trials, a group receiving the standard population recommendation was included [33]) did not allow to account for changes unrelated to the intervention (i.e. ageing or secular trends). The VDOP study included relatively healthy older adults and excluded those with known renal disease and an eGFR < 30 mL/min/1.73 m^2 at screening. The eGFR categorisation was solely based on eGFR (predominantly reflecting functional abnormalities), without data regarding albuminuria (indicative of kidney damage). We could therefore not evaluate whether any relationships and/or the response to supplementation was influenced by structural abnormalities or damage. 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 an heterogenous nature. Participants of the VDOP study were predominantly of white origin and therefore results may not be generalisable 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.

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) [74]. 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 [74,75].

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 [29,76], 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.

A large number of comparisons were conducted in this study, without correction for multiple testing. Group sizes were unequal; the group with the lower eGFR had limited numbers compared to the group with the higher eGFR. Jointly this will have increased the chance of type 1 errors. Research specifically designed and powered to confirm our findings is therefore required.

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 [77–79], it cannot be excluded that directly measured concentrations would have provided different findings.

5. Conclusion

This study showed that a moderate decline in eGFR is associated with alterations 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 with both normal and impaired renal function. The response depended on renal function. Supplementation increased concentrations of Vitamin D metabolites, including $1,25(OH)_2D$ 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/1.73 m^2.

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Declaration of Competing Interest

Marilena Christodoulou has no interests to disclose. Terence J. Aspray has served on an advisory board and received lecture fees from Internis. Isabelle Piec, Christopher Washbourne, Jonathan C. Y. Tang have no interests to disclose. William D. Fraser has received research grants, sat on advisory boards, and given lectures on behalf of Eli Lilly, NPS Pharmaceuticals, Shirel, Entera Bio Ltd. and Nycomed. Inez Schoenmakers has served on advisory boards.

Data availability

Data will be made available on request.

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Role of the funder

The study design of the VDOP trial primary outcomes and secondary analyses were internationally peer reviewed as part of the funding-decision process by the respective funding bodies. The funders had no involvement in the design, analyses, interpretation and publication of the results.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jsbmb.2023.106267.

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