Association between vitamin D deficiency and exercise capacity in patients with CKD, a cross-sectional analysis.

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32 Abbreviations

- 33 1-RM – 1 repetition maximum
- 34 $1\alpha,25(\text{OH})_2\text{D}_3$ – 1,25-dihydroxyvitamin D₃
- 35 $24,25(\text{OH})_2\text{D}_3$ - 24,25-dihydroxyvitamin D₃
- 36 25OHD₂ - 25-hydroxyvitamin D₂
- 37 25OHD₃ - 25-hydroxyvitamin D₃
- 38 3-epi-25OHD₃ - 3-epi-25-hydroxyvitamin D₃,
- 39 5-RM – 5 repetition maximum
- 40 ALM - Appendicular lean mass
- 41 BIA – Bioelectrical impedance analysis
- 42 BSA - Bovine serum albumin
- 43 CFSE - Carboxyfluorescein succinimidyl ester
- 44 CKD - Chronic kidney disease
- 45 DM – Differentiation medium
- 46 FBS – fetal bovine serum
- 47 eGFR – estimated glomerular filtration rate
- 48 GM – Growth medium
- 49 IL-6 – Interleukin-6
- 50 ISWT – Incremental shuttle walk test
- 51 LC-MS/MS - liquid chromatography-tandem mass spectrometry
- 52 MCP-1 – monocyte chemoattractant protein-1
- 53 MRI – Magnetic resonance imaging
- 54 MRM - Multiple reaction monitoring
- 55 MAFbx – Muscle atrophy F-box

- 56 MuRF-1 – Muscle ring finger-1
- 57 MyHC – Myosin heavy chain
- 58 MyoD – Myoblast determination protein 1
- 59 NKF-KDOQI - National Kidney Foundation Kidney Disease Outcome Quality Initiative
- 60 PBS – Phosphate buffered saline
- 61 STS60 – sit-to-stand 60
- 62 TNF- α - Tumour necrosis factor alpha
- 63 $\dot{V}O_{2peak}$ – Peak oxygen uptake
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Abstract

Evidence is growing for a role of vitamin D in regulating skeletal muscle mass, strength and functional capacity. Given the role the kidneys play in activating total vitamin D, and the high prevalence of vitamin D deficiency in Chronic Kidney Disease (CKD), it is possible that deficiency contributes to the low levels of physical function and muscle mass in these patients. This is a secondary cross-sectional analysis of previously published interventional study, with *ex vivo* follow up work. 34 CKD patients at stages G3b-5 (eGFR 25.5 ± 8.3 ml/min/1.73m²; age 61 ± 12 years) were recruited, with a sub-group (n=20) also donating a muscle biopsy. Vitamin D and associated metabolites were analysed in plasma by liquid chromatography tandem-mass spectroscopy and correlated to a range of physiological tests of muscle size, function, exercise capacity and body composition. The effects of $1\alpha,25(\text{OH})_2\text{D}_3$ supplementation on myogenesis and myotube size was investigated in primary skeletal muscle cells from vitamin D deficient donors. *In vivo*, there was no association between total or active vitamin D and muscle size or strength, but a significant correlation with $\dot{V}\text{O}_{2\text{Peak}}$ was seen with the total form. *Ex vivo*, $1\alpha,25(\text{OH})_2\text{D}_3$ supplementation reduced IL-6 mRNA expression, but had no effect upon proliferation, differentiation or myotube diameter. This early preliminary work suggests that vitamin D deficiency is not a prominent factor driving the loss of muscle mass in CKD, but may play a role in reduced exercise capacity.

Key Words: Skeletal muscle, Vitamin D, Physical function, supplementation.

Introduction

Patients with chronic kidney disease (CKD) commonly experience skeletal muscle wasting, reduced exercise capacity and lower levels of physical function (1-3). These appear early in the disease process (4) and are associated with adverse clinical outcomes and reduced quality of life (5-10). The factors driving loss of muscle mass and physical function are not yet fully understood but are likely to be multifactorial with large heterogeneity. Gaps in our understanding have meant that there are currently no viable therapies to protect or restore muscle mass and physical function in CKD.

The classical effects of vitamin D focus around calcium homeostasis and bone health, but it is becoming increasingly accepted that it may also have a role in skeletal muscle function (11, 12) and exercise capacity (13). Studies involving both humans and animals have shown that vitamin D deficiency is associated with muscle atrophy affecting predominately type II fibres (14, 15), which can be reversed following vitamin D supplementation (16). Vitamin D deficiency is also associated with an increased number of falls (17), which in some cases can be prevented with supplementation (18, 19). Community-based cross-sectional studies have shown vitamin D deficiency is associated with reduced measures of physical functioning such as gait speed and rising from a chair (20, 21). However, studies in both healthy and clinical populations have not always demonstrated improvements in physical functioning following vitamin D supplementation (22-24). Therefore, the role of vitamin D in the maintenance or improvement of physical function requires further examination.

Vitamin D obtained from sunlight or through dietary sources is relatively inactive and must be converted to the active form, of which the final step occurs in the kidney by 1α -hydroxylase. Given the impairment of kidney function, vitamin D deficiency is highly prevalent in CKD patients (25). Despite this, there is limited and conflicting data regarding the association between vitamin D and physical function in the CKD population. One study of CKD patients failed to find an association between the active metabolite, $1,25\text{-dihydroxyvitamin D}_3$ ($1\alpha,25(\text{OH})_2\text{D}_3$), and muscle function (26), whilst others in both non-dialysis CKD (27) and end-stage renal disease (28) have reported associations between $1\alpha,25(\text{OH})_2\text{D}_3$ muscle strength and physical functioning.

Mature skeletal muscle cells are terminally differentiated, and by themselves, are capable of limited repair and regeneration. Therefore for repair to occur, cells are reliant on a population

of stem cells, termed satellite cells, that support repair and regeneration through a process called myogenesis (29). Myogenesis is thought to be dysfunctional in CKD (29) and may contribute to skeletal muscle wasting. A role is also emerging for vitamin D in skeletal muscle repair (30) where it has been shown to influence satellite cell proliferation and differentiation (31, 32). Therefore, it is possible that vitamin D deficiency contributes to atrophy through inhibition of myogenesis in CKD, but this is yet to be investigated.

Vitamin D status is generally based upon the analysis of the inactive total form of vitamin D (25OHD). However, related metabolites have also been shown to be clinically important (33), demonstrating the importance to also consider the vitamin D metabolome alongside total vitamin D, which by itself provides only a limited view of vitamin D status.

The aims of this study were: 1) to perform *in vivo* analysis to determine the relationship between serum vitamin D and its metabolites and skeletal muscle mass and function in patients with CKD not requiring dialysis; and 2) to determine *ex vivo* the effect of $1\alpha,25(\text{OH})_2\text{D}_3$ supplementation on myoblast proliferation, differentiation, and hypertrophy using human-derived skeletal muscle cells isolated from CKD vitamin D deficient donors. We hypothesised that plasma $1\alpha,25(\text{OH})_2\text{D}_3$ would be associated with muscle size, strength and exercise capacity and that supplementation of vitamin D deficient cells with $1\alpha,25(\text{OH})_2\text{D}_3$ would increase myotube size.

Material and Methods

Patients and study design

This study was a cross-sectional observational design. Patients in this report are from two separate cohorts. Physical function data is taken from the ExTra CKD study (34) (ISRCTN: 36489137), whilst patients who donated biopsies used in the *ex vivo* study took part in the Explore CKD study (ISRCTN: 18221837). Sample size was based upon available data from these participants. All patients were recruited from nephrology outpatient clinics at Leicester General Hospital, UK between December 2013 – April 2017. Exclusion criteria were age <18years, pregnancy, disability that prevented patients from undertaking exercise, insufficient command of English, or an inability to give informed consent. Ethical approval was given by the National Research Ethics Committee (13/EM/0344; 15/EM/0467). All patients gave written informed consent and the trial was conducted in accordance with the Declaration of Helsinki.

Physiological assessments

Muscle size

Muscle size was determined using two methods: (i) quadriceps volume of the right leg measured by Magnetic Resonance Imaging (MRI) (34) acquired using a 3T Siemens Skyra HD MRI scanner in the axial plane using a T1 turbo spin-echo sequence and (ii) rectus femoris cross-sectional area of the right leg measured by 2-D B-mode ultrasound. These techniques have previously been described by our group (35).

Muscle strength

Quadriceps strength was assessed by leg extension exercise using a 5-Repetition Maximum (5-RM) test (34). Prediction equations were then used to estimate 1-Repetition Maximum (1-RM) (36).

Exercise capacity

Patients underwent the incremental shuttle walk test (ISWT) (34) during which patients walked along a 10m course in time with externally paced beeps that become progressively quicker until volitional fatigue. This is a valid and reliable method to determine peak exercise capacity ($\dot{V}O_{2peak}$) (37). Patients also underwent an incremental Cardiopulmonary Exercise Test to measure $\dot{V}O_{2peak}$ performed on an electrically-braked cycle ergometer (34).

Physical Function

Physical function was determined using the sit-to-stand 60 (STS60) test, a surrogate marker of muscular endurance (37).

Body composition

Body fat percentage and appendicular lean mass (ALM), was estimated using multi-frequency bioelectrical impedance analysis (BIA) (InBody 370, CA,USA). This device has been validated against dual-energy x-ray absorptiometry (38).

Blood sampling and Vitamin D metabolite analysis

Venous blood samples were taken from 38 CKD patients (Table 1) into a plain tube and left undisturbed at room temperature for 30min to allow the blood to clot. The blood was then centrifuged at 1500 g for 10min at 4°C. Resulting serum was collecting and stored at -80°C until subsequent analysis. Serum concentrations of vitamin D metabolites were analysed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) as previously described (39). Briefly, 200µl serum was extracted prior to analysis by protein precipitation followed by supportive liquid-liquid extraction. Analysis was performed on a Waters Acquity UPLC coupled to a Waters Xevo TQ-XS mass spectrometer. Analysis was carried out in multiple reaction monitoring (MRM) for the following analytes: 25-hydroxyvitamin D3 (25OHD3), 3-epi-25-hydroxyvitamin D3 (3-epi-25OHD3), 24,25-dihydroxyvitamin D3 (24,25(OH)₂D3), 1,25-dihydroxyvitamin D3 (1α,25(OH)₂D3) and 25-hydroxyvitamin D2 (25OHD2). The LC-MS/MS method for vitamin D quantification was validated for serum analysis as previously described for accuracy, precision, recovery and matrix effects (39). Vitamin D metabolites were purchased from Supleco Sigma Aldrich. LC-MS grade methanol and water were purchased from Greyhound Chromatography and Thermo Fisher respectively. Supportive liquid-liquid extraction plates were purchased from Phenomenex.

Muscle biopsy collection

Vastus lateralis muscle biopsies were taken from 20 patients using the micro biopsy technique after an overnight fast (40). Biopsy specimens from five of these patients deemed to be vitamin D deficient (25(OH)D <20ng/ml) were also used to establish primary cultures as described below in which the effect of vitamin D repletion could be more closely studied. After dissection of any visible fat and connective tissue, samples were placed into liquid nitrogen

(RNA extraction) or 5mL ice-cold Hams F10 media containing 1% penicillin streptomycin and 1% Gentamycin (cell culture).

Satellite cell isolation procedure and cell treatments

Muscle tissue was washed in HamsF10 (containing 1% penicillin streptomycin and 1% Gentamycin), minced into small fragments and enzymatically digested in two incubations with collagenase IV (1mg/mL), Bovine Serum Albumin (BSA) (5mg/mL) and trypsin (500µl/mL) at 37°C with gentle agitation. The resultant supernatant was added to Foetal Bovine Serum (FBS), strained through a 70µm nylon filter and centrifuged at 800 g for 7min. The cells were washed in Hams F10 with 1% penicillin streptomycin and 1% Gentamycin and pre-plated on uncoated 9cm² petris in 3mL growth media (GM; Hams F10 Glutamax, 20% FBS, 1% Penicillin Streptomycin, 1% fungazone) for 3h. The cell suspension was then moved to collagen I coated 25cm² flasks and kept at 37°C under humidified 95% air and 5% CO₂ until cells had achieved approximately 70% confluence. For experiments, cells were plated at a density of 3x10⁴ and grown until 70% confluent. For experiments using myoblasts, cells were exposed to either high dose of exogenous 1α,25(OH)₂D₃ (100nmol), low dose (10nmol), or control vehicle (95% EtOH), and proliferation rates determined after 72h. For experiments using myotubes, GM was replaced with differentiation medium (DM; DMEM 4.5g/L glucose, 1% Penicillin Streptomycin, 10% horse serum) for five days by which time multinucleated muscle fibres had formed. Cells were again exposed to either high dose of exogenous 1α,25(OH)₂D₃ (100nmol), low dose (10nmol), or control vehicle (95% EtOH) to investigate effects differentiation, determined after five days (immunofluorescence and PCR).

Proliferation assay

Carboxyfluorescein succinimidyl ester (CFSE) dye (Thermo Fisher, UK) was added to human skeletal muscle cells in suspension (1ml HBSS) at a concentration of 5µm and incubated at 37°C for 20min. Staining was quenched by the addition of five volumes of GM and incubated at 37°C for a further 5min. Cells were rinsed and seeded onto collagen I coated 6-well plates and collected 72h later. Cells were then re-suspended in Phosphate Buffered Saline (PBS) and analysed using a FACSCelesta instrument (BD Biosciences). Data were analysed using FlowJo 10.2 (FlowJo LLC, USA).

Immunofluorescence

Cells were fixed in 4% paraformaldehyde for 20min at room temperature, washed three times with PBS and blocked and permeabilized in PBS containing 5% goat serum and 0.25% Triton X-100 for 1h. Cells were incubated with rabbit anti-desmin primary antibody (1/400; cell signalling) at 4°C overnight, washed three times in PBS, and incubated with Alexa Flour 488-labelled goat anti-rabbit IgG (1/400; Thermo Fisher) for 2h at room temperature. DAPI (100ng/mL) was used to visualise the nuclei. Ten random fields were acquired per condition using a FLoid imaging system (Thermo Fisher) and images analysed using ImageJ. Myotube diameter was assessed at three points on each cell. Fusion indexes were defined by the number of DAPI positive nuclei within myotubes (desmin positive cell containing 3 or more nuclei) divided by the total number of DAPI positive nuclei.

Quantitative RT-PCR

Total RNA was extracted from skeletal muscle tissue (10mg wet weight) and primary cells using Trizol® (Invitrogen, UK) and 1µg RNA was reverse transcribed to cDNA using an AMV reverse transcription system (Promega, Madison, WI, USA). Primers, probes and internal controls for all genes were supplied as Taqman gene expression assays (Applied Biosystems, Warrington, UK) Vitamin D receptor: Hs01045843_m1, Myogenin: Hs01072232_m1, MyoD: Hs02330075_g1, Myf5: Hs00929416_g1, Pax7: Hs00242962_m1, MAFbx: Hs00369714_m1, MuRF-1: Hs00822397_m1, IL-6: Hs00985639_m1, MCP-1: Hs00234140_m1, TNF-α: Hs01113624_g1, Myostatin: Hs00976237_m1, MYHC1: Hs00428600_m1, MYHC2: Hs00430042_m1, MyHC3: Hs01074230_m1, MYHC7: Hs01110632_m1, MYHC8: Hs00267293_m1 and 18s: Hs99999901_s1 was used as an internal control. All reactions were carried out in a 20 µl volume, 1 µl cDNA, 10 µl 2X Taqman Mastermix, 8 µl water, 1 µl primer/probe on an Agilent Biosystem Light Cycler with the following conditions, 95°C 15s, followed by 40X at 95°C for 15s and 60°C for 1 min. The Ct values from the target gene were normalized to 18s and expression levels calculated according to $2^{-\Delta\Delta Ct}$ method to determine fold changes.

Statistical analysis

All data were tested for normality using the Shapiro-Wilk test. If data were not normally distributed, analysis was performed on log-transformed data, or non-parametric tests were used as appropriate. Partial correlations, controlled for age and gender, were performed to determine the relationship between vitamin D metabolites and physiological outcomes. Spearman's or

Pearson's bivariate correlations were performed as appropriate to determine the relationship between vitamin D metabolites and skeletal muscle gene expression data. The LC/MS-MS lower detection limit for $1\alpha,25(\text{OH})_2\text{D}_3$ analysis is 32pg/ml. A sensitivity analysis was performed removing cases that fell below this threshold. For all PCR data, one-way repeated measures ANOVA was performed on the ΔCT values with predetermined pairwise comparisons (vehicle vs 10nm $1\alpha,25(\text{OH})_2\text{D}_3$, vehicle vs 100nm $1\alpha,25(\text{OH})_2\text{D}_3$ and 10 vs 100nm $1\alpha,25(\text{OH})_2\text{D}_3$). Differences in outcome measures for patients classified as 'deficient' vs 'insufficient' were analysed by linear regression with age and gender added into the model as covariates. For tissue culture work data were expressed as fold change compared to the vehicle control condition ($2^{-\Delta\Delta\text{CT}}$). Effect sizes were estimated using Cohens d or eta squared (η^2) statistic as appropriate (d; interpreted small ≥ 0.20 , medium ≥ 0.50 , large ≥ 0.80 ; η^2 ; interpreted small ≥ 0.01 , medium ≥ 0.06 , large ≥ 0.14). Missing data was analysed using Little's test, to test the assumption of missing completely at random (MCAR). This showed that missing data was MCAR and so a complete case analysis was performed All statistical analyses were performed using IBM SPSS 25 software (IBM, Chicago, IL). Statistical significance was accepted as $P < 0.05$.

Results

Patient characteristics and vitamin D status

Patient characteristics for the *in vivo* study can be found in Table 1. In summary, median age was 63 (57-69 years), 19/34 patients were females, median eGFR was 24 (20-31 ml/min/1.73m²). Of these, 28/34 (82%) patients were vitamin D deficient (25(OH)D: <20ng/ml), and a further 6 (18%) were insufficient (25(OH)D: 21-29ng/ml). No patients exhibited sufficient vitamin D levels (25(OH)D: >30ng/ml) according to the Endocrine Society guideline criteria (41). Using the National Kidney Foundation Kidney Disease Outcome Quality Initiative (NKF KDOQI) guidelines, 4/34 (12%) patients were severely deficient (25(OH)D: <5ng/ml), 20/34 (59%) had a mild deficiency (25(OH)D: 5-15ng/ml), and 10/34 (29%) were insufficient (25(OH)D: 16-30ng/ml). No patients were classified as having sufficient vitamin D levels (25(OH)D: >30ng/ml) (42). Regardless of which criteria are used, all patients fell below the cut-offs defined for intervention (41).

In vivo investigation of the association between vitamin D and its metabolites and measures of muscle mass and physical function

Associations with physiological assessments

Correlations between vitamin D metabolites and physiological assessments can be found in Table 3. Positive, although small, correlations were seen between total vitamin D and both $\dot{V}O_{2Peak}$ ($\rho = 0.41$, $p = 0.04$) and STS60 performance ($\rho = 0.45$, $p = 0.02$), as well as between total vitamin D and ISWT ($\rho = 0.37$, $p = 0.06$), e1-RM ($\rho = 0.36$, $p = 0.07$), and percentage body fat ($\rho = -0.39$, $p = 0.05$). We saw a moderate association between the active form of vitamin D and $\dot{V}O_{2Peak}$ ($\rho = 0.53$, $p = 0.005$), e-1RM ($\rho = 0.50$, $p = 0.008$) and STS 60 performance ($\rho = 0.49$, $p = 0.01$). No meaningful association was seen with RF-CSA ($\rho = 0.35$, $p = 0.08$). However, all these relationships disappeared when individuals with values <32pg/ml were removed from the analysis ($n = 19$). When the cohort was split for 'deficiency' compared to 'insufficiency' based upon NKF-KDOQI guidelines, there was a significant difference in $\dot{V}O_{2peak}$ between the groups (deficiency: 17.4 (15.0-20.5) vs insufficiency: 22.3 (17.7-28.4ml/min/kg; $p = 0.006$) but not for performance in the ISWT (deficiency: 350 (262-495) vs insufficiency: 395 (345-672m; $p = 0.16$). Characteristics for patients within these groups can be found in supplementary table 1.

Associations with skeletal muscle gene expression

Characteristics of the 20 patients who donated muscle biopsies used in this *ex vivo* analysis can be found in Table 2. Partial correlations can be found in Table 4. A negative association was seen between 25OHD and Activin type II receptor ($\rho = -0.69$, $p = 0.03$), MuRF-1 ($\rho = -0.75$, $p = 0.01$) and MAFbx ($\rho = -0.79$, $p = 0.006$). No other correlations were observed.

Ex vivo investigation of the effect of Vitamin D on human derived skeletal muscle cells

Characteristics of those patients ($n=5$) used in this investigation can be found in Table 2.

Effects on inflammation, protein degradation and myogenesis

Doses of both 10 and 100nm $1\alpha,25(\text{OH})_2\text{D}_3$ reduced IL-6 mRNA expression in myotubes 2-fold compared to the vehicle condition ($p = 0.03$, $d = 1.5$; $p = 0.02$, $d = 0.9$ respectively), which were both large effects. However, there was no significant effect of either dose on TNF- α ($p = 0.35$; $d = 0.18$; Figure 1). There was a trend for $1\alpha,25(\text{OH})_2\text{D}_3$ to reduce expression of myostatin by 1.6-fold (10nm) and 2-fold (100nm) compared to the vehicle, but this was only a small effect ($p = 0.07$, $\eta^2 = 0.47$). There was also a trend to reduce expression of MuRF-1 by 1.8-fold (10nm) and 1.1-fold (100nm; $p = 0.08$, $\eta^2 = 0.57$). No effect was seen of either dose on MAFbx expression ($p = 0.32$, $\eta^2 = 0.25$; Figure 2). When the expression of the myogenic regulatory factors was determined, there was a trend for $1\alpha,25(\text{OH})_2\text{D}_3$ to reduce myogenin expression by 1.4-fold (10nm) and 2-fold (100nm) ($p = 0.08$, $\eta^2 = 0.46$), but MyoD expression was unchanged ($p = 0.10$, $\eta^2 = 0.44$). There was no effect of $1\alpha,25(\text{OH})_2\text{D}_3$ on pax7 expression ($p = 0.42$, $\eta^2 = 0.21$; Figure 3). 10nm $1\alpha,25(\text{OH})_2\text{D}_3$ was seen to significantly reduce expression of MYHC1 compared to the vehicle condition by 5-fold ($p = 0.04$, $d = 0.57$) and a similar 5-fold reduction was seen for 100nm ($p = 0.09$, $d = 1.0$). An effect of 10nm $1\alpha,25(\text{OH})_2\text{D}_3$ was also seen on MyHC8 expression, which was reduced by 2.5-fold ($p = 0.03$, $d = 0.53$), a similar reduction was seen with 100nm ($p = 0.07$, $d = 1.01$; Figure 4). No effect of either dose was seen on MYHC2 ($p = 0.12$, $\eta^2 = 0.41$), MYHC3 ($p = 0.07$, $\eta^2 = 0.58$) or MYHC7 ($p = 0.23$, $\eta^2 = 0.31$) mRNA expression. There was no effect of $1\alpha,25(\text{OH})_2\text{D}_3$ at either dose on mRNA expression of the Vitamin D receptor ($p = 0.39$, $\eta^2 = 0.13$).

Effects on morphology

There was no effect of either 10nm or 100nm $1\alpha,25(\text{OH})_2\text{D}_3$ compared to vehicle on myotube

diameter (10nm = 18.2 ± 3.1 vs 100nm = 19.7 ± 4.4 vs vehicle = $16.5 \pm 2.4\mu\text{m}$; $p = 0.37$, $\eta^2 = 0.22$), or fusion index (10nm = 23.0 ± 9.5 vs 100nm = 23.8 ± 12.8 vs vehicle = $22.9 \pm 6.4\%$; $p = 0.85$, $\eta^2 = 0.05$). 100nm $1\alpha,25(\text{OH})_2\text{D}_3$ resulted in significantly fewer myotubes per field of view compared to the vehicle ($p = 0.03$, $d = 0.84$), with no differences between vehicle vs 10nm ($p = 0.11$, $d = 0.28$) or 10nm vs 100nm ($p = 0.24$, $d = 0.38$) (10nm = 4 ± 4 vs 100nm = 3 ± 2 vs vehicle = 5 ± 3 myotubes per field of view; Figure 5).

Effect on cell proliferation

There was no effect of $1\alpha,25(\text{OH})_2\text{D}_3$ on myoblast proliferation, with no difference in geometric mean fluorescent intensity of CFSE in response to either 10nm or 100nm dose, compared to vehicle ($P = 0.77$, $\eta^2 = 0.09$).

Discussion

The aims of this study were two-fold. Firstly, to perform *in vivo* analysis to determine the relationship between serum vitamin D and its metabolites with skeletal muscle mass and function in CKD patients not requiring dialysis and to establish if vitamin D deficiency might contribute to reduced physical function. Secondly, to determine if $1\alpha,25(\text{OH})_2\text{D}_3$ supplementation in human-derived skeletal muscle cells established from CKD vitamin D deficient donors, could improve myoblast proliferation, differentiation, and myotube size.

Our data demonstrates a high prevalence of vitamin D deficiency, with all patients meeting the guidelines for initiation of vitamin D supplementation (25(OH)D levels: $<30\text{ng/ml}$). This is in line with previous reports (41, 43) and highlights the need to understand the physiological effects of such a deficiency. Data from healthy population cohorts demonstrate a relationship between vitamin D and muscle function. In particular, vitamin D levels are associated with muscular strength (44) and physical function (21), with vitamin D supplementation reducing the risk of falls in elderly populations (45). However, the influence of vitamin D status on muscle mass or size is less clear. A study in vitamin D receptor knock out mice found smaller muscle fibres compared to their sham littermates (46), but a study of nearly 700 healthy men and women was unable to find an association between total or active vitamin D and muscle mass (47). The authors concluded that the link between falls and vitamin D may be due to effects upon neuromuscular function rather than muscle mass.

There is a scarcity of data available on the role of vitamin D in muscle mass/function in the CKD population. Gordon and colleagues reported that active vitamin D was associated with gait speed, sit to stand tests, 6-minute walk test, and isokinetic muscle strength in CKD patients at stage G3-4 (27). They also saw an association with muscle size when calcium and physical activity levels were controlled for. A similar association has also been observed between total vitamin D (although not active vitamin D), muscle strength, and falls also in CKD patients not requiring dialysis (28). Further, vitamin D supplementation in CKD patients at stages G3-4 has been shown to improve measures of physical functioning (48). We observed a significant association between both the total and active forms of vitamin D with and VO_{2Peak} , and performance in the STS-60. This tentatively suggests a role for vitamin D in determining exercise capacity and physical function in these patients. An association between serum vitamin D and cardiorespiratory fitness has been reported recently in the healthy population (49), which may be driven through effects upon oxidative metabolism (50, 51). Interestingly, we have recently shown in the same cohort of patients as those reported on here, reduced mitochondrial number compared to a healthy control cohort (52). It would, therefore, be important to know the effect of vitamin D on skeletal muscle mitochondrial function in these patients. It is also possible that this association of vitamin D with exercise capacity and physical function is acting through an effect upon patient's symptom and fatigue perception rather than a direct effect upon skeletal muscle *per se*. This is an interesting observation that warrants closer investigation.

We also report some associations between vitamin D metabolites and the physiological outcome measures. However, there is limited previously published data on these relationships (53) and more research is required to understand the importance of these associations. These relatively ambiguous results highlight the need for more definitive studies to better understand the relationship between the vitamin D metabolome and physical function and muscle function and mass and the relative importance of supplementation in this group.

As it was thought vitamin D may play a role in the maintenance of muscle mass (46), we hypothesised that the addition of $1\alpha,25(OH)_2D_3$ to primary skeletal muscle cells from vitamin D deficient donors would reduce myoblast proliferation and increase myoblast differentiation and myotube size. Studies of C2C12 cells and primary skeletal muscle cells have shown that Vitamin D has anti-proliferative effects (32, 54). However, we saw no effect of either dose of

1 α ,25(OH)₂D₃ on myoblast proliferation rates after 72h of treatment, which has been reported previously in C2C12 cells (55), although a lower dose of 1nm was used in this study. There was also no effect of supplementation on differentiation, fusion index, desmin positivity, or myotube diameter and no change in the mRNA expression of MyoD or myogenin, which are usually required for myotube fusion. Rather, we observed a reduction in the mRNA expression of MyHC1 (type IIX) and MyHC8 (perinatal) with 10nm 1 α ,25(OH)₂D₃, which is in contrast to previous reports (54). We did, however, observe a decrease in the number of myotubes formed following 100nm 1 α ,25(OH)₂D₃, which has been reported before in both primary human skeletal muscle cells and C2C12 cells (30, 31). Therefore, the reduction in MyHC expression may just be an artefact of the reduced number of myotubes in culture. Vitamin D has also been reported to have hypertrophic effects *ex vivo* (30, 56), with 1 α ,25(OH)₂D₃ supplementation resulting in increases in myotube diameter. However, no evidence of 1 α ,25(OH)₂D₃ stimulated hypertrophy was seen here. The reasons for these discrepancies are currently unclear. A lack of an effect on hypertrophy may be the result of the absence of a change in myostatin mRNA expression. Both *in vitro* and *in vivo* studies have demonstrated that vitamin D supplementation reduces skeletal muscle myostatin mRNA expression (32, 54). It is possible that CKD has induced a higher myostatin expression in these cells (57), which is not modifiable by 1 α ,25(OH)₂D₃ exposure and that this higher expression level prevents hypertrophy driven by other mechanisms. The lack of any large effect on our human-derived skeletal muscle cells is in agreement with our *in vivo* data, which failed to find any associations between vitamin D and measures of muscle size or strength. This lends support to the notion that vitamin D does not directly affect skeletal muscle in these patients.

Interestingly, IL-6 mRNA expression was reduced with both doses of 1 α ,25(OH)₂D₃, which is in contrast to *in vivo* results, where there was no relationship seen between any form of vitamin D and skeletal muscle IL-6 mRNA expression. There has been little reported regarding the anti-inflammatory properties of vitamin D in skeletal muscle, but there is evidence from other systems (58). The effect on IL-6 was seen in the absence of any reduction of TNF- α expression, and was, therefore, unlikely to have resulted in significant anti-inflammatory affects. Given the role IL-6 is known to play in skeletal muscle wasting (59), this does warrant further investigation.

There are a few limitations of this study that should be taken into account. Firstly, this is a secondary analysis of an earlier study and was therefore not powered to detect relationships

between vitamin D deficiency and physical function or muscle mass. Given the prevalence of vitamin D deficiency in these patients and the role this plays in physical function in other groups, a suitably powered study is warranted to better understand its implications. We suspect that many of the discrepancies in the *ex vivo* results presented here might be explained by differences in the models used in the experiments, human vs rodent and immortalised vs primary cell lines. The effect of vitamin D administration in human-derived skeletal muscle cells from CKD donors has not been investigated before. Given the complicated and diverse effects of CKD upon skeletal muscle physiology, it is likely that vitamin D supplementation is not sufficient to overcome more potent effects imposed by the illness. These results are only based on five patients that exhibit a large degree of variation which is likely masking real effects. A larger sample size might provide more definitive results and as such these results should only be considered preliminary. Finally, this study has performed individual metabolite analysis only. It may be interesting to apply modelling strategies that take into account the interplay between the different metabolites (60) to get a full overview of the effect of the vitamin D metabolome on physical function and muscle mass in these patients.

In conclusion, we have seen no strong evidence for a role of total or active vitamin D in determining the level of muscle size or strength in these patients. At the cellular level, our preliminary data suggests there is no effect of $1\alpha,25(\text{OH})_2\text{D}_3$ supplementation on myoblast proliferation, differentiation or hypertrophy. We did, however, see an association between total vitamin D and $\text{VO}_{2\text{Peak}}$ and STS60 performance which was also seen with active vitamin D before the sensitivity analysis. This suggests that vitamin D deficiency is not a prominent factor driving the loss of muscle mass in CKD, but may have a role to play in the poor exercise tolerance and low exercise capacity seen in these patients. In light of the high prevalence of vitamin D deficiency in this patient population, further investigation is warranted to understand the role of this hormone on skeletal muscle physiology in CKD.

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Conflict of interest statement

No authors have any conflicts to disclose.

Author contributions

EW, TWJ, DWG, MH, CJ, AP and AS were involved in study conception and plan. EW, TJW, SX, MGB, RM, and DWG were involved in patient recruitment and performed assessments. EW, LB, DWG, TOS, CJ, MH and AP were involved in laboratory analysis, data analysis interpretation. EW, LB, TJW, AP and AS were responsible for preparing the manuscript for submission. All authors approve this submission.

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Tables

699 **Table 1.** Patient characteristics for the *in vivo* study

Characteristic	N	Full Cohort (n=34)	N	Biopsied Patients (n=20)
Age (years)	38	63 (57-69)	20	63 (58-71)
Gender (female)	38	19 (59%)	20	14 (70%)
eGFR (ml/min/1.73m ²)	38	24 (20-31)	20	24 (21-32)
VO _{2Peak} (ml/min/kg)	34	18.1 (15.7-23.5)	20	17.7 (15.7-25.4)
ISWT (m)	36	360 (270-520)	18	480 (350-655)
STS60 (reps)	35	26 (22-30)	19	27 (23-45)
e1RM (kg)	34	48 (34-58)	17	49 (37-57)
Muscle volume (cm ³)	33	931.7 (752.5-1091.9)	17	960 (858-1139)
Rectus femoris CSA (cm ²)	36	8.2(5.7-10.2)	18	9.4 (8.0-10.6)
Body fat (%)	31	36.9 (29.2-47.7)	18	35.2 (28.5-44.1)
Appendicular lean mass (kg)	33	19.6 (18.6-23.8)	18	21.1 (19.1-24.9)
25(OH)D (ng/ml)	38	10.8 (7.9-18.0)	20	12.6 (8.7-19.1)
25(OH)D2 (ng/ml)	38	0.5 (0.3-1.0)	20	0.6 (0.3-1.0)
25(OH)D3 (ng/ml)	38	11.1 ± 6.8	20	10.9 (7.2-12.2)
24,25(OH) ₂ D3 (ng/ml)	38	0.4 (0.3-0.7)	20	0.5 (0.3-0.7)
3-Epi-25(OH)D3 (ng/ml)	38	0.9 (0.8-1.1)	20	1.0 (0.8-1.1)

1 α ,25(OH) ₂ D ₃ (pg/ml)	38	32.8 (26.8-44.7)	20	37.5 (28.6-47.0)
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Note: unless otherwise stated all data are presented as median and interquartile range.

Abbreviations: CSA, cross-sectional area; e1RM, estimated 1 repetition maximum; eGFR, estimated glomerular filtration rate; ISWT, incremental shuttle walk test; STS60, Sit to stand 60.

Table 2. Patient characteristics for *ex vivo* biopsy analysis and cell culture experiments.

Characteristic	Donors used in biopsy PCR analysis	Donors used in primary cell culture experiments
n	20	5
Age (years)	58 (57-63)	57 (52-58)
Gender (female)	4 (80%)	4 (80%)
eGFR (ml/min/1.73m ²)	23 (19-29)	23 (19-29)
25(OH)D (ng/ml)	10.0 (9.2-12.0)	10.0 (9.2-12.0)
VO ₂ Peak (ml/min/kg)	16.7 (15.9-24.5)	16.1 (15.7-27.0)
ISWT (m)	540 (370-560)	575 (495-635)
STS60 (reps)	27 (26-39)	28 (27-38)
e1RM (kg)	48 (44-53)	53 (50-54)
Muscle volume (cm ³)	973 (955-1039)	960 (916-1026)
Rectus femoris CSA (cm ²)	8.7 (7.5-9.5)	9.0 (8.2-9.6)
Body fat (%)	34 (30-43)	39.3 (35.9-44.2)

Note: unless otherwise stated all data are presented as median and interquartile range.

Abbreviations: CSA, cross-sectional area; e1RM, estimated 1 repetition maximum; eGFR, estimated glomerular filtration rate; ISWT, incremental shuttle walk test; STS60, Sit to stand 60.

Table 3. Bivariate correlations between serum vitamin D and vitamin D metabolites and physiological outcome measuresData are correlation coefficients with p values in brackets. * denotes $p < 0.05$

Physiological outcome measure	25(OH)D	25OHD2	25OHD3	24,25(OH) ₂ D3	3-Epi-25OHD3	1 α ,25(OH) ₂ D3	1 α ,25(OH) ₂ D3 (>32pg/ml [§])
eGFR (ml/min/1.72m ²)	0.017 (p = 0.99)	-0.15 (p = 0.55)	-0.008 (p = 0.97)	0.22 (p = 0.28)	0.47 (p = 0.01)	0.14 (p = 0.48)	-0.13 (p = 0.66)
VO _{2Peak} (ml/min/kg)	0.41* (p = 0.04)	0.38 (p = 0.14)	0.36 (p = 0.70)	0.40* (p = 0.04)	0.25 (p = 0.21)	0.53* (p = 0.005)	0.25 (p = 0.39)
ISWT (m)	0.37 (p = 0.06)	0.08 (p = 0.75)	0.36 (p = 0.07)	0.40* (p = 0.04)	0.41* (p = 0.04)	0.25* (p = 0.20)	-0.04 (p = 0.89)
e-1RM (kg)	0.36 (p = 0.07)	-0.26 (p = 0.30)	0.36 (p = 0.07)	0.33 (p = 0.10)	0.43* (p = 0.03)	0.50* (p = 0.008)	0.31 (p = 0.28)
RF-CSA (cm ²)	0.28 (p = 0.16)	-0.45 (p = 0.07)	0.28 (p = 0.16)	0.33 (p = 0.11)	0.47* (p = 0.02)	0.35 (p = 0.08)	0.04 (p = 0.89)
Quadriceps volume (cm ³)	0.03 (p = 0.89)	-0.50* (p = 0.04)	0.04 (p = 0.83)	0.16 (p = 0.43)	0.22 (p = 0.27)	0.24 (p = 0.24)	-0.04 (p = 0.89)
Appendicular lean mass (kg)	0.27 (p = 0.17)	-0.60 (p = 0.10)	0.30 (p = 0.13)	0.31 (p = 0.12)	0.47* (p = 0.02)	0.36 (p = 0.10)	0.12 (p = 0.67)
Body fat (%)	-0.39 (p = 0.05)	-0.15 (p = 0.58)	-0.38 (p = 0.06)	-0.22 (p = 0.28)	-0.21 (p = 0.29)	-0.07 (p = 0.70)	-0.25 (p = 0.39)
STS60 (reps)	0.45* (p = 0.02)	0.20 (p = 0.45)	0.43* (p = 0.03)	0.36 (p = 0.07)	0.15 (p = 0.45)	0.49* (p = 0.01)	0.47 (p = 0.09)

Abbreviations: e-1RM, estimated 1-repetition maximum; eGFR, estimated glomerular filtration rate; ISWT, incremental shuttle walk test; RF-CSA, rectus femoris cross-sectional area. STS60, sit to stand 60.

[§] Cases <32pg/ml removed as below the LC/MS-MS lower detection limit

Gene	25(OH)D	25OHD2	25OHD3	24,25(OH) ₂ D3	3-Epi-25OHD3	1 α ,25(OH) ₂ D3
Myostatin	0.06 (p = 0.83)	-0.48 (p = 0.16)	0.17 (p = 0.49)	-0.04 (p = 0.87)	-0.05 (p = 0.86)	0.06 (p = 0.84)
Activin type II receptor	-0.08 (p = 0.79)	-0.69* (p = 0.03)	0.13 (p = 0.57)	-0.04 (p = 0.89)	-0.03 (p = 0.92)	0.19 (p = 0.53)
MuRF-1	0.07 (p = 0.82)	-0.75* (p = 0.01)	0.12 (p = 0.64)	0.12 (p = 0.69)	-0.03 (p = 0.90)	0.29 (p = 0.32)
MAFbx	-0.06 (p = 0.83)	-0.79* (p = 0.006)	-0.02 (p = 0.92)	0.02 (p = 0.92)	0.06 (p = 0.84)	0.24 (p = 0.41)
MyoD	-0.30 (p = 0.31)	-0.49 (p = 0.14)	-0.17 (p = 0.45)	-0.12 (p = 0.68)	-0.02 (p = 0.93)	-0.21 (p = 0.47)
Myf5	0.12 (p = 0.69)	-0.23 (p = 0.51)	0.10 (p = 0.68)	0.18 (p = 0.55)	0.11 (p = 0.70)	-0.11 (p = 0.70)
Myogenin	-0.16 (p = 0.60)	-0.59 (p = 0.07)	-0.04 (p = 0.87)	-0.10 (p = 0.72)	0.08 (p = 0.97)	-0.04 (p = 0.89)
Pax7	-0.13 (p = 0.67)	-0.63 (p = 0.05)	-0.11 (p = 0.65)	0.02 (p = 0.92)	-0.05 (p = 0.85)	0.17 (p = 0.56)
TNF- α	-0.33 (p = 0.26)	0.002 (p = 0.99)	-0.28 (p = 0.24)	-0.27 (p = 0.35)	-0.38 (p = 0.20)	-0.11 (p = 0.71)
IL-6	-0.36 (p = 0.23)	-0.55 (p = 0.10)	-0.28 (p = 0.24)	-0.35 (p = 0.24)	-0.33 (p = 0.26)	0.02 (p = 0.93)
MCP-1	-0.18 (p = 0.54)	-0.55 (p = 0.09)	0.02 (p = 0.93)	-0.10 (p = 0.73)	-0.01 (p = 0.95)	-0.29 (p = 0.33)

Table 4. Partial correlations between serum vitamin D and vitamin D metabolites and expression levels of genes involved in processes of maintenance of muscle mass.

Data are correlation coefficients with p values in brackets. * denotes p<0.05

Abbreviations: IL-6, Interleukin-6; MAFbx, muscle atrophy F box; MCP-1, monocyte chemoattractant protein-1; MuRF-1. Muscle RING finger 1; myf5, myogenic factor 5; myoD, myoblast determination protein; TNF- α , tumor necrosis factor alpha.

Figure Legends

Figure 1. Effect of 10nm and 100nm 1 α ,25(OH) $_2$ D3 vs vehicle on inflammatory cytokine mRNA expression. a) IL-6; b) TNF- α . Real time PCR data presented as $2^{-\Delta\Delta CT}$ relative to vehicle. * denotes P<0.05 vs baseline.

Figure 2. Effect of 10nm and 100nm 1 α ,25(OH) $_2$ D3 vs vehicle on mRNA expression of proteins relating to muscle protein breakdown. a) Myostatin; b) MAFbx; c) MuRF-1.

Figure 3. Effect of 10nm and 100nm 1 α ,25(OH) $_2$ D3 vs vehicle on mRNA expression of proteins relating to myogenesis. a) MyoD; b) myogenin; c) Pax7.

Figure 4. Effect of 10nm and 100nm 1 α ,25(OH) $_2$ D3 vs vehicle on mRNA expression of myosin heavy chain isoforms. a) MyH1; b) MyH2; c) MyH3; d) MyH7; e) MyH8. * denotes P<0.05 vs vehicle.

Figure 5. Effect of 10nm and 100nm 1 α ,25(OH) $_2$ D3 vs vehicle on myotube morphology.

a) myotube diameter (μ m); b) myotube number per field; c) fusion index (%); d) desmin positive cells (%); e) representative immunofluorescent images. Cells are counterstained for desmin (green) and DAPI (blue). Scale bar represents 100 μ m. * denotes P<0.05 vs vehicle.

753 Figure 1

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Figure 3

Figure 4

Figure 5