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

Emma L Watson[1], Thomas J Wilkinson[2], Tom F O'Sullivan[3], Luke A Baker[2] Douglas W Gould[1], Soteris Xenophontos[2], Matthew PM Graham-Brown[1], Rupert W Major[2], Carl Jenkinson[4], Martin Hewison[4,5], Andrew Philp[6,7] and Alice C Smith[2].

[1] Department of Cardiovascular Sciences, University of Leicester, United Kingdom

[2] Department of Health Sciences, University of Leicester, United Kingdom

[3] Department of Infection, Immunity and Inflammation, University of Leicester, United Kingdom

[4] Institute of Metabolism and Systems Research, University of Birmingham, Birmingham, United Kingdom

[5] Centre for Endocrinology, Diabetes and Metabolism, Birmingham Health Partners, Birmingham, United Kingdom

[6] Garvan Institute of Medical Research, New South Wales, Australia

[7] UNSW Medicine, UNSW Sydney, New South Wales, Australia

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Corresponding author:

Emma L Watson

Leicester Kidney Lifestyle Team

Dept of Cardiovascular Sciences

Medical Sciences Building

University of Leicester

Leicester

LE1 9HN

UK

Tel: (+44-116) 252 5033

Fax: (+44-116) 252 5030

email: emma.watson@le.ac.uk

32   Abbreviations

33   1-RM – 1 repetition maximum

34    $1\alpha,25(OH)_2D3$ – 1,25-dihydroxyvitamin D3

35    $24,25(OH)_2D3$ - 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

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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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80   Abstract

81   Evidence is growing for a role of vitamin D in regulating skeletal muscle mass, strength and

82   functional capacity. Given the role the kidneys play in activating total vitamin D, and the high

83   prevalence of vitamin D deficiency in Chronic Kidney Disease (CKD), it is possible that

84   deficiency contributes to the low levels of physical function and muscle mass in these patients.

85   This is a secondary cross-sectional analysis of previously published interventional study, with

86   *ex vivo* follow up work. 34 CKD patients at stages G3b-5 (eGFR 25.5 ± 8.3ml/min/1.73m2;

87   age 61 ± 12 years) were recruited, with a sub-group (n=20) also donating a muscle biopsy.

88   Vitamin D and associated metabolites were analysed in plasma by liquid chromatography

89   tandem-mass spectroscopy and correlated to a range of physiological tests of muscle size,

90   function, exercise capacity and body composition. The effects of $1\alpha,25(OH)2D3$

91   supplementation on myogenesis and myotube size was investigated in primary skeletal muscle

92   cells from vitamin D deficient donors. *In vivo*, there was no association between total or active

93   vitamin D and muscle size or strength, but a significant correlation with $\dot{V}O_{2Peak}$ was seen with

94   the total form. *Ex vivo*, $1\alpha,25(OH)_2D3$ supplementation reduced IL-6 mRNA expression, but

95   had no effect upon proliferation, differentiation or myotube diameter. This early preliminary

96   work suggests that vitamin D deficiency is not a prominent factor driving the loss of muscle

97   mass in CKD, but may play a role in reduced exercise capacity.

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

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109   Introduction

110   Patients with chronic kidney disease (CKD) commonly experience skeletal muscle wasting,

111   reduced exercise capacity and lower levels of physical function (1-3). These appear early in the

112   disease process (4) and are associated with adverse clinical outcomes and reduced quality of

113   life (5-10). The factors driving loss of muscle mass and physical function are not yet fully

114   understood but are likely to be multifactorial with large heterogeneity. Gaps in our

115   understanding have meant that there are currently no viable therapies to protect or restore

116   muscle mass and physical function in CKD.

117   The classical effects of vitamin D focus around calcium homeostasis and bone health, but it is

118   becoming increasingly accepted that it may also have a role in skeletal muscle function (11,

119   12) and exercise capacity (13). Studies involving both humans and animals have shown that

120   vitamin D deficiency is associated with muscle atrophy affecting predominately type II fibres

121   (14, 15), which can be reversed following vitamin D supplementation (16). Vitamin D

122   deficiency is also associated with an increased number of falls (17), which in some cases can

123   be prevented with supplementation (18, 19). Community-based cross-sectional studies have

124   shown vitamin D deficiency is associated with reduced measures of physical functioning such

125   as gait speed and rising from a chair (20, 21). However, studies in both healthy and clinical

126   populations have not always demonstrated improvements in physical functioning following

127   vitamin D supplementation (22-24). Therefore, the role of vitamin D in the maintenance or

128   improvement of physical function requires further examination.

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130   Vitamin D obtained from sunlight or through dietary sources is relatively inactive and must be

131   converted to the active form, of which the final step occurs in the kidney by 1α-hydroxylase.

132   Given the impairment of kidney function, vitamin D deficiency is highly prevalent in CKD

133   patients (25). Despite this, there is limited and conflicting data regarding the association

134   between vitamin D and physical function in the CKD population. One study of CKD patients

135   failed to find an association between the active metabolite, 1,25-dihydroxyvitamin D3

136   ($1α,25(OH)_2D3$), and muscle function (26), whilst others in both non-dialysis CKD (27) and

137   end-stage renal disease (28) have reported associations between $1α,25(OH)_2D3$ muscle

138   strength and physical functioning.

139   Mature skeletal muscle cells are terminally differentiated, and by themselves, are capable of

140   limited repair and regeneration. Therefore for repair to occur, cells are reliant on a population

141 of stem cells, termed satellite cells, that support repair and regeneration through a process
142 called myogenesis (29). Myogenesis is thought to be dysfunctional in CKD (29) and may
143 contribute to skeletal muscle wasting. A role is also emerging for vitamin D in skeletal muscle
144 repair (30) where it has been shown to influence satellite cell proliferation and differentiation
145 (31, 32). Therefore, it is possible that vitamin D deficiency contributes to atrophy through
146 inhibition of myogenesis in CKD, but this is yet to be investigated.

147 Vitamin D status is generally based upon the analysis of the inactive total form of vitamin D
148 (25OHD). However, related metabolites have also been shown to be clinically important (33),
149 demonstrating the importance to also consider the vitamin D metabolome alongside total
150 vitamin D, which by itself provides only a limited view of vitamin D status.

151 The aims of this study were: 1) to perform *in vivo* analysis to determine the relationship
152 between serum vitamin D and its metabolites and skeletal muscle mass and function in patients
153 with CKD not requiring dialysis; and 2) to determine *ex vivo* the effect of $1\alpha,25(OH)_2D3$
154 supplementation on myoblast proliferation, differentiation, and hypertrophy using human-
155 derived skeletal muscle cells isolated from CKD vitamin D deficient donors. We hypothesised
156 that plasma $1\alpha,25(OH)_2D3$ would be associated with muscle size, strength and exercise
157 capacity and that supplementation of vitamin D deficient cells with $1\alpha,25(OH)_2D3$ would
158 increase myotube size.

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169   Material and Methods

170   Patients and study design

171   This study was a cross-sectional observational design. Patients in this report are from two

172   separate cohorts. Physical function data is taken from the ExTra CKD study (34) (ISRCTN:

173   36489137), whilst patients who donated biopsies used in the *ex vivo* study took part in the

174   Explore CKD study (ISRCTN: 18221837). Sample size was based upon available data from

175   these participants. All patients were recruited from nephrology outpatient clinics at Leicester

176   General Hospital, UK between December 2013 – April 2017. Exclusion criteria were age

177   <18years, pregnancy, disability that prevented patients from undertaking exercise, insufficient

178   command of English, or an inability to give informed consent. Ethical approval was given by

179   the National Research Ethics Committee (13/EM/0344; 15/EM/0467). All patients gave written

180   informed consent and the trial was conducted in accordance with the Declaration of Helsinki.

181   Physiological assessments

182   *Muscle size*

183   Muscle size was determined using two methods: (i) quadriceps volume of the right leg

184   measured by Magnetic Resonance Imaging (MRI) (34) acquired using a 3T Siemens Skyra HD

185   MRI scanner in the axial plane using a T1 turbo spin-echo sequence and (ii) rectus femoris

186   cross-sectional area of the right leg measured by 2-D B-mode ultrasound. These techniques

187   have previously been described by our group (35).

188   *Muscle strength*

189   Quadriceps strength was assessed by leg extension exercise using a 5-Repetition Maximum (5-

190   RM) test (34). Prediction equations were then used to estimate 1-Repetition Maximum (1-RM)

191   (36).

192   *Exercise capacity*

193   Patients underwent the incremental shuttle walk test (ISWT) (34) during which patients walked

194   along a 10m course in time with externally paced beeps that become progressively quicker until

195   volitional fatigue. This is a valid and reliable method to determine peak exercise capacity

196   ($\dot{V}O_{2peak}$) (37). Patients also underwent an incremental Cardiopulmonary Exercise Test to

197   measure $\dot{V}O_{2peak}$ performed on an electrically-braked cycle ergometer (34).

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199   *Physical Function*

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

201   muscular endurance (37).

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203   *Body composition*

204   Body fat percentage and appendicular lean mass (ALM), was estimated using multi-frequency

205   bioelectrical impedance analysis (BIA) (InBody 370, CA,USA). This device has been validated

206   against dual-energy x-ray absorptiometry (38).

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208   Blood sampling and Vitamin D metabolite analysis

209   Venous blood samples were taken from 38 CKD patients (Table 1) into a plain tube and left

210   undisturbed at room temperature for 30min to allow the blood to clot. The blood was then

211   centrifuged at 1500 g for 10min at 4°C. Resulting serum was collecting and stored at -80°C

212   until subsequent analysis. Serum concentrations of vitamin D metabolites were analysed by

213   liquid chromatography-tandem mass spectrometry (LC-MS/MS) as previously described (39).

214   Briefly, 200µl serum was extracted prior to analysis by protein precipitation followed by

215   supportive liquid-liquid extraction. Analysis was performed on a Waters Acquity UPLC

216   coupled to a Waters Xevo TQ-XS mass spectrometer. Analysis was carried out in multiple

217   reaction monitoring (MRM) for the following analytes: 25-hydroxyvitamin D3 (25OHD3), 3-

218   epi-25-hydroxyvitamin D3 (3-epi-25OHD3), 24,25-dihydroxyvitamin D3 ($24,25(OH)_2D3$),

219   1,25-dihydroxyvitamin D3 ($1\alpha,25(OH)_2D3$) and 25-hydroxyvitamin D2 (25OHD2). The LC-

220   MS/MS method for vitamin D quantification was validated for serum analysis as previously

221   described for accuracy, precision, recovery and matrix effects (39). Vitamin D metabolites

222   were purchased from Supleco Sigma Aldrich. LC-MS grade methanol and water were

223   purchased from Greyhound Chromatography and Thermo Fisher respectively. Supportive

224   liquid-liquid extraction plates were purchased from Phenomenex.

225   Muscle biopsy collection

226   Vastus lateralis muscle biopsies were taken from 20 patients using the micro biopsy technique

227   after an overnight fast (40). Biopsy specimens from five of these patients deemed to be vitamin

228   D deficient (25(OH)D <20ng/ml) were also used to establish primary cultures as described

229   below in which the effect of vitamin D repletion could be more closely studied. After

230   dissection of any visible fat and connective tissue, samples were placed into liquid nitrogen

231   (RNA extraction) or 5mL ice-cold Hams F10 media containing 1% penicillin streptomycin and

232   1% Gentamycin (cell culture).

Satellite cell isolation procedure and cell treatments

234   Muscle tissue was washed in HamsF10 (containing 1% penicillin streptomycin and 1%

235   Gentamycin), minced into small fragments and enzymatically digested in two incubations with

236   collagenase IV (1mg/mL), Bovine Serum Albumin (BSA) (5mg/mL) and trypsin (500μl/mL)

237   at 37°C with gentle agitation. The resultant supernatant was added to Foetal Bovine Serum

238   (FBS), strained through a 70μm nylon filter and centrifuged at 800 g for 7min. The cells were

239   washed in Hams F10 with 1% penicillin streptomycin and 1% Gentamycin and pre-plated on

240   uncoated $9cm^2$ petris in 3mL growth media (GM; Hams F10 Glutamax, 20% FBS, 1%

241   Penicillin Streptomycin, 1% fungazone) for 3h. The cell suspension was then moved to

242   collagen I coated $25cm^2$ flasks and kept at 37°C under humidified 95% air and 5% CO_2 until

243   cells had achieved approximately 70% confluence. For experiments, cells were plated at a

244   density of $3x10^4$ and grown until 70% confluent. For experiments using myoblasts, cells were

245   exposed to either high dose of exogenous $1\alpha,25(OH)_2D_3$ (100nmol), low dose (10nmol), or

246   control vehicle (95% EtOH), and proliferation rates determined after 72h. For experiments

247   using myotubes, GM was replaced with differentiation medium (DM; DMEM 4.5g/L glucose,

248   1% Penicillin Streptomycin, 10% horse serum) for five days by which time multinucleated

249   muscle fibres had formed. Cells were again exposed to either high dose of exogenous

250     $1\alpha,25(OH)_2D_3$ (100nmol), low dose (10nmol), or control vehicle (95% EtOH) to investigate

251   effects differentiation, determined after five days (immunofluorescence and PCR).

Proliferation assay

253   Carboxyfluorescein succinimidyl ester (CFSE) dye (Thermo Fisher, UK) was added to human

254   skeletal muscle cells in suspension (1ml HBSS) at a concentration of 5μm and incubated at

255   37°C for 20min. Staining was quenched by the addition of five volumes of GM and incubated

256   at 37°C for a further 5min. Cells were rinsed and seeded onto collagen I coated 6-well plates

257   and collected 72h later. Cells were then re-suspended in Phosphate Buffered Saline (PBS) and

258   analysed using a FACSCELESTA instrument (BD Biosciences). Data were analysed using

259   FlowJo 10.2 (FlowJo LLC, USA).

Immunofluorescence

261 Cells were fixed in 4% paraformaldehyde for 20min at room temperature, washed three times
262 with PBS and blocked and permeabilized in PBS containing 5% goat serum and 0.25% Triton
263 X-100 for 1h. Cells were incubated with rabbit anti-desmin primary antibody (1/400; cell
264 signalling) at 4°C overnight, washed three times in PBS, and incubated with Alexa Flour 488-
265 labelled goat anti-rabbit IgG (1/400; Thermo Fisher) for 2h at room temperature. DAPI
266 (100ng/mL) was used to visualise the nuclei. Ten random fields were acquired per condition
267 using a FLoid imaging system (Thermo Fisher) and images analysed using ImageJ. Myotube
268 diameter was assessed at three points on each cell. Fusion indexes were defined by the number
269 of DAPI positive nuclei within myotubes (desmin positive cell containing 3 or more nuclei)
270 divided by the total number of DAPI positive nuclei.

271 Quantitative RT-PCR

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

287 Statistical analysis

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

292   Pearson's bivariate correlations were performed as appropriate to determine the relationship

293   between vitamin D metabolites and skeletal muscle gene expression data. The LC/MS-MS

294   lower detection limit for $1\alpha,25(OH)_2D3$ analysis is 32pg/ml. A sensitivity analysis was

295   performed removing cases that fell below this threshold. For all PCR data, one-way repeated

296   measures ANOVA was performed on the ΔCT values with predetermined pairwise

297   comparisons (vehicle vs 10nm $1\alpha,25(OH)_2D3$, vehicle vs 100nm $1\alpha,25(OH)_2D3$ and 10 vs

298   100nm $1\alpha,25(OH)_2D3$. Differences in outcome measures for patients classified as 'deficient'

299   vs 'insufficient' were analysed by linear regression with age and gender added into the model

300   as covariates. For tissue culture work data were expressed as fold change compared to the

301   vehicle control condition ($2^{-\Delta\Delta CT}$). Effect sizes were estimated using Cohens d or eta squared

302   (n^2) statistic as appropriate (d; interpreted small ≥ 0.20, medium ≥ 0.50, large ≥ 0.80; n^2;

303   interpreted small ≥ 0.01, medium ≥ 0.06, large ≥ 0.14). Missing data was analysed using Little's

304   test, to test the assumption of missing completely at random (MCAR). This showed that

305   missing data was MCAR and so a complete case analysis was performed All statistical analyses

306   were performed using IBM SPSS 25 software (IBM, Chicago, IL). Statistical significance was

307   accepted as $P<0.05$.

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320 Results

321 *Patient characteristics and vitamin D status*

322 Patient characteristics for the *in vivo* study can be found in Table 1. In summary, median age
323 was 63 (57-69 years), 19/34 patients were females, median eGFR was 24 (20-31
324 ml/min/1.73m^2). Of these, 28/34 (82%) patients were vitamin D deficient (25(OH)D:
325 <20ng/ml), and a further 6 (18%) were insufficient (25(OH)D: 21-29ng/ml). No patients
326 exhibited sufficient vitamin D levels (25(OH)D: >30ng/ml) according to the Endocrine Society
327 guideline criteria (41). Using the National Kidney Foundation Kidney Disease Outcome
328 Quality Initiative (NKF KDOQI) guidelines, 4/34 (12%) patients were severely deficient
329 (25(OH)D: <5ng/ml), 20/34 (59%) had a mild deficiency (25(OH)D: 5-15ng/ml), and 10/34
330 (29%) were insufficient (25(OH)D: 16-30ng/ml). No patients were classified as having
331 sufficient vitamin D levels (25(OH)D: >30ng/ml) (42). Regardless of which criteria are used,
332 all patients fell below the cut-offs defined for intervention (41).

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

335 Associations with physiological assessments

336 Correlations between vitamin D metabolites and physiological assessments can be found in
337 Table 3. Positive, although small, correlations were seen between total vitamin D and both
338 VO$_{2Peak}$ (rho = 0.41, p = 0.04) and STS60 performance (rho = 0.45, p = 0.02), as well as between
339 total vitamin D and ISWT (rho = 0.37 p = 0.06), e1-RM (rho = 0.36, p = 0.07), and percentage
340 body fat (rho = -0.39, p = 0.05). We saw a moderate association between the active form of
341 vitamin D and $\dot{V}$O$_{2Peak}$ (rho = 0.53, p = 0.005), e-1RM (rho = 0.50, p = 0.008) and STS 60
342 performance (rho = 0.49, p = 0.01). No meaningful association was seen with RF-CSA (rho =
343 0.35, p = 0.08). However, all these relationships disappeared when individuals with values
344 <32pg/ml were removed from the analysis (n = 19). When the cohort was split for 'deficiency'
345 compared to 'insufficiency' based upon NKF-KDOQI guidelines, there was a significant
346 difference in $\dot{V}$O$_{2peak}$ between the groups (deficiency: 17.4 (15.0-20.5) vs insufficiency: 22.3
347 (17.7-28.4ml/min/kg; p = 0.006) but not for performance in the ISWT (deficiency: 350 (262-
348 495) vs insufficiency: 395 (345-672m; p = 0.16). Characteristics for patients within these
349 groups can be found in supplementary table 1.

350   Associations with skeletal muscle gene expression

351   Characteristics of the 20 patients who donated muscle biopsies used in this *ex vivo* analysis can

352   be found in Table 2. Partial correlations can be found in Table 4. A negative association was

353   seen between 25OHD and Activin type II receptor (rho -0.69, p = 0.03), MuRF-1 (rho -0.75, p

354   = 0.01) and MAFbx (rho -0.79, p = 0.006). No other correlations were observed.

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

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

357   Effects on inflammation, protein degradation and myogenesis

358   Doses of both 10 and 100nm $1\alpha,25(OH)_2D3$ reduced IL-6 mRNA expression in myotubes 2-

359   fold compared to the vehicle condition (p = 0.03, d = 1.5; p = 0.02, d = 0.9 respectively), which

360   were both large effects. However, there was no significant effect of either dose on TNF-α (p =

361   0.35; d = 0.18; Figure 1). There was a trend for $1\alpha,25(OH)_2D3$ to reduce expression of

362   myostatin by 1.6-fold (10nm) and 2-fold (100nm) compared to the vehicle, but this was only a

363   small effect (p = 0.07, η^2 = 0.47). There was also a trend to reduce expression of MuRF-1 by

364   1.8-fold (10nm) and 1.1-fold (100nm; p = 0.08, η^2 = 0.57). No effect was seen of either dose

365   on MAFbx expression (p = 0.32, η^2 = 0.25; Figure 2). When the expression of the myogenic

366   regulatory factors was determined, there was a trend for $1\alpha,25(OH)_2D3$ to reduce myogenin

367   expression by 1.4-fold (10nm) and 2-fold (100nm) (p = 0.08, η^2 = 0.46), but MyoD expression

368   was unchanged (p = 0.10, η^2 = 0.44). There was no effect of $1\alpha,25(OH)_2D3$ on pax7 expression

369   (p = 0.42, η^2 = 0.21; Figure 3). 10nm $1\alpha,25(OH)_2D3$ was seen to significantly reduce

370   expression of MYHC1 compared to the vehicle condition by 5-fold (p = 0.04, d = 0.57) and a

371   similar 5-fold reduction was seen for 100nm (p = 0.09, d = 1.0). An effect of 10nm

372    $1\alpha,25(OH)_2D3$ was also seen on MyHC8 expression, which was reduced by 2.5-fold (p = 0.03,

373   d = 0.53), a similar reduction was seen with 100nm (p = 0.07, d = 1.01; Figure 4). No effect of

374   either dose was seen on MYHC2 (p = 0.12, η^2 = 0.41), MYHC3 (p = 0.07, η^2 = 0.58) or MYHC7

375   (p = 0.23, η^2 = 0.31) mRNA expression. There was no effect of $1\alpha,25(OH)_2D3$ at either dose

376   on mRNA expression of the Vitamin D receptor (p = 0.39, η^2 = 0.13).

377   Effects on morphology

378   There was no effect of either 10nm or 100nm $1\alpha,25(OH)_2D3$ compared to vehicle on myotube

379   diameter (10nm = 18.2 ± 3.1 vs 100nm = 19.7 ± 4.4 vs vehicle = $16.5 \pm 2.4\mu m$; p = 0.37, η^2 =

380   0.22), or fusion index (10nm = 23.0 ± 9.5 vs 100nm = 23.8 ± 12.8 vs vehicle = $22.9 \pm 6.4\%$; p

381   = 0.85, η^2 = 0.05). 100nm $1\alpha,25(OH)_2D3$ resulted in significantly fewer myotubes per field of

382   view compared to the vehicle (p = 0.03, d = 0.84), with no differences between vehicle vs 10nm

383   (p = 0.11, d = 0.28) or 10nm vs 100nm (p = 0.24, d = 0.38) (10nm = 4 ± 4 vs 100nm = 3 ± 2

384   vs vehicle = 5 ± 3 myotubes per field of view; Figure 5).

385   <u>Effect on cell proliferation</u>

386   There was no effect of $1\alpha,25(OH)2D3$ on myoblast proliferation, with no difference in

387   geometric mean fluorescent intensity of CFSE in response to either 10nm or 100nm dose,

388   compared to vehicle (P = 0.77, η^2 = 0.09).

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390   <u>Discussion</u>

391   The aims of this study were two-fold. Firstly, to perform *in vivo* analysis to determine the

392   relationship between serum vitamin D and its metabolites with skeletal muscle mass and

393   function in CKD patients not requiring dialysis and to establish if vitamin D deficiency might

394   contribute to reduced physical function. Secondly, to determine if $1\alpha,25(OH)_2D3$

395   supplementation in human-derived skeletal muscle cells established from CKD vitamin D

396   deficient donors, could improve myoblast proliferation, differentiation, and myotube size.

397   Our data demonstrates a high prevalence of vitamin D deficiency, with all patients meeting the

398   guidelines for initiation of vitamin D supplementation (25(OH)D levels: <30ng/ml). This is in

399   line with previous reports (41, 43) and highlights the need to understand the physiological

400   effects of such a deficiency. Data from healthy population cohorts demonstrate a relationship

401   between vitamin D and muscle function. In particular, vitamin D levels are associated with

402   muscular strength (44) and physical function (21), with vitamin D supplementation reducing

403   the risk of falls in elderly populations (45). However, the influence of vitamin D status on

404   muscle mass or size is less clear. A study in vitamin D receptor knock out mice found smaller

405   muscle fibres compared to their sham littermates (46), but a study of nearly 700 healthy men

406   and women was unable to find an association between total or active vitamin D and muscle

407   mass (47). The authors concluded that the link between falls and vitamin D may be due to

408   effects upon neuromuscular function rather than muscle mass.

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

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

435

436   As it was thought vitamin D may play a role in the maintenance of muscle mass (46), we
437   hypothesised that the addition of 1α,25(OH)$_2$D3 to primary skeletal muscle cells from vitamin
438   D deficient donors would reduce myoblast proliferation and increase myoblast differentiation
439   and myotube size. Studies of C2C12 cells and primary skeletal muscle cells have shown that
440   Vitamin D has anti-proliferative effects (32, 54). However, we saw no effect of either dose of

441   1α,25(OH)$_2$D3 on myoblast proliferation rates after 72h of treatment, which has been reported

442   previously in C2C12 cells (55), although a lower dose of 1nm was used in this study. There

443   was also no effect of supplementation on differentiation, fusion index, desmin positivity, or

444   myotube diameter and no change in the mRNA expression of MyoD or myogenin, which are

445   usually required for myotube fusion. Rather, we observed a reduction in the mRNA expression

446   of MyHC1 (type IIX) and MyHC8 (perinatal) with 10nm 1α,25(OH)$_2$D3, which is in contrast

447   to previous reports (54). We did, however, observe a decrease in the number of myotubes

448   formed following 100nm 1α,25(OH)$_2$D3, which has been reported before in both primary

449   human skeletal muscle cells and C2C12 cells (30, 31). Therefore, the reduction in MyHC

450   expression may just be an artefact of the reduced number of myotubes in culture. Vitamin D

451   has also been reported to have hypertrophic effects *ex vivo* (30, 56), with 1α,25(OH)$_2$D3

452   supplementation resulting in increases in myotube diameter. However, no evidence of

453   1α,25(OH)$_2$D3 stimulated hypertrophy was seen here. The reasons for these discrepancies are

454   currently unclear. A lack of an effect on hypertrophy may be the result of the absence of a

455   change in myostatin mRNA expression. Both *in vitro* and *in vivo* studies have demonstrated

456   that vitamin D supplementation reduces skeletal muscle myostatin mRNA expression (32, 54).

457   It is possible that CKD has induced a higher myostatin expression in these cells (57), which is

458   not modifiable by 1α,25(OH)$_2$D3 exposure and that this higher expression level prevents

459   hypertrophy driven by other mechanisms. The lack of any large effect on our human-derived

460   skeletal muscle cells is in agreement with our *in vivo* data, which failed to find any associations

461   between vitamin D and measures of muscle size or strength. This lends support to the notion

462   that vitamin D does not directly affect skeletal muscle in these patients.

463   Interestingly, IL-6 mRNA expression was reduced with both doses of 1α,25(OH)$_2$D3, which is

464   in contrast to *in vivo* results, where there was no relationship seen between any form of vitamin

465   D and skeletal muscle IL-6 mRNA expression. There has been little reported regarding the

466   anti-inflammatory properties of vitamin D in skeletal muscle, but there is evidence from other

467   systems (58). The effect on IL-6 was seen in the absence of any reduction of TNF-α expression,

468   and was, therefore, unlikely to have resulted in significant anti-inflammatory affects. Given the

469   role IL-6 is known to play in skeletal muscle wasting (59), this does warrant further

470   investigation.

471   There are a few limitations of this study that should be taken into account. Firstly, this is a

472   secondary analysis of an earlier study and was therefore not powered to detect relationships

473   between vitamin D deficiency and physical function or muscle mass. Given the prevalence of

474   vitamin D deficiency in these patients and the role this plays in physical function in other

475   groups, a suitably powered study is warranted to better understand its implications. We suspect

476   that many of the discrepancies in the *ex vivo* results presented here might be explained by

477   differences in the models used in the experiments, human vs rodent and immortalised vs

478   primary cell lines. The effect of vitamin D administration in human-derived skeletal muscle

479   cells from CKD donors has not been investigated before. Given the complicated and diverse

480   effects of CKD upon skeletal muscle physiology, it is likely that vitamin D supplementation is

481   not sufficient to overcome more potent effects imposed by the illness. These results are only

482   based on five patients that exhibit a large degree of variation which is likely masking real

483   effects. A larger sample size might provide more definitive results and as such these results

484   should only be considered preliminary. Finally, this study has performed individual metabolite

485   analysis only. It may be interesting to apply modelling strategies that take into account the

486   interplay between the different metabolites (60) to get a full overview of the effect of the

487   vitamin D metabolome on physical function and muscle mass in these patients.

488   In conclusion, we have seen no strong evidence for a role of total or active vitamin D in

489   determining the level of muscle size or strength in these patients. At the cellular level, our

490   preliminary data suggests there is no effect of $1\alpha,25(OH)_2D3$ supplementation on myoblast

491   proliferation, differentiation or hypertrophy. We did, however, see an association between total

492   vitamin D and VO_{2Peak} and STS60 performance which was also seen with active vitamin D

493   before the sensitivity analysis. This suggests that vitamin D deficiency is not a prominent

494   factor driving the loss of muscle mass in CKD, but may have a role to play in the poor exercise

495   tolerance and low exercise capacity seen in these patients. In light of the high prevalence of

496   vitamin D deficiency in this patient population, further investigation is warranted to understand

497   the role of this hormone on skeletal muscle physiology in CKD.

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511

512   Conflict of interest statement

513   No authors have any conflicts to disclose.

514

515   Author contributions

516   EW, TWJ, DWG, MH, CJ, AP and AS were involved in study conception and plan. EW, TJW,

517   SX, MGB, RM, and DWG were involved in patient recruitment and performed assessments.

518   EW, LB, DWG, TOS, CJ, MH and AP were involved in laboratory analysis, data analysis

519   interpretation. EW, LB, TJW, AP and AS were responsible for preparing the manuscript for

520   submission. All authors approve this submission.

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697

698   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)

$1\alpha,25(OH)_2D3$ (pg/ml)	38	32.8 (26.8-44.7)	20	37.5 (28.6-47.0)

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

701   Abbreviations: CSA, cross-sectional area; e1RM, estimated 1 repetition maximum; eGFR,

702   estimated glomerular filtration rate; ISWT, incremental shuttle walk test; STS60, Sit to stand

703   60.

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705

706   **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$_{2Peak}$ (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)

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

708   Abbreviations: CSA, cross-sectional area; e1RM, estimated 1 repetition maximum; eGFR,

709   estimated glomerular filtration rate; ISWT, incremental shuttle walk test; STS60, Sit to stand

710   60.

711

712 **Table 3.** Bivariate correlations between serum vitamin D and vitamin D metabolites and physiological outcome measures

713 Data 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	1α,25(OH)$_2$D3	1α,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)

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

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

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Gene	25(OH)D	25OHD2	25OHD3	24,25(OH)$_2$D3	3-Epi-25OHD3	1α,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)

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

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

726 Abbreviations: IL-6, Interleukin-6; MAFbx, muscle atrophy F box; MCP-1, monocyte chemoattractant protein-1; MuRF-1. Muscle RING finger
727 1; myf5, myogenic factor 5; myoD, myoblast determination protein; TNF-α, tumor necrosis factor alpha.

728   Figure Legends

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

732   **Figure 2. Effect of 10nm and 100nm 1α,25(OH)₂D3 vs vehicle on mRNA expression of**
733   **proteins relating to muscle protein breakdown.** a) Myostatin; b) MAFbx; c) MuRF-1.
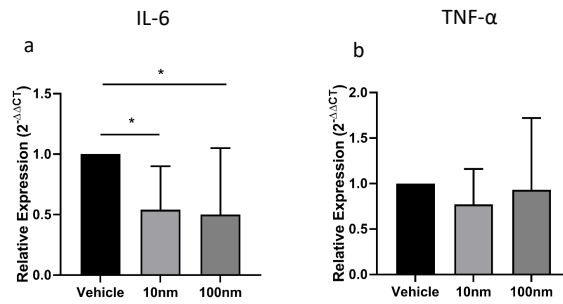
734   **Figure 3. Effect of 10nm and 100nm 1α,25(OH)₂D3 vs vehicle on mRNA expression of**
735   **proteins relating to myogenesis.** a) MyoD; b) myogenin; c) Pax7.

736   **Figure 4. Effect of 10nm and 100nm 1α,25(OH)₂D3 vs vehicle on mRNA expression of**
737   **myosin heavy chain isoforms.** a) MyH1; b) MyH2; c) MyH3; d) MyH7; e) MyH8. * denotes
738   P<0.05 vs vehicle.

739   **Figure 5. Effect of 10nm and 100nm 1α,25(OH)₂D3 vs vehicle on myotube morphology.**
740   a) myotube diameter (μm); b) myotube number per field; c) fusion index (%); d) desmin
741   positive cells (%); e) representative immunofluroscent images. Cells are counterstained for
742   desmin (green) and DAPI (blue). Scale bar represents 100μm. * denotes P<0.05 vs vehicle.
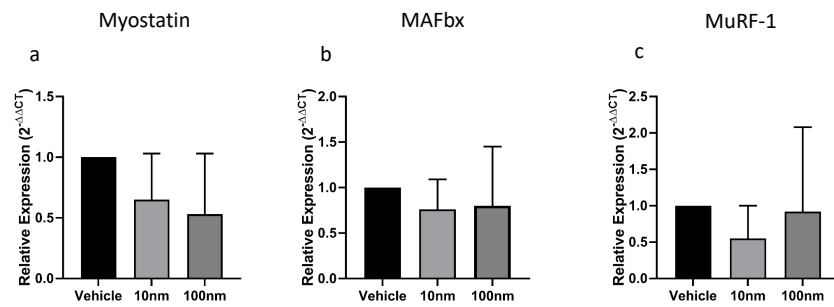
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753   Figure 1

IL-6

TNF-α

a

b

Relative Expression ($2^{-\Delta\Delta CT}$)

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756   Figure 2

Myostatin

MAFbx

MuRF-1

a

b

c

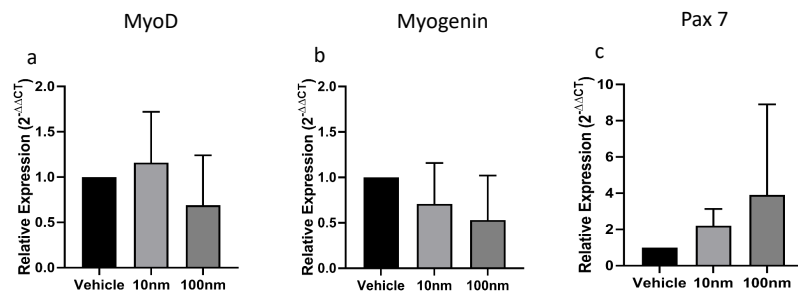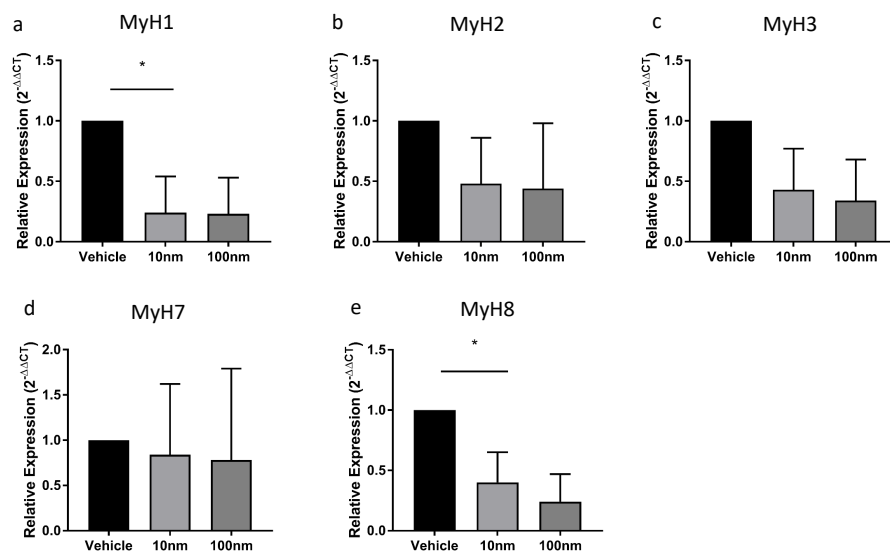Relative Expression ($2^{-\Delta\Delta CT}$)

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760   Figure 3

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762   Figure 4

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766   Figure 5

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