



## Vitamin D metabolites are associated with physical performance in young healthy adults

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1 **Vitamin D metabolites are associated with physical performance in young healthy**  
2 **adults**

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9 **ABSTRACT**

10 **Purpose.** To determine vitamin D metabolites and vitamin D receptor (VDR) single-  
11 nucleotide polymorphisms (SNPs) relationships with physical performance.

12 **Methods.** In 1205 men and 322 women (94.8% white Caucasian,  $22.0 \pm 2.8$  years)  
13 commencing military training, we measured: serum vitamin D metabolites (25-  
14 hydroxyvitamin D (25(OH)D) and 24,25-dihydroxyvitamin D (24,25(OH)<sub>2</sub>D) by high-  
15 performance liquid chromatography tandem mass spectrophotometry, and 1,25-  
16 dihydroxyvitamin D (1,25(OH)<sub>2</sub>D) by immunoassay); VDR SNPs (rs2228570, rs4516035,  
17 and rs7139166 by polymerase chain reaction genotyping); and endurance performance by 2.4  
18 km run, muscle strength by maximal dynamic lift, and muscle power by maximal vertical  
19 jump.

20 **Results.** Serum 25(OH)D was negatively associated with 2.4 km run time and positively  
21 associated with muscle power ( $\beta = -12.0$  and  $90.1$ ), 1,25(OH)<sub>2</sub>D was positively associated  
22 with run time and negatively associated with strength and muscle power ( $\beta = 5.6$ ,  $-1.06$ , and  
23  $-38.4$ ), and 24,25(OH)<sub>2</sub>D was negatively associated with run time ( $\beta = -8.9$ ;  $P < 0.01$ ), after  
24 controlling for age, sex, smoking, alcohol, physical activity, time outdoors, season, and BMI.  
25 Vitamin D metabolites (25(OH)D, 1,25(OH)<sub>2</sub>D, and 24,25(OH)<sub>2</sub>D) together explained  
26 variances of 5.0% in run time, 0.7% in strength, and 0.9% in muscle power ( $\Delta F P < 0.001$ ).  
27 All performance measures were superior with low 1,25(OH)<sub>2</sub>D:24,25(OH)<sub>2</sub>D ratio ( $P < 0.05$ ).  
28 VDR SNPs were not associated with physical performance ( $\Delta F P \geq 0.306$ ).

29 **Conclusion.** Vitamin D metabolites accounted for a small portion of variance in physical  
30 performance. Associations between vitamin D metabolites and run time were the most  
31 consistent. VDR SNPs explained no variance in performance. Greater conversion of  
32 25(OH)D to 24,25(OH)<sub>2</sub>D, relative to 1,25(OH)<sub>2</sub>D (*i.e.*, low 1,25(OH)<sub>2</sub>D:24,25(OH)<sub>2</sub>D ratio),

33 was favourable for performance, indicating 24,25(OH)<sub>2</sub>D may have a role in optimising  
34 physical performance.

35 **Key words:** Vitamin D, exercise, endurance, muscle strength, muscle power,  
36 polymorphisms.

37

## 38 INTRODUCTION

39 Serum 25-hydroxyvitamin D (25(OH)D) concentration is the recommended, and widely used,  
40 indicator of an individual's vitamin D status (25(OH)D  $\geq$ 50 nmol·L<sup>-1</sup> is deemed sufficiency  
41 (1)) due to its abundance and longer half-life relative to other circulating vitamin D  
42 metabolites (1, 2). 1,25-dihydroxyvitamin D (1,25(OH)<sub>2</sub>D)—synthesized from 25(OH)D by  
43 1 $\alpha$ -hydroxylase—is the most biologically active vitamin D metabolite in humans.  
44 1,25(OH)<sub>2</sub>D circulates in pmol·L<sup>-1</sup> concentrations, with its actions mediated by vitamin D  
45 receptors (VDRs) (3). Despite their proximity in the metabolic pathway, there is no direct  
46 correlation between serum 25(OH)D and 1,25(OH)<sub>2</sub>D due to the tight regulation of  
47 hydroxylation enzymes (4). A dynamic relationship exists between 25(OH)D and  
48 1,25(OH)<sub>2</sub>D when expressed as a relative ratio with 24,25-dihydroxyvitamin D  
49 (24,25(OH)<sub>2</sub>D) (4). 24,25(OH)<sub>2</sub>D is synthesized from 25(OH)D by 24-hydroxylase and like  
50 25(OH)D, circulates in nmol·L<sup>-1</sup> concentrations. Although 24,25(OH)<sub>2</sub>D has been labeled as a  
51 purely catabolic metabolite of vitamin D, potential biological roles and the possible existence  
52 of a 24,25(OH)<sub>2</sub>D specific receptor have emerged (5-8). Individuals with low 25(OH)D,  
53 normal 1,25(OH)<sub>2</sub>D, but increased 1,25(OH)<sub>2</sub>D:24,25(OH)<sub>2</sub>D ratio have higher parathyroid  
54 hormone (PTH) concentrations than those at the opposite end of the spectrum with high  
55 25(OH)D, normal 1,25(OH)<sub>2</sub>D, and decreased 1,25(OH)<sub>2</sub>D:24,25(OH)<sub>2</sub>D ratio (4). High  
56 24,25(OH)<sub>2</sub>D relative to 1,25(OH)<sub>2</sub>D (*i.e.*, low 1,25(OH)<sub>2</sub>D:24,25(OH)<sub>2</sub>D) may reduce the  
57 bioactivity of 25(OH)D and 1,25(OH)<sub>2</sub>D, downregulating PTH secretion, whilst maintaining

58 1,25(OH)<sub>2</sub>D within strict boundaries. Low 24,25(OH)<sub>2</sub>D relative to 1,25(OH)<sub>2</sub>D (*i.e.*, high  
59 1,25(OH)<sub>2</sub>D:24,25(OH)<sub>2</sub>D) may upregulate PTH secretion and enhance the effects of vitamin  
60 D (4). On the other hand, if 24,25(OH)<sub>2</sub>D is itself biologically active, low  
61 1,25(OH)<sub>2</sub>D:24,25(OH)<sub>2</sub>D may be beneficial. How this recently identified inverse  
62 exponential relationship between serum 25(OH)D and 1,25(OH)<sub>2</sub>D:24,25(OH)<sub>2</sub>D relates to  
63 physiological outcomes, such as physical performance, remains unexplored.

64

65 Beyond regulating calcium and phosphate homeostasis and augmenting bone mineralisation  
66 (1), extra-skeletal functions of vitamin D and its metabolites have emerged following the  
67 discovery of the VDR in almost all human tissues (3, 9). 1,25(OH)<sub>2</sub>D stimulates skeletal  
68 muscle protein synthesis by VDR-mediated signaling (9), and may improve cardiac, skeletal  
69 muscle, and endothelial function (10-14). Avoiding low serum 25(OH)D and achieving  
70 vitamin D sufficiency (1) may, therefore, be important for muscle strength and endurance  
71 type exercise (15, 16). Cross-sectional studies investigating the influence of vitamin D on  
72 physical performance in young healthy adults have reported both positive and no associations  
73 between circulating 25(OH)D and physical performance (17-20), when controlling for  
74 variables that influence performance (*e.g.*, sex, body composition, smoking, physical activity,  
75 and season (21-23)). In contrast, improving vitamin D status by increasing serum 25(OH)D  
76 with oral vitamin D<sub>3</sub> supplementation or increased sunlight exposure has not enhanced  
77 physical performance in randomized controlled trials (24, 25). This inconsistency between  
78 observational and interventional studies may be due to a focus on serum 25(OH)D as a  
79 measure of vitamin D status, and not examining the relative concentrations of vitamin D  
80 metabolites. Rather than simply increasing serum 25(OH)D, shifting  
81 1,25(OH)<sub>2</sub>D:24,25(OH)<sub>2</sub>D from high to low may be necessary for a beneficial effect on  
82 performance to emerge.

83 Several single-nucleotide polymorphisms (SNPs) within vitamin D pathway-related genes are  
84 associated with circulating 25(OH)D and may be responsible for some inter-individual  
85 variability in the vitamin D endocrine system (26). SNPs in the gene that encodes for the  
86 VDR have been studied in relation to muscle strength and function in mostly elderly and  
87 sedentary adults with equivocal results (26). The relationship between rs2228570, rs4516035,  
88 and rs7139166 VDR polymorphisms and physical performance remains to be determined in  
89 young, physically active adults.

90

91 The purpose of the study was to examine the relationship: i) between vitamin D metabolites  
92 (serum 25(OH)D, 1,25(OH)<sub>2</sub>D, and 24,25(OH)<sub>2</sub>D) and physical performance; and ii) between  
93 VDR polymorphisms (rs2228570, rs4516035, and rs7139166) and physical performance. We  
94 hypothesized a three-dimensional model of vitamin D metabolites incorporating serum  
95 25(OH)D and 1,25(OH)<sub>2</sub>D:24,25(OH)<sub>2</sub>D would have a dynamic relationship with physical  
96 performance; and SNPs in the VDR would be associated with variance in performance.

97

## 98 **METHODS**

### 99 **Participants**

100 1527 British Army recruits (1205 men and 322 women, 94.8% white Caucasian; Table 1)  
101 voluntarily participated in the study, after providing informed written consent and passing a  
102 physician-screened military medical assessment. All experimental procedures were  
103 completed during week one of initial Army training. Participants were recruited during week  
104 one of initial Army training between April 2013 and May 2017 from three military training  
105 populations: male infantry recruits at Infantry Training Centre, Catterick; standard entry  
106 female recruits at Army Training Centre, Pirbright; and male and female officer cadets at  
107 Royal Military Academy, Sandhurst—thereby providing a representative sample of all

108 individuals commencing Army training in the UK. A subset of these data have been  
109 published (25). The present study includes unpublished vitamin D metabolite and SNP data,  
110 and is from a larger sample, more representative of all individuals commencing Army  
111 training. The study received ethical approval from the UK Ministry of Defence Research  
112 Ethics Committee (protocol number 165/Gen/10) and was conducted in accordance with the  
113 Declaration of Helsinki (2013).

114

## 115 **Study design**

116 A cross-sectional, observational study design was used to determine whether serum vitamin  
117 D metabolites and VDR SNPs were associated with physical performance in young healthy  
118 adults. All assessments were performed during week one of initial Army training and are  
119 listed here. Venous blood samples were obtained for analysis of: serum 25(OH)D, and its  
120 metabolites 1,25(OH)<sub>2</sub>D and 24,25(OH)<sub>2</sub>D; and VDR SNPs in whole blood (rs2228570,  
121 rs4516035, and rs7139166). Physical performance was assessed by a maximal effort 2.4 km  
122 run, and tests of maximal dynamic lift strength and vertical jump peak power output. Body  
123 mass and height (Seca, Hamburg, Germany) were measured in light clothing and without  
124 shoes. Participants self-reported their alcohol intake; smoking habits; physical activity levels;  
125 and typical time spent outdoors, using questionnaires.

126

## 127 **Experimental procedures**

### 128 *Blood collection and handling*

129 Whole blood samples were obtained by venipuncture from a prominent vein in the  
130 antecubital fossa into one serum vacutainer and one EDTA vacutainer (Becton Dickinson,  
131 Oxford, UK). Whole blood in the EDTA vacutainer was immediately frozen at -80°C for  
132 later analysis. Whole blood in the serum vacutainer was left to clot in a vacutainer rack at

133 room temperature for 1 h before being centrifuged at 1500 g for 10 min at 4°C, with serum  
134 aliquots immediately frozen at -80°C for later analysis.

135

### 136 *Biochemical analysis*

137 Total serum 25(OH)D (25(OH)D<sub>2</sub> and 25(OH)D<sub>3</sub>) and total 24,25(OH)<sub>2</sub>D (24,25(OH)<sub>2</sub>D<sub>2</sub> and  
138 24,25(OH)<sub>2</sub>D<sub>3</sub>) were measured with high-performance liquid chromatography tandem mass  
139 spectrophotometry using a Micromass Quattro Ultima Pt electrospray ionisation mass  
140 spectrometer, as described previously (27). Serum 1,25(OH)<sub>2</sub>D was measured by  
141 chemiluminescent immunoassay using a DiaSorin LIAISON® XL analyser (Stillwater,  
142 Minnesota, USA). The measurement ranges of the assays were 0–200 nmol·L<sup>-1</sup> for 25(OH)D<sub>2</sub>  
143 and 25(OH)D<sub>3</sub>, 0–25 nmol·L<sup>-1</sup> for 24,25(OH)<sub>2</sub>D<sub>2</sub> and 24,25(OH)<sub>2</sub>D<sub>3</sub>, and 12–480 pmol·L<sup>-1</sup> for  
144 1,25(OH)<sub>2</sub>D. The mean coefficient of variation (CV) for intra-assay imprecision across the  
145 measuring range of the assays was 4.9% for 25(OH)D<sub>2</sub>, 8.3% for 25(OH)D<sub>3</sub>, 7.7% for  
146 24,25(OH)<sub>2</sub>D<sub>2</sub>, 9.0% for 24,25(OH)<sub>2</sub>D<sub>3</sub>, and 7.4% for 1,25(OH)<sub>2</sub>D. The cumulative inter-  
147 assay CVs were ≤7.4% for 25(OH)D<sub>2</sub>, ≤9.6% for 25(OH)D<sub>3</sub>, ≤10.6% for 24,25(OH)<sub>2</sub>D<sub>2</sub>,  
148 ≤8.9% for 24,25(OH)<sub>2</sub>D<sub>3</sub>, and ≤9.3% for 1,25(OH)<sub>2</sub>D. All biochemical analyses were  
149 undertaken by the Good Clinical Laboratory Practice and Vitamin D External Quality  
150 Assessment Scheme certified Bioanalytical Facility at the University of East Anglia.

151

### 152 *Single-nucleotide polymorphisms*

153 Whole blood samples in EDTA vacutainers were defrosted and resuspended for 15 min on a  
154 rotating wheel. Genomic DNA was isolated from whole blood using the ReliaPrep™ Blood  
155 gDNA Miniprep System (Promega, Southampton, UK) according to the manufacturer's  
156 instructions. Using samples of DNA, Kompetitive Allele Specific PCR (KASP™, LGC



157 Genomics, Teddington, Middlesex, UK) genotyping was used for SNP genotyping of  
158 rs2228570, rs4516035, and rs7139166 in the VDR gene.

159

### 160 *Endurance performance*

161 Endurance performance was assessed as the time to complete a maximal effort 2.4 km run,  
162 recorded to the nearest second. After an 800 m warm up, the 2.4 km run was performed on a  
163 standardized running course at each training site. The time to complete a 2.4 km run is  
164 indicative of maximal aerobic capacity (28) and is assessed during selection, training, and  
165 throughout a military career. All participants were accustomed to performing this test from  
166 selection before commencing military training. Faster 2.4 km run times indicated better  
167 endurance performance. Therefore, negative associations with run time indicated improved  
168 endurance performance, and positive associations with run time indicated worsened  
169 endurance performance.

170

### 171 *Muscle strength*

172 Maximal dynamic lift strength was determined as the maximal weight lifted using an  
173 incremental lift machine that simulates a power clean weightlifting movement, as described  
174 previously (29). The device consisted of a vertically moving carriage with handgrips  
175 positioned 0.30 m above the ground. Participants lifted the weight (20 kg starting mass) to a  
176 height where the handgrips were 1.45 m from the ground, the height of a British Army four  
177 tonne truck. With each successful lift, the weight was increased by 5 kg. The test was  
178 terminated when participants failed to lift the weight to 1.45 m on their second attempt.  
179 Differences in body height may have affected the participants ability to lift weight to the  
180 same absolute height, however, this measure of maximal dynamic lift strength was chosen  
181 because it correlates with and predicts success in military and functional tasks (28).

## 182 *Muscle power*

183 Vertical jump peak power output was assessed by countermovement vertical jump using a  
184 jump mat (Takei Scientific Instruments, Tokyo, Japan) and validated equation (30): peak  
185 power (W) = (51.9 x maximal vertical jump height (cm)) + (48.9 x body mass (kg)) – 2007,  
186 as described previously (29). We analyzed this estimate of muscle power rather than jump  
187 height because lower body power is important for the performance of military specific tasks  
188 (28). A belt was fitted around the waist of each participant and secured to a rubber mat.  
189 Participants were instructed to jump as high as possible three times, with their hands placed  
190 on their hips. A fourth jump was performed if jump height increased across the three  
191 attempts, indicative of a learning effect. Maximal vertical jump height was recorded as the  
192 highest score achieved. Test-retest reliability of  $r \geq 0.90$  has been reported for these  
193 performance tests (29).

194

## 195 **Statistical analysis**

196 Hierarchical multiple linear regression was used to examine the association between vitamin  
197 D metabolites and physical performance. Age, sex, smoking, alcohol intake, physical activity,  
198 time spent outdoors, season, and body mass index (BMI) were included in regression models  
199 as covariates (21-23, 31). The association between vitamin D metabolites and physical  
200 performance was analyzed in two steps. Serum 1,25(OH)<sub>2</sub>D and 24,25(OH)<sub>2</sub>D were included  
201 in step one, and 25(OH)D was added in step two, so the relationship between serum  
202 1,25(OH)<sub>2</sub>D and 24,25(OH)<sub>2</sub>D, and physical performance could be examined, with and  
203 without 25(OH)D. Given the large inter-individual differences in metabolites, these variables  
204 were standardized by scaling them relative to their standard deviation to improve the  
205 interpretation of beta coefficients. Cohen's  $f^2$  effect sizes were calculated using a standard  
206 formula (32). No signs of strong heteroscedasticity or deviations from a normal distribution

207 were detected for model residuals. Sensitivity analyses (data not shown) conducted with  
208 vitamin D metabolites log transformed or categorized into tertiles, as described previously  
209 (33), resulted in no substantive changes to the null-hypotheses tests or effect sizes. Variance  
210 inflation factor for all multiple regression models was  $<4.2$ , indicating no presence of  
211 multicollinearity (34). The association between vitamin D metabolites and physical  
212 performance was also explored by clustering participants into groups based on two  
213 dimensions of serum 25(OH)D and 1,25(OH)<sub>2</sub>D:24,25(OH)<sub>2</sub>D ratio. Clustering was  
214 performed using a k-means technique and the Bayesian information criterion to select the  
215 number of clusters, with the  $n$  in each cluster determined by the algorithm (35). Pairwise  
216 comparisons of the mean differences in physical performance across clusters were made  
217 using the t-distribution. Multiple linear regression models were also used to investigate the  
218 association between VDR SNPs and physical performance. Separate models were fitted for  
219 each of the SNPs whilst controlling for the same variables used to assess the association  
220 between vitamin D metabolites and physical performance. Associations were evaluated by  
221 conducting F-tests for nested linear models. Pairwise comparisons of the mean differences  
222 between vitamin D metabolites across seasons were made using the t-distribution. All  
223 statistical tests were conducted within a general linear model framework and using R 3.6.2 (R  
224 Foundation for Statistical Computing, Vienna, Austria). Statistical significance was accepted  
225 at  $P < 0.05$ .

226

## 227 **RESULTS**

### 228 **Vitamin D metabolites and season**

229 There was some seasonal variation in vitamin D metabolites ( $P < 0.001$ , Table 2). Across all  
230 seasons, 66.7% of participants were vitamin D sufficient, 21.4% were insufficient, and 11.9%

231 were deficient. During winter, 30.7% were vitamin D sufficient, 38.6% insufficient, and  
232 30.7% deficient.

233

## 234 **Vitamin D metabolite predictors of physical performance**

### 235 *Endurance performance*

236 Serum 1,25(OH)<sub>2</sub>D was positively associated, and 24,25(OH)<sub>2</sub>D was negatively associated  
237 with 2.4 km run time after controlling for age, sex, smoking, alcohol intake, physical activity,  
238 time spent outdoors, season, and BMI ( $P < 0.01$  and  $P < 0.001$ , respectively; Table 3). These  
239 relationships remained following the addition of serum 25(OH)D as a predictor ( $P < 0.001$   
240 and  $P < 0.01$ , respectively), with 25(OH)D negatively associated with 2.4 km run time ( $P <$   
241  $0.001$ ). Vitamin D metabolites (25(OH)D, 1,25(OH)<sub>2</sub>D, and 24,25(OH)<sub>2</sub>D) together  
242 explained 5.0% of the variance in 2.4 km run time (significant  $\Delta F P < 0.001$ ).

243

### 244 *Muscle strength*

245 Serum 1,25(OH)<sub>2</sub>D was negatively associated, and 24,25(OH)<sub>2</sub>D was positively associated  
246 with maximal dynamic lift strength (muscle strength) after controlling for age, sex, smoking,  
247 alcohol intake, physical activity, time spent outdoors, season, and BMI ( $P < 0.01$  and  $P <$   
248  $0.001$ , respectively; Table 3). Following the addition of serum 25(OH)D as a predictor,  
249 1,25(OH)<sub>2</sub>D remained negatively associated with muscle strength ( $P < 0.001$ ), but neither  
250 24,25(OH)<sub>2</sub>D nor 25(OH)D were associated with muscle strength ( $P = 0.126$  and  $P = 0.093$ ,  
251 respectively). Vitamin D metabolites (25(OH)D, 1,25(OH)<sub>2</sub>D, and 24,25(OH)<sub>2</sub>D) together  
252 explained 0.7% of the variance in muscle strength (significant  $\Delta F P < 0.001$ ).

253

254

255

256 *Muscle power*

257 Serum 1,25(OH)<sub>2</sub>D was negatively associated, and 24,25(OH)<sub>2</sub>D was positively associated  
258 with vertical jump peak power output (muscle power) after controlling for age, sex, smoking,  
259 alcohol intake, physical activity, time spent outdoors, season, and BMI ( $P < 0.05$  and  $P <$   
260  $0.001$ , respectively; Table 3). Following the addition of serum 25(OH)D as a predictor,  
261 1,25(OH)<sub>2</sub>D remained negatively associated ( $P < 0.01$ ), 24,25(OH)<sub>2</sub>D was not associated ( $P$   
262  $= 0.791$ ), and 25(OH)D was positively associated with muscle power ( $P < 0.001$ ). Vitamin D  
263 metabolites (25(OH)D, 1,25(OH)<sub>2</sub>D, and 24,25(OH)<sub>2</sub>D) together explained 0.9% of the  
264 variance in muscle power (significant  $\Delta F P < 0.001$ ).

265

266 **Relative concentrations of vitamin D metabolites and physical performance**

267 *Endurance performance*

268 Run times were faster in participants within clusters 3 to 6 vs cluster 1 and 2, and participants  
269 within clusters 4 to 6 vs cluster 3 ( $P < 0.05$ , Fig. 1A).

270

271 *Muscle strength*

272 Maximal dynamic lift strength was higher in participants within clusters 4 to 6 vs cluster 1,  
273 and participants within clusters 4 and 6 vs cluster 2 ( $P < 0.05$ , Fig. 1B).

274

275 *Muscle power*

276 Vertical jump peak power output was higher in participants within clusters 3 to 6 vs cluster 1  
277 ( $P < 0.05$ , Fig. 1C).

278

279

280

281 **Vitamin D receptor polymorphisms and physical performance**

282 Vitamin D receptor SNPs did not explain any of the variance in 2.4 km run time, muscle  
283 strength, or muscle power after controlling for age, sex, smoking, alcohol intake, physical  
284 activity, time spent outdoors, season, and BMI (significant  $\Delta F P \geq 0.306$ , Table 4). There  
285 were no between genotype differences in 2.4 km run time, muscle strength, or muscle power  
286 when no confounding factors were controlled for ( $P \geq 0.086$ ).

287

288 **DISCUSSION**

289 Serum 25(OH)D, 1,25(OH)<sub>2</sub>D, and 24,25(OH)<sub>2</sub>D were associated with 2.4 km run time;  
290 1,25(OH)<sub>2</sub>D was associated with muscle strength; and 25(OH)D and 1,25(OH)<sub>2</sub>D were  
291 associated with muscle power, in young healthy adult men and women. Other factors  
292 contributing to physical performance (age, sex, smoking, alcohol intake, physical activity,  
293 time spent outdoors, season, and BMI) were controlled for as covariates using hierarchical  
294 multiple linear regression. Vitamin D metabolites (25(OH)D, 1,25(OH)<sub>2</sub>D, and 24,25(OH)<sub>2</sub>D)  
295 together explained variances of 5.0% in 2.4 km run time, 0.7% in muscle strength, and 0.9%  
296 in muscle power. In terms of practical significance, the magnitude of the association between  
297 vitamin D metabolites and physical performance can be considered small (Cohen's  $f^2$  effect  
298 sizes  $< 0.15$ ). Nevertheless, in real-world terms, for every 1 SD increase in 25(OH)D (+28.0  
299 nmol·L<sup>-1</sup>), 2.4 km run time was 12 s faster and vertical jump peak power output 90 W higher;  
300 for every 1 SD increase in 24,25(OH)<sub>2</sub>D (+3.3 nmol·L<sup>-1</sup>), 2.4 km run time was 9 s faster; and  
301 for every 1 SD increase in 1,25(OH)<sub>2</sub>D (+36.5 pmol·L<sup>-1</sup>), 2.4 km run time was 6 s slower,  
302 maximal dynamic lift strength 1 kg lower, and vertical jump peak power output 38 W lower.  
303  
304 As hypothesized, serum 25(OH)D and 1,25(OH)<sub>2</sub>D:24,25(OH)<sub>2</sub>D had a dynamic relationship  
305 with physical performance: 2.4 km run times were faster, and muscle strength and muscle

306 power were greater in men and women with proportionally greater conversion of 25(OH)D to  
307 24,25(OH)<sub>2</sub>D relative to 1,25(OH)<sub>2</sub>D (*i.e.*, low 1,25(OH)<sub>2</sub>D:24,25(OH)<sub>2</sub>D ratio). Examining  
308 this relationship between vitamin D metabolites provides a unique insight into how the  
309 vitamin D metabolic pathway is related to physical performance and suggests that  
310 24,25(OH)<sub>2</sub>D may have role in optimising physical performance. Contrary to our hypothesis,  
311 VDR SNPs (rs2228570, rs4516035, and rs7139166) were not associated with physical  
312 performance.

313

### 314 **Vitamin D metabolite predictors of physical performance**

315 The negative association between serum 24,25(OH)<sub>2</sub>D and 2.4 km run time, and positive  
316 associations between 24,25(OH)<sub>2</sub>D and muscle strength and muscle power, were weaker or  
317 absent when 25(OH)D was added as a predictor because of the tight correlation between  
318 these metabolites (4). Serum 25(OH)D was itself negatively associated with 2.4 km run time  
319 and positively associated with muscle power. In contrast, 1,25(OH)<sub>2</sub>D was positively  
320 associated with 2.4 km run time and negatively associated with muscle strength and muscle  
321 power, even when 25(OH)D was included as a predictor. However, serum 1,25(OH)<sub>2</sub>D alone  
322 does not reflect vitamin D reserves or status because it is tightly regulated by the  
323 hydroxylation enzymes expressed by CYP27B1 and CYP24A1, with 1,25(OH)<sub>2</sub>D production  
324 upregulated by PTH and downregulated by fibroblast growth factor 23 (FGF23) (36). No  
325 metabolites were associated with muscle strength when controlling for age and sex in the  
326 only published study to examine 25(OH)D, 1,25(OH)<sub>2</sub>D, and 24,25(OH)<sub>2</sub>D relationships with  
327 muscle function (37). This non-significant finding may be explained by the small sample size  
328 (116 adults, 20–74 years) (37), relative to the present study.

329

330

331 **Relative concentrations of vitamin D metabolites and physical performance**

332 An inverse exponential relationship exists between serum 25(OH)D and  
333 1,25(OH)<sub>2</sub>D:24,25(OH)<sub>2</sub>D ratio (4). As the availability of 25(OH)D as a precursor  
334 diminishes, the conversion of 25(OH)D to 24,25(OH)<sub>2</sub>D is reduced, resulting in a  
335 proportional increase in 1,25(OH)<sub>2</sub>D (38). Superior physical performance in adults with low  
336 1,25(OH)<sub>2</sub>D:24,25(OH)<sub>2</sub>D ratio suggests 24,25(OH)<sub>2</sub>D is not a purely catabolic metabolite.  
337 Given 1,25(OH)<sub>2</sub>D is the most biologically active vitamin D metabolite, greater circulating  
338 concentrations of 1,25(OH)<sub>2</sub>D relative to 24,25(OH)<sub>2</sub>D (*i.e.*, high 1,25(OH)<sub>2</sub>D:24,25(OH)<sub>2</sub>D)  
339 might be expected to be favourable for physical performance (16). Our novel finding that  
340 proportionally greater conversion of 25(OH)D to 24,25(OH)<sub>2</sub>D relative to 1,25(OH)<sub>2</sub>D (*i.e.*,  
341 low 1,25(OH)<sub>2</sub>D:24,25(OH)<sub>2</sub>D ratio) was better for endurance performance, muscle strength,  
342 and muscle power, suggests 24,25(OH)<sub>2</sub>D might influence physical performance. Rather than  
343 being a catabolic waste product, emerging evidence indicates 24,25(OH)<sub>2</sub>D has a role in  
344 osteoblastic differentiation and bone development (39), promotion of fracture healing (7, 8,  
345 40), and protection against cartilage damage (6). The existence of 24,25(OH)<sub>2</sub>D receptors,  
346 and their possible function relevant to physical performance needs to be examined.

347

348 Vitamin D metabolites may improve physical performance by increasing the delivery of  
349 oxygenated blood to muscle through improved endothelial function (13, 14) and maintenance  
350 of normotension (41). Vitamin D metabolites can also increase mitochondrial oxidative  
351 function, potentially attenuating the development of skeletal muscle fatigue (12). By  
352 potentially increasing aerobic capacity, these mechanisms could account for why the  
353 associations between vitamin D metabolites and 2.4 km run time were the most consistent.  
354 Whether similar relationships exist between vitamin D metabolites and performance in  
355 endurance events of longer duration is an interesting area for future study. Vitamin D



356 metabolites may exert beneficial downstream effects on physical performance by modulating  
357 muscle remodelling, since the VDR/retinoid X receptor signaling pathway is upregulated  
358 during the early stages of hypertrophy (42). Vitamin D can enhance skeletal muscle repair  
359 following damaging exercise and help to protect against infection by supporting aspects of  
360 innate and acquired immunity (16). By doing so, vitamin D may help to minimise the number  
361 of training sessions missed by athletes and military personnel, and thus potentially lead to  
362 improved physical performance.

363

### 364 **Vitamin D receptor polymorphisms and physical performance**

365 This is the largest study to examine the relationship between VDR SNPs (rs2228570,  
366 rs4516035, and rs7139166) and physical performance in young adults, with no associations  
367 observed. Previously, the rs2228570 allele related to increased VDR function was associated  
368 with weaker muscle strength in older adults (men and women, mean 62 years (43); men, 58–  
369 93 years (44)). Increased VDR function may increase CYP24A1 expression, leading to the  
370 degradation and decreased availability of 1,25(OH)<sub>2</sub>D (9). Associations between the  
371 rs2228570 polymorphism and quadriceps strength were no longer statistically significant  
372 after controlling for fat-free mass in women (mean 42 years) (45) and men (58–93 years)  
373 (44), suggesting this polymorphism may influence muscle mass rather than strength *per se*. In  
374 contrast to the present study, strength differed between groups of children (mean 10 years)  
375 with different rs4516035 alleles, but no differences emerged for rs2228570 alleles (46). The  
376 range of muscle or other performance assessments used, and differences in participants'  
377 fitness and age (skeletal muscle VDR expression decreases with age (47)) have contributed to  
378 these equivocal findings.

379

380

381 **Perspectives**

382 This study provides a unique insight into the dynamic relationship between vitamin D  
383 metabolites and demonstrates that the relative circulating concentrations of 1,25(OH)<sub>2</sub>D and  
384 24,25(OH)<sub>2</sub>D are related to physical performance. Randomized controlled trials have shown  
385 vitamin D<sub>3</sub> supplementation does not improve physical performance (24), however, in these  
386 studies vitamin D status has been assessed using 25(OH)D in isolation and the relative  
387 concentrations of 1,25(OH)<sub>2</sub>D and 24,25(OH)<sub>2</sub>D have not been considered. Shifting  
388 1,25(OH)<sub>2</sub>D:24,25(OH)<sub>2</sub>D ratio from high to low may be necessary for a beneficial effect on  
389 performance to occur. Whether oral vitamin D<sub>3</sub> supplementation can correct high  
390 1,25(OH)<sub>2</sub>D:24,25(OH)<sub>2</sub>D ratio and achieve high 25(OH)D and low  
391 1,25(OH)<sub>2</sub>D:24,25(OH)<sub>2</sub>D ratio—and thereby enhance physical performance—remains to be  
392 determined. How much and how often oral vitamin D<sub>3</sub> is needed to achieve a steady state of  
393 vitamin D metabolites needs to be examined. Avoiding high serum 24,25(OH)<sub>2</sub>D has been  
394 recommended because 24,25(OH)<sub>2</sub>D may act to block the activity of the VDR (48). This  
395 recommendation, however, assumes 24,25(OH)<sub>2</sub>D is purely a catabolic waste product—a  
396 hypothesis that warrants further evaluation given that relatively high 24,25(OH)<sub>2</sub>D was  
397 associated with superior performance.

398

399 Avoiding a relative increase in serum 1,25(OH)<sub>2</sub>D may be beneficial for performance,  
400 therefore, supplementation with alfacalcidol (1-hydroxyvitamin D<sub>3</sub>) or calcitriol  
401 (1,25(OH)<sub>2</sub>D) are unlikely to be effective for enhancing physical performance—especially  
402 because serum 1,25(OH)<sub>2</sub>D is maintained within a tight range, despite fluctuations in  
403 25(OH)D and 24,25(OH)<sub>2</sub>D (4, 36). Supplementation that increases 1,25(OH)<sub>2</sub>D beyond its  
404 normal plateau, thereby increasing the 1,25(OH)<sub>2</sub>D:24,25(OH)<sub>2</sub>D ratio, could be detrimental.

405

406 Genome wide studies have identified CYP24A1 as one of the major genetic determinants of  
407 variability in vitamin D metabolism (49). Increased CYP24A1 activity increases  
408 24,25(OH)<sub>2</sub>D and decreases 1,25(OH)<sub>2</sub>D and PTH (36). Whether supplementation, dietary or  
409 lifestyle interventions can be used in individuals with genetically lower CYP24A1 activity (as  
410 indicated by increased 1,25(OH)<sub>2</sub>D:24,25(OH)<sub>2</sub>D ratio), to manage their vitamin D  
411 metabolism and enhance physical performance requires future study.

412

413 The present study is limited by its cross-sectional design. Associations between vitamin D  
414 metabolites and physical performance could be explained by reverse causation, *i.e.*, fitter,  
415 more physically active individuals spend more time outdoors exposed to sunlight and, in-turn,  
416 had higher serum concentrations of vitamin D metabolites. However, we included  
417 participants' self-reported physical activity levels and typical time spent outdoors as  
418 covariates in our regression models. Almost all of the participants in the present study were  
419 white Caucasian. Whether the vitamin D metabolites and SNPs we examined are associated  
420 with physical performance in other ethnic groups is unknown and warrants further study.

421

## 422 **Conclusions**

423 Serum 25(OH)D, 1,25(OH)<sub>2</sub>D, and 24,25(OH)<sub>2</sub>D were associated with 2.4 km run time;  
424 1,25(OH)<sub>2</sub>D was associated with muscle strength; and 25(OH)D and 1,25(OH)<sub>2</sub>D were  
425 associated with muscle power, after controlling for covariates. Vitamin D metabolites  
426 accounted for a small portion of variance in physical performance. Polymorphisms in the  
427 VDR were not associated with physical performance. Faster 2.4 km run times and greater  
428 muscle strength and muscle power in adults with proportionally greater conversion of  
429 25(OH)D to 24,25(OH)<sub>2</sub>D relative to 1,25(OH)<sub>2</sub>D (*i.e.*, low 1,25(OH)<sub>2</sub>D:24,25(OH)<sub>2</sub>D ratio)  
430 indicates 24,25(OH)<sub>2</sub>D may have a role in optimising physical performance.

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435

436 **CONFLICT OF INTEREST**

437 The authors have nothing to disclose. The results of the present study do not constitute  
438 endorsement by the ACSM. The results of the study are presented clearly, honestly, and  
439 without fabrication, falsification, or inappropriate data manipulation.

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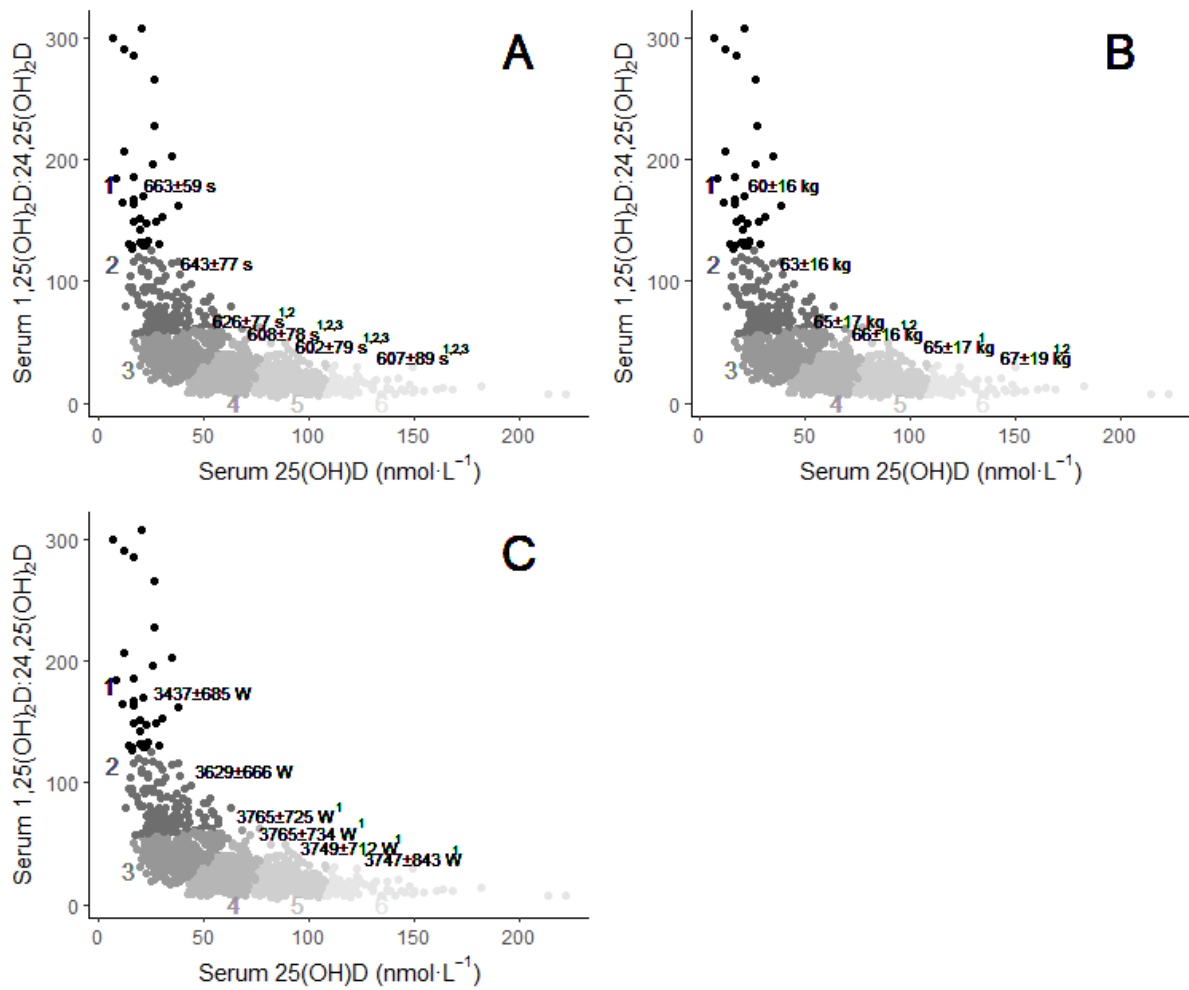
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- 569



570 **FIGURE CAPTIONS**

571 **Figure 1.** Dynamic relationships between vitamin D metabolites and physical performance.  
 572 Participants (1 per filled circle) are categorized into one of six clusters, with each cluster's  
 573 mean  $\pm$  SD physical performance shown. Panel A, 2.4 km run time; Panel B, maximal  
 574 dynamic lift strength; and Panel C, vertical jump peak power output.  $^1P < 0.05$  vs cluster 1;  
 575  $^2P < 0.05$  vs cluster 2;  $^3P < 0.05$  vs cluster 3.



576

**Table 1.** Demographic, anthropometric, lifestyle behavior, and physical performance characteristics.

<b>Demographics</b>				
Age (years)	22.0 ± 2.8			
Sex (% men)	78.9			
<b>Ethnicity</b>				
White Caucasian (%)	94.8			
Other (%)	5.2			
<b>Anthropometrics</b>				
Body mass (kg)	74.1 ± 10.4			
Height (m)	1.75 ± 0.08			
BMI (kg·m <sup>-2</sup> )	24.1 ± 2.5			
<b>Lifestyle behaviors</b>				
Smoker (%)	36.6			
Alcohol user (%)	89.5			
Physical activity (h·week <sup>-1</sup> )	9.6 ± 11.2			
Time spent outdoors (h·week <sup>-1</sup> )	<1	1–3.5	3.5–6	>6
April-September (%)	2.3	28.7	33.1	35.9
October-March (%)	6.7	39.0	26.7	27.6
<b>Physical performance</b>				
2.4 km run time (s)	617 ± 79			
Maximal dynamic lift strength (kg)	65 ± 17			
Vertical jump peak power output (W)	3702 ± 748			

BMI, body mass index. Data are mean ± SD or percent.

**Table 2.** Seasonal variation in vitamin D status and serum vitamin D metabolites.

	Spring <i>n</i> = 358	Summer <i>n</i> = 472	Fall <i>n</i> = 394	Winter <i>n</i> = 303	All Seasons <i>n</i> = 1527
<b>Vitamin D status</b>					
Sufficient (%)	66.8	85.6	71.8	30.7	66.7
Insufficient (%)	22.9	11.9	18.0	38.6	21.4
Deficient (%)	10.3	2.5	10.2	30.7	11.9
<b>25(OH)D (nmol·L<sup>-1</sup>)</b>	62.3 ± 26.8 <sup>a</sup>	76.8 ± 25.8	65.2 ± 26.0 <sup>a</sup>	43.5 ± 22.7 <sup>a,b,c</sup>	63.8 ± 28.0
<b>1,25(OH)<sub>2</sub>D (pmol·L<sup>-1</sup>)</b>	141.8 ± 34.8	142.9 ± 36.5	132.1 ± 37.1 <sup>a,c</sup>	131.1 ± 35.8 <sup>a,c</sup>	137.5 ± 36.5
<b>24,25(OH)<sub>2</sub>D (nmol·L<sup>-1</sup>)</b>	5.1 ± 3.0 <sup>a,b</sup>	6.6 ± 3.0	6.5 ± 3.2	3.8 ± 3.2 <sup>a,b,c</sup>	5.6 ± 3.3
<b>1,25(OH)<sub>2</sub>D:24,25(OH)<sub>2</sub>D</b>	40.2 ± 32.1 <sup>d</sup>	28.3 ± 21.6 <sup>c,d</sup>	26.9 ± 20.6 <sup>c,d</sup>	52.8 ± 41.3	35.6 ± 30.5
<b>25(OH)D:24,25(OH)<sub>2</sub>D</b>	13.8 ± 4.3	12.7 ± 3.8 <sup>c,d</sup>	11.1 ± 3.3 <sup>a,c,d</sup>	13.7 ± 5.4	12.8 ± 4.3

Vitamin D sufficient, serum 25(OH)D  $\geq 50$  nmol·L<sup>-1</sup>; insufficient, serum 25(OH)D 30–<50 nmol·L<sup>-1</sup>; and deficient, serum 25(OH)D <30 nmol·L<sup>-1</sup>. Data are mean ± SD or percent. a, lower than summer; b, lower than fall; c, lower than spring; d, lower than winter,  $P < 0.001$ .

**Table 3.** Serum 1,25(OH)<sub>2</sub>D, 24,25(OH)<sub>2</sub>D, and 25(OH)D predictors of 2.4 km run time (endurance), muscle strength (maximal dynamic lift), and muscle power (vertical jump peak power output).

		Serum vitamin D				
	metabolites	Beta	R <sup>2</sup>	ΔR <sup>2</sup>	Sig. ΔF	f <sup>2</sup>
<b>2.4 km run time</b>	1,25(OH) <sub>2</sub> D	4.1**	0.488	0.044	<0.001	0.09
	24,25(OH) <sub>2</sub> D	-18.2***				
	1,25(OH) <sub>2</sub> D	5.6***	0.494	0.050	<0.001	0.10
	24,25(OH) <sub>2</sub> D	-8.9**				
	25(OH)D	-12.0***				
<b>Muscle strength</b>	1,25(OH) <sub>2</sub> D	-0.95**	0.668	0.007	<0.001	0.02
	24,25(OH) <sub>2</sub> D	1.41***				
	1,25(OH) <sub>2</sub> D	-1.06***	0.668	0.007	<0.001	0.02
	24,25(OH) <sub>2</sub> D	0.75				
	25(OH)D	0.86				
<b>Muscle power</b>	1,25(OH) <sub>2</sub> D	-27.8*	0.672	0.006	<0.001	0.02
	24,25(OH) <sub>2</sub> D	63.7***				
	1,25(OH) <sub>2</sub> D	-38.4**	0.675	0.009	<0.001	0.03
	24,25(OH) <sub>2</sub> D	-5.5				
	25(OH)D	90.1***				

After controlling for covariates (age, sex, smoking, alcohol intake, physical activity, time spent outdoors, season, and BMI) serum 1,25(OH)<sub>2</sub>D and 24,25(OH)<sub>2</sub>D were entered in step one, and 25(OH)D was entered in step two as predictors of physical performance. Beta, standardized beta coefficient; Sig. ΔF, significant F change *P* value; f<sup>2</sup>, Cohen's f<sup>2</sup> effect size, f<sup>2</sup> ≥0.02, ≥0.15 and ≥0.35 represent small, medium and large effect sizes, respectively (32).

\**P* < 0.05, \*\**P* < 0.01 and \*\*\**P* < 0.001.

**Table 4.** VDR SNP (rs2228570, rs4516035, and rs7139166) predictors of 2.4 km run time (endurance), muscle strength (maximal dynamic lift), and muscle power (vertical jump peak power output).

	VDR SNP	Beta	Beta	R <sup>2</sup>	ΔR <sup>2</sup>	Sig. ΔF	f <sup>2</sup>
		CC vs alternate	CC vs alternate				
		allele 1	allele 2				
<b>2.4 km run time</b>	rs2228570	0.48	-4.0	0.429	0.000	0.610	<0.001
	rs4516035	0.59	-0.23	0.429	0.000	0.716	<0.001
	rs7139166	0.71	0.56	0.429	0.000	0.978	<0.001
<b>Muscle strength</b>	rs2228570	0.39	0.52	0.650	0.000	0.743	<0.001
	rs4516035	0.57	0.86	0.650	0.000	0.575	<0.001
	rs7139166	-0.29	-0.88	0.650	0.000	0.599	<0.001
<b>Muscle power</b>	rs2228570	19.4	19.0	0.666	0.000	0.610	<0.001
	rs4516035	39.2	51.1	0.666	0.000	0.306	0.002
	rs7139166	-12.2	-50.8	0.666	0.000	0.312	0.002

After controlling for covariates (age, sex, smoking, alcohol intake, physical activity, time spent outdoors, season, and BMI), VDR SNP genotypes were entered as predictors of physical performance. rs2228570: 40%, 44%, and 16% of participants had CC, CT (alternate allele 1), and TT (alternate allele 2) genotypes; rs4516035: 18%, 46%, and 36% of participants had CC, TC (alternate allele 1), and TT (alternate allele 2) genotypes; rs7139166: 36%, 46%, and 18% of participants had CC, GC (alternate allele 1), and GG (alternate allele 2) genotypes. VDR, vitamin D receptor; SNP, single-nucleotide polymorphism; Beta, standardized beta coefficient; Sig. ΔF, significant F change *P* value; f<sup>2</sup>, Cohen's f<sup>2</sup> effect size, f<sup>2</sup> ≥0.02, ≥0.15 and ≥0.35 represent small, medium and large effect sizes, respectively (32).