Effect of vitamin D supplementation on CVD risk factors and exercise performance in healthy subjects; a randomised placebo controlled preliminary study

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Abstract

Background and objectives: Evidence suggests associations between vitamin D deficiency and cardiovascular risk factors, including hypertension and excessive cortisol levels. Also, vitamin D levels may impact exercise performance. Thus, we aimed to investigate the effects of vitamin D intake on cardiovascular risk factors, free urinary cortisol and exercise performance.

Methods: A randomised placebo controlled single-blinded parallel trial was conducted in healthy subjects (n=15). They received 2000IU (50µg) vitamin D3 per day (n=9) or placebo (lactose) (n=6) for 14 days. Body composition, systolic (SBP) and diastolic blood pressure (DBP) and arterial elasticity (PWV) were recorded at baseline, day 7 and day 14 of intervention. Two 24h urine samples were collected to estimate free cortisol and cortisone levels. Exercise performance was assessed at baseline and day 14 of intervention using a bike ergometer in which BP and PWV were measured before and after exercise. The distance cycled in 20 minutes and Borg rate of exertion scale were recorded.

Results: In the intervention arm, at day 14, vitamin D supplementation significantly reduced SBP and DBP from 115.8±17.1 and 75.4±10.3 at baseline to 106.3±10.9 (p=0.022) and 68.5±10.1mmHg (p=0.012) respectively. Also arterial stiffness was markedly reduced in vitamin D group (from 7.45±1.55 to 6.11±1.89, p=0.049). Urinary free cortisol levels and cortisol/cortisone ratio were significantly reduced from 162.65±58.9 nmole/day and 2.22±0.7 to 96.4±37.2 (p=0.029) and 1.04±0.4 (p=0.017) respectively. Exercise-induced systolic and diastolic BP were significantly reduced post vitamin D intake from 130.7±12.2 to 116.1±8.1 (p=0.012) and from 76.2±8.4 to 70.5±7.7 mmHg (p=0.042) respectively. The distance cycled in 20 minutes significantly increased from 4.98±2.65 to 6.51±2.28km (p=0.020), whilst the Borg rate of exertion scale reduced from 5.13±1.36 to 4.25±0.71RPE (P=0.021). In the placebo arm, no significant effects on CVD risk factors and exercise performance were observed.

Conclusions: These results suggest that daily vitamin D supplementation may ameliorate CVD risk factors including a decrease in 11β-HSD1 activity as evidenced by the decrease in the cortisol/cortisone ratio, and improve exercise performance in healthy individuals. However, large scale studies are required to verify our findings.

Keywords: vitamin D, cardiovascular disease, exercise performance, blood pressure, oxidative stress, pulse wave velocity
Introduction

There is an interesting body of evidence for the multiplicity of roles of vitamin D in the human body exerted through vitamin D receptors (VDR), which is present in many tissues including endothelial cells (Martins et al. 2014). VDR have a role in the conversion of 25(OH)D₃ to its active form 1,25(di-OH)D This has many functions including anti-poliferative effects on vascular smooth muscle, immune modulation, stimulating release of inflammatory cytokines and modulates renin-angiotensin-aldosterone system (RAS) (Giallauria et al. 2012). The discovery of vitamin D receptors in many tissues has provided new insights into the broad classical and non-classical functions of vitamin D and the adverse effects of its deficiency (Kendrick et al. 2009). Vitamin D deficiency is highly prevalent worldwide. Low levels of serum vitamin D are present in as many as 30-50% of otherwise healthy
adults (Wang et al, 2008a). Limited synthesis due to inadequate sun exposure, low intake of Vitamin D rich food, pigmented skin, indoor lifestyle and use of sun-screen creams are the main causes of low serum vitamin D levels, while a poor dietary habits and intake of vitamin D in food or supplement also contributes to the risk of deficiency. Hypovitaminosis D is very common in winter months in the UK; synthesis of vitamin D3 is almost impossible and the majority of the UK population might be vitamin D deficient (Close et al, 2013). Hypovitaminosis D is defined as a serum vitamin D level of <40 nmol/L (Hypponen et al, 2007), though some researchers recommend a level of >50 nmol/L to be adequate (Zitterman et al, 2009). The Endocrine Society Clinical Practice Guidelines (2011) recommended levels for optimal vitamin D of >75 nmol/L.

Cardiovascular diseases (CVD) such as heart attack, congenital heart disease and stroke are a major cause of morbidity and mortality. An estimated £19 billion is spent on CVD related treatment (Kendrick et al. 2009; British Heart Foundation 2015). Recent literature has implicated vitamin D deficiency as a risk factor for CVD and its deficiency has developed to be a key biological predictor of increased rates of CVD (Mheid et al. 2011; Gotsman et al. 2012). Vitamin D inadequacy has also been associated with hypertension, obesity, atherosclerosis, diabetes mellitus type 2 and oxidative stress (Anderson et al. 2010; Giallauria et al. 2012; Gotsman et al. 2012; Antoniades et al, 2009). For instance, the Framingham offspring study showed that low levels of vitamin D are independently related to CVD incidence (Wang et al. 2008a). Additionally, low vitamin D levels have been linked with reduced exercise performance. Vitamin D deficiency has been associated with reduced exercise performance in athletes Fitzgerald et al, 2015). Researchers have investigated whether vitamin D may benefit exercise performance (Koundourakins et al, 2014), and reported that in trained athletes, vitamin D intake showed improvement in their performance. However, the understanding of the effect of vitamin D supplementation on both CVD risk factors and exercise performance has been compromised by limited prospective studies, the suboptimal dosing of vitamin D and the lack of understanding of the mechanism by which vitamin D mediates benefit to CVD risk factors and exercise performance (Giallauria et al. 2012).

In humans, vitamin D known as "sunshine vitamin" is a fat-soluble unique vitamin because it can be synthesised by the body in the skin from sun exposure (UVB light). The sun contributes to 80-90% of vitamin D supply Zitterman et al, 2005), while the diet is a poor source of vitamin D with only 20% of vitamin D supply (Zitterman et al, 2009). Food sources which do contain some vitamin D include oily fish, egg, fortified spreads and breakfast cereals (Wang et al, 2008a). The term ‘vitamin D’ is an umbrella term for the two forms of the vitamin: vitamin D3, which is also known as cholecalciferol and vitamin D2, which is also known as ergocalciferol. Both of these forms of the vitamin are biologically inactive and so must undergo metabolism to their active form before they can exert their beneficial effects on the body. The aims of this study are to investigate the effects of vitamin D supplementation on
cardiovascular risk factors, urinary free glucocorticoids and exercise performance in healthy volunteers.

Materials and Methods

Subject Recruitment
Subject recruitment was held at Queen Margaret University (QMU), and following the approval of the Research Ethics Committee was granted for the project, a recruitment email was sent via the university’s internal email system. Eligibility criteria were assessed using a health status questionnaire. The inclusion criteria included both healthy males and females aged 19-53 years. The exclusion criteria included people suffering from CVD, hyperglycaemia, primary hyperthyroidism, renal failure, diabetes mellitus and vitamin D intolerance. Additionally, individuals who were already taking Vitamin D supplements and pregnant and breastfeeding women were excluded. Information sheets were presented to the subjects and once participants decided to participate they were asked to sign the consent form, and all data collected from the volunteers was kept anonymous. All measurements took place in QMU between 15th Feb.2015 and 1st May 2015.

Study Design
A parallel single blinded randomised placebo-controlled trial was conducted with duration of two weeks (Table 1). Subjects were advised to avoid vitamin supplementation and not to change their dietary habits. In order to decrease intra-patients variability, and thus increase statistical power, the cardiovascular risk factors BP and PVW were measured in the beginning, middle and at the end of the intervention (Vickers 2003). All parameters were recorded 3 times at each visit and the average reading was taken. Subjects taking Vitamin D were given 2000 IU (50μg in tablet form contains also 100mg lactose) daily for two weeks, and a placebo tablets (containing 100mg lactose) which resemble vitamin D tablet were given to the placebo group. A total of 15 subjects were recruited; 9 people were decided to take vitamin D and 6 subjects for the placebo group.

Blood pressure, pulse wave velocity and BMI measurements
A digital sphygmomanometer (Omron M5-I) was used to measure BP while the subjects lied down comfortably in a 30-degree position and were allowed to relax for 10 minutes in a quiet room to avoid “white coat” hypertension (Mheid et al. 2011). The pulse wave velocity (PWV) was measured to assess arterial stiffness using a noninvasive devise, VicorderTM (Skidmore Medical, Bristol, UK). Participants were rested in supine position in a quiet room and the PWV was measured by
simultaneously inflating both cuffs to 60mmHg. PWV is calculated by measuring pulse transient time and distance between the two sites. The distance (cm) between sites was measured manually using a tape measure (Weber et al, 2009). Height and weight were measured using standard techniques and BMI was calculated. Samples of urine collected as 24 hour urine collections to assess urinary cortisol and cortisone levels using specific ELISA methods.

**Exercise Performance**
Exercise performance was investigated using a bike ergometer (Ronge 1952). BP and PWV were measured before and after exercise. The intensity of the bike ergometer was set to 100watts to reduce inter and intra individual variability. The distance was recorded after 20 minutes of exercise. The same bike was used at both sessions to minimise variance between different bike ergometers. To investigate the strain of exercise the Borg scale was used for ratings of perceived excretion as described previously (Borg 1982). Diet diaries and health status questionnaires were measured before and after intervention to ensure any changes in CVD risk factors and exercise performance were due to vitamin D supplementation.

**Table 1. Outline of the study design**

<table>
<thead>
<tr>
<th>Day</th>
<th>Scheduled activities</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day -5</td>
<td>5 day washout period</td>
</tr>
<tr>
<td></td>
<td>2 day diet diary to be completed</td>
</tr>
<tr>
<td></td>
<td>Health status questionnaire</td>
</tr>
<tr>
<td>Day -1</td>
<td>24 hour urine collection</td>
</tr>
<tr>
<td>Day 0</td>
<td>Baseline BP, PWV, height, weight, BMI all recorded</td>
</tr>
<tr>
<td></td>
<td>Baseline distance for 20 minutes cycling</td>
</tr>
<tr>
<td></td>
<td>Vitamin D supplementation or Placebo starts</td>
</tr>
<tr>
<td>Day 8-9</td>
<td>BP, PWV recorded</td>
</tr>
<tr>
<td>Day 13</td>
<td>24 hour urine collection</td>
</tr>
<tr>
<td></td>
<td>2 day diet diary to be completed</td>
</tr>
<tr>
<td></td>
<td>Health status questionnaire</td>
</tr>
<tr>
<td>Day 14-15</td>
<td>Final BP, PWV, height, weight, BMI measured</td>
</tr>
<tr>
<td></td>
<td>Distance for 20 minutes cycling recorded</td>
</tr>
</tbody>
</table>

**Biochemical markers**
24 hour urine collections were obtained at baseline and at day 14 to assess oxidative stress, cortisol and cortisone levels. All samples were weighed and stored at -20ºC to avoid bacterial and fungal growth and to reduce polyphenol oxidation.
TBARS assay was performed to measure lipid peroxidation (oxidative stress) (Yagi 1984). The TBARS method is used for screening and monitoring lipid peroxidation, which is a major indicator of cellular injury and thus considered to be an oxidative stress marker (Amstrong and Browne 1994). At present, the TBARS assay is the most widely employed method for assessing plasma lipid peroxidation that allows comparison with other published studies. In brief, 0.1mL of urine sample, 0.2mL of Tris buffer and 0.1mL of Ascorbic acid was added to the incubation tube. After 15 minutes of incubation at 37°C, 0.4mL of TCA and 0.8mL of TBA solution were added into the tubes. Tubes were then incubated at 99°C for 15 minutes and centrifuged at 4000 rpm for 10 minutes. The absorbance of the samples was read at 532nm. The data were calculated from standard curve constructed simultaneously. The within individual run coefficient of variation was 1.8-3.3%, which is fairly specific and acceptable (Amstrong and Browne 1994).

Urinary free cortisol and cortisone levels were measured using highly specific and sensitive ELISA methods previously described by Al-Dujaili et al. (2012). The urine samples were extracted by taking 100μL of urine samples and 1.5mL of ether and the tubes were vortex mixed for 10 minutes. Tubes were then placed in a −80°C freezer for 10 minutes and the upper layer was dried in glass tubes and reconstituted in 1mL assay buffer. Briefly, the ELISA plate was coated with the steroid-BSA conjugate and left in the fridge overnight. The plate was washed with wash buffer and blocked using 200μL of blocking buffer. The plate was then incubated at 37°C for one hour. After emptying the buffer, 50μL of sample or standard was added into the assigned wells, followed by the addition of 100μL of antibody in assay buffer. The plate was incubated at room temperature for 2 hours and washed. Enzyme coupled to a double antibody solution was added followed by 1 hour incubation at room temperature. The plate was then washed and 100μL of substrate was added and incubated for 15 minutes, 50μL of stop solution was then added and the plate was read at 450nm in the MRX ELISA reader (Dynex, USA).

**Statistical analysis**

All statistics were performed using SPSS statistics version 19.0 and numerical variables were first tested for normal distribution. Differences in baseline characteristics were examined using independent t tests for scale data and χ2-test for categorical variables. A one way ANOVA was performed to assess changes between baseline, day 7 and day 14 in BP and PWV. The models included the main effects of treatment and post-hoc Bonferroni adjustment was used to account for multiple testing. Paired t-tests were used to establish changes between baseline and day 14 in exercise performance, oxidative stress and cortisol/cortisone levels. Values were presented as the mean±sd or sem and P ≤0.05 (Field 2005).
Results

Subjects Characteristics
Fifteen healthy non-smokers aged 19-53 were recruited; the subjects were randomly allocated either to receive vitamin D supplementation (n=9) or placebo (n=6) (Table 2). Six out of all the 15 subjects were regular exercisers with mean±SD of 2.77±3.59 hours/week and no subjects were on contraceptive drugs. The baseline characteristics of the subjects are presented in Table 2.

Physiological markers
In the intervention arm, vitamin D supplementation significantly reduced both SBP and DBP from 115.8±17.1 and 75.4±10.3 to 109.1±11.3 (p=0.022) and 70.3±11.6mmHg (p=0.014) respectively at day 7. PWV was only reduced slightly from 7.45±1.55 to 6.79±1.28 (p=0.08). At day 14 of intervention SBP and DBP were further reduced to 106.3±10.9 (p=0.02) and 68.5±10.1mmHg (p=0.01) respectively. Arterial stiffness as PWV was markedly reduced from 7.45±1.55 to 6.11±1.89 (p=0.04) (figure 1).

In the placebo arm, baseline SBP and DBP were slightly reduced at day 7 but was not significant; from 116.7±9.3 and 73.7±7.9mmHg to 114.4±8.1 (p=0.306) and 72.4±5.8 (p=0.689) respectively, and again no significant reduction was noted between baseline and day 14 (114.8±7.6; p=0.3 and 72.9±8.3; p=0.26) (figure 1). One way ANOVA were not significant for SBP, DBP baseline between the vitamin D and placebo groups. The Δ changes in SBP, DBP and PWV in vitamin D subjects versus the placebo group are shown in figure 2.

Biochemical Markers
At baseline the biochemical markers were calculated by analysing 24 hour urine collections to measure oxidative stress using TBARS (3.82±1.12μM). ELISA was used to measure stress levels through cortisol (162.6±58.9nmole/day) and cortisone (74.2±76.9nmole/day). Following intervention, cortisol levels were significantly reduced to 96.4±37.42nmole/day (p=0.029). However, no significant change in TBARS (3.38±1.30μM), or cortisone levels (92.6±52.6nmole/day) were found. Cortisol/cortisone ratio was significantly reduced from 2.22±0.7 to 1.04±0.4 (p=0.017). In the placebo group, no significant changes were evident (Table 3).
<table>
<thead>
<tr>
<th>Baseline Characteristics</th>
<th>Vitamin D</th>
<th>Placebo</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ± SD</td>
<td>Mean ± SD</td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td>27.75 ± 11.3</td>
<td>25.20 ± 3.1</td>
<td>0.6^</td>
</tr>
<tr>
<td>BMI (kg/m^2)</td>
<td>22.39 ± 4.2</td>
<td>22.76 ± 4.3</td>
<td>0.4^</td>
</tr>
<tr>
<td>Physical Activity (Hours/week)</td>
<td>4.2 ± 3.9</td>
<td>2.80 ± 2.8</td>
<td>0.2^</td>
</tr>
<tr>
<td>Alcohol (Units/week)</td>
<td>3.75 ± 11.7</td>
<td>3.2 ± 2.9</td>
<td>0.07^</td>
</tr>
<tr>
<td></td>
<td>n (%)</td>
<td>n (%)</td>
<td></td>
</tr>
<tr>
<td>Gender</td>
<td>9 (100)</td>
<td>6 (100)</td>
<td>0.5^</td>
</tr>
<tr>
<td>Male</td>
<td>2 (22.2)</td>
<td>2 (33.3)</td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>7 (77.7)</td>
<td>4 (66.6)</td>
<td></td>
</tr>
<tr>
<td>Ethnicity</td>
<td>9 (100)</td>
<td>6 (100)</td>
<td>0.7^</td>
</tr>
<tr>
<td>White</td>
<td>4 (44.4)</td>
<td>3 (50.0)</td>
<td></td>
</tr>
<tr>
<td>Chinese</td>
<td>2 (22.2)</td>
<td>0 (0)</td>
<td></td>
</tr>
<tr>
<td>Pakistani</td>
<td>3 (33.3)</td>
<td>3 (50.0)</td>
<td></td>
</tr>
<tr>
<td>Caffeine</td>
<td>9 (100)</td>
<td>6 (100)</td>
<td>0.07^</td>
</tr>
<tr>
<td>Never/1-3 times per week</td>
<td>6 (42.4)</td>
<td>4 (66.6)</td>
<td></td>
</tr>
<tr>
<td>1-2 Cups/day</td>
<td>1 (7.3)</td>
<td>1 (16.6)</td>
<td></td>
</tr>
<tr>
<td>2-3 Cups/day</td>
<td>1 (7.3)</td>
<td>1 (16.6)</td>
<td></td>
</tr>
<tr>
<td>4 Cups/day</td>
<td>1 (7.3)</td>
<td>0 (0)</td>
<td></td>
</tr>
</tbody>
</table>

^1 Independent t-test; ^2 χ^2 test. The percentages were calculated out of the total number of volunteers in the study.
Table 3, Changes in TBARS, cortisol, cortisone and cortisol/cortisone ratio between baseline and intervention at day 14 (mean±SD) following vitamin D supplementation.

<table>
<thead>
<tr>
<th>Marker</th>
<th>Baseline (mean±SD)</th>
<th>Intervention (mean±SD)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>TBARS</td>
<td>3.82±1.12</td>
<td>3.38±1.30</td>
<td>0.190</td>
</tr>
<tr>
<td>Cortisol</td>
<td>162.6±58.9</td>
<td>96.4±37.2</td>
<td>0.029</td>
</tr>
<tr>
<td>Cortisone</td>
<td>74.2±76.9</td>
<td>92.6±52.58</td>
<td>0.210</td>
</tr>
<tr>
<td>Cortisol: Cortisone Ratio</td>
<td>2.22±0.70</td>
<td>1.04±0.42</td>
<td>0.017</td>
</tr>
</tbody>
</table>

Placebo

<table>
<thead>
<tr>
<th>Marker</th>
<th>Baseline (mean±SD)</th>
<th>Intervention (mean±SD)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>TBARS</td>
<td>3.24±0.56</td>
<td>4.74±0.32</td>
<td>0.250</td>
</tr>
<tr>
<td>Cortisol</td>
<td>238.8±105.3</td>
<td>187.6±125.7</td>
<td>0.494</td>
</tr>
<tr>
<td>Cortisone</td>
<td>75.4±39.67</td>
<td>65.1±14.50</td>
<td>0.339</td>
</tr>
<tr>
<td>Cortisol:Cortisone Ratio</td>
<td>3.16±1.24</td>
<td>2.89±2.37</td>
<td>0.788</td>
</tr>
</tbody>
</table>

P-value calculated using paired t-test.

Table 4. Dietary intake of subjects at baseline and intervention phase (mean±SD).

<table>
<thead>
<tr>
<th>Dietary Intake</th>
<th>Baseline (mean±SD)</th>
<th>Intervention (mean±SD)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fat (g)</td>
<td>59.65±16.66</td>
<td>55.05±12.78</td>
<td>0.383</td>
</tr>
<tr>
<td>CHO (g)</td>
<td>167.65±42.91</td>
<td>138.98±49.02</td>
<td>0.141</td>
</tr>
<tr>
<td>Protein (g)</td>
<td>79.95±46.52</td>
<td>74.16±40.59</td>
<td>0.877</td>
</tr>
<tr>
<td>Total Energy (kcal)</td>
<td>1413.96±334.51</td>
<td>1281.46±240.60</td>
<td>0.156</td>
</tr>
<tr>
<td>Vitamin D (µg)</td>
<td>0.87±0.41</td>
<td>0.88±0.27</td>
<td>0.937</td>
</tr>
<tr>
<td>BMI</td>
<td>22.54±4.08</td>
<td>22.65±4.01</td>
<td>0.358</td>
</tr>
</tbody>
</table>

P-value calculated using paired t-test.

Effect on exercise performance
The results of this study showed a significant reduction in pre-post exercise SBP and DBP following 2 weeks of vitamin D intervention. At baseline, the BP measurements
after exercise were; SBP (130.7±12.6mmHg) and DBP (76.2±8.4 mmHg) in subjects receiving vitamin D supplementation and SBP (128.7±8.7mmHg), DBP (74.1±7.5mmHg) in the placebo group. After two weeks of vitamin D supplementation the exercise-induced rise in SBP was reduced to 116.1±8.2mmHg (p=0.012) and DBP reduced to 70.5±7.7mmHg (p=0.04) after 20 minutes exercise (figure 3). However, there was no significant changes in the placebo group as SBP was 127.2±11.1mmHg (p=0.2) and DBP 73.7±5.9mmHg (p=0.827).

The vitamin D group showed a statistically significant increase in the distance cycled from 4.98±2.65km to 6.51±2.28km (p=0.02) and a significant reduction in the Borg scale values of exertion from 5.13±0.80RPE to 4.25±0.70RPE (p=0.02) following two weeks of intervention. No statistical significant changes were seen in the placebo group for either the distance cycled (4.14±2.06km to 4.83±1.17km, p=0.24) or the Borg scale values (5.8±1.89 RPE to 5.80±1.79RPE, p= 0.98) (figures 4A and 4B).

**Diet**

Results from the food diaries collected from all participants showed no significant changes in the macronutrients and energy intake for vitamin D group at baseline and during intervention. BMI was also calculated and no significant difference was noted between baseline and intervention phase (Table 4). Additionally, analysis of the health status questionnaires showed no significant difference in the amount of exercise per week, total alcohol consumption and coffee consumption.
Figure 1. Effects of vitamin D supplementation on basal Systolic & Diastolic BP (mean±sem). *p=0.02, 95% CI (0.4-11.6); **p=0.01, 95% CI (0.3-7.9).

Figure 2. The Δ changes in SBP, DBP and PWV following vitamin D and placebo intervention at day 14.
Figure 3. Effects of vitamin D supplementation on exercise-induced systolic and diastolic BP (mean±sem). *p=0.042; 95% CI (0.3-21.3); **p=0.012; 95% CI (0.1-10.1)
Figure 4. Effects of vitamin D supplementation on exercise performance measured as distance (4A), and Borg scale of exertion (4B).

Results are expressed as mean±sem; 4A: Subjects distance cycled at baseline and day 14 of intervention, *p=0.02; 95% CI [(-2.74) to (-0.320)]; 4B: Subjects rate of exhaustion after exercise calculated using Borg scale, p=0.02; 95% CI [(-2.74) to (-0.32)].

Discussion

This pilot study found that healthy adults supplemented with vitamin D had both lower blood pressure and lower levels of the stress hormone cortisol in their urine compared to those given a placebo. Furthermore, the vitamin D group increased the distance cycled by 1.5Km (30%) in 20 minutes and showed lower signs of physical exertion than the placebo group following two weeks of intervention.

Around 10 million people in the UK may have low vitamin D levels. On average, one in ten adults has low levels of vitamin D in the summer, compared to two in five in winter. Because people with darker skin are less efficient at using sunlight to make vitamin D, up to three out of four adults with dark skin are deficient in winter. Our pilot study suggests that taking vitamin D supplements can improve fitness levels and lower cardiovascular risk factors such as blood pressure and pulse wave velocity. Our study adds to the body of evidence showing the importance of tackling this widespread problem.

A significant reduction was noted in SBP and DBP between baseline and after vitamin D intervention. A similar improvement in BP was seen in a prospective double blind study in 283 black individuals with 1000, 2000 and 4000IU of vitamin D supplementation (Forman et al. 2013). Vitamin D exerts antihypertensive effects
through the inhibition of the RAS system and the association between vitamin D levels and renin activity was first established in 1986 (Resnick et al. 1986; Wu et al. 2010). RAS is a vital regulator of BP through renin activity, in which renin cleaves angiotensin I to angiotensin II and once bound to the receptor, it exerts regulatory effects on BP. Inappropriate stimulation of RAS proceeds to hypertension suggesting that the inhibition of RAS by vitamin D may reduce BP (Li et al. 2002). Conversely, a number of studies have reported no reduction in BP with vitamin D. One study (Zittermann et al. 2009) reported that 83μg vitamin D daily showed no reduction in BP in 200 subjects. However, the study showed marked variations and was confined as the BP was measured only at two occasions limiting the detection of small but significant changes in BP. More recently, 100,000IU vitamin D supplements every 2 months failed to reduce BP in 75 subjects, however the dose is too large to be given at once which might disappear in the adipose tissue and the daily available vitamin D is reported to be insufficient to exert the biological effects (Heaney et al. 2005; Witham et al. 2013). Additionally, concomitant medication has prevented vitamin D from exerting beneficial effects, explaining no effect on BP in many studies (Witham et al. 2012).

Arterial stiffness, the reduced ability of an artery to respond to blood pressure changes, has been associated with vitamin D deficiency (Giallauria et al. 2012; Pirro et al. 2012). Vitamin D deficiency causes arterial stiffness through the stimulation of vascular smooth muscle proliferation, macrophage invasion and calcification (Mheid et al. 2011). In the current study vitamin D supplementation induced a small but statically reduction in PWV, which is the gold standard index for measuring arterial stiffness (Pirro et al. 2012). Similarly, 2500IU vitamin D daily exerted no effect on arterial stiffness in 114 participants, although average circulating levels of 25(OH)D were increased (Gepner et al. 2012). More recently, arterial stiffness in 199 subjects was non-significantly affected by 50,000IU and 100,000IU single intramuscular vitamin D (McGreevy et al. 2015). In contrast, 2 other studies supplemented with 2500IU vitamin D daily for 4 months and 100,000IU vitamin D for 3 months significantly reduced PWV in African individuals (Dong et al. 2010; Martins et al. 2014). However, data from both studies cannot be extrapolated on the general population as both studies included black individuals living in high latitudes and skin colour Africans have a higher prevalence of vitamin D deficiency. Vasoprotective functions of vitamin D are believed to be exerted by direct and indirect effects on vascular cells and suppression of RAS (Dong et al. 2010). The reduction in PWV might also impact favourably the development of CVD later in adulthood (Dong et al. 2010). Additionally, this study did not have sufficient power to detect a moderate but significant change in PWV. The method of assessing PWV may have introduced possible errors in the study as PWV is advised to be measured in a dark, temperature controlled environment (Mheid et al. 2011). Moreover, the intervention period was insufficient as improvement in calcification may take longer than 3 months to the point where arterial compliance may improve and make structural or functional changes to the arteries (Witham et al. 2012; Ryu et al. 2014). Therefore,
longer trials investigating the potential of vitamin D in stimulating favourable alterations in cardiovascular function are warranted.

Vitamin D has been associated with CVD due to its effects on oxidative stress. Tarcin et al. (2009) demonstrated that vitamin D deficient individuals presented higher plasma TBARS. Free radical oxidation of cellular components in CVD has been widely recognised and oxidative modification of low density lipoprotein and cellular constituents subsidises mechanism of atherogenesis (Canale et al. 2014). Additionally, Reactive oxygen species promote oxidative stress, since they induce specific post translational modifications which alter the function of vital cellular proteins and signalling pathways to the heart (Ho et al. 2013). Biomarkers of oxidation, specifically malondialdehyde (MDA), often measured using TBARS, are elevated in relationship with CVD risk factors. Since the 1990’s the association between vitamin D supplementation and TBARS has been thoroughly investigated (Wiseman 1993; Kuzmenko et al. 1997). In this particular study, no change in TBARS was observed in samples taken before exercise suggesting oxidative stress was unaffected by vitamin D supplements or post urine samples should have been collected to test the effect following the challenge of exercise. A study supplemented with 300,000IU vitamin D monthly, for three months, effectively reduced TBARS in 23 asymptomatic subjects, possibly due to an improvement of endothelial function with vitamin D supplementation, whereas measurements of flow mediated dilation were significantly lower in 25(OH)D-deficient subjects (Tarcin et al., 2009). Activated T cells induce oxidative stress and studies have found that the effect of vitamin D on RAS inhibit release of inflammatory cytokines from activated T-cells, thus, reducing oxidative stress (Judd et al. 2010). In addition, Wiseman (1993) suggested that the hydrophobic sections of 1,25(OH)D₃ has the capability to impair the viscosity of the membrane, thus, protecting the cell membrane from lipid peroxidation and harmful effects of free radicals suggesting a role for vitamin D as a potential antioxidant.

Vitamin D supplementation significantly reduced cortisol levels and cortisol/cortisone ratio but has non-significant effect on cortisone. Cortisol is vital for the body’s recovery form stress, however excessive levels of cortisol has direct effects on cardiac output and has been associated with hypertension (Vogelzangs et al. 2010). Cortisol release is stimulated by the activation of HPA which promotes the release of CRH and ACTH. Also the enzyme 11β-HSD1 exerts an important role in modulating the levels of active cortisol. The reduction in the ratio of urinary cortisol/cortisone indicated an inhibition of 11β-HSD1 activity, thus the decrease in cortisol levels. Hyperactivity of HPA results in an excessive production of cortisol and once cortisol is bound to the mineralocorticoid receptor, pro-inflammatory effects and vascular cell calcification is promoted causing damaging effects in CVD (Bhathena et al. 1991; Vogelzangs et al. 2010: Kumari et al. 2011). Cortisone and cortisol/cortisone ratio has been linked with increase CVD events (Quinkler and Stewart 2003). To date no study has investigated the potential effect of vitamin D supplementation on cortisol levels, although a limited number of studies have investigated the effects of vitamin
E and C on cortisol levels (Peters et al. 2001). For instance, vitamin C supplementation significantly reduces cortisol levels after ultramarathon racing in 45 participants (Peters et al. 2001). The current study indicated that vitamin D has the potential to reduce cortisol levels and cortisol/cortisone ratio.

Suboptimal levels of vitamin D have been associated with impaired exercise performance, as it reduces muscle action and skeletal mineralisation (Wyon et al. 2014; Fitzgerald et al. 2015). Many studies have argued that vitamin D status is linked with muscle strength, aerobic capacity and speed, however, the specific mechanism by which vitamin D exerts its effects on performance is debated (Wyon et al. 2014; Koundouakis et al. 2014). One proposed mechanism is that vitamin D supplementation increases ATP content in muscle, while others argue that vitamin D improves muscle function through the presence of VDR in muscle fibres by controlling serum calcium concentration (Wyon et al. 2014). Additionally, vitamin D influences VO2 max via effects on erythropoiesis, thus modifying the oxygen supply to exercising muscles, consequently improving aerobic exercise performance (Koundouakis et al. 2014). In the current study, vitamin D supplementation significantly reduced SBP and DBP, and a slight reduction in PWV thus, improving performance and tolerance as high BP induces fatigue, dyspnea, mild exertion and excessive ventilator response to any load of exercise (D’Alonzo et al. 1987). This was first suggested when acute intravenous verapamil reduced systolic and arterial stiffness, thus, enhanced aerobic exercise performance (Chen et al. 1999). It may be possible to detect a greater reduction in PWV if the duration of vitamin D intervention was increased as intervention may take 3 months before a marked improvement in arterial stiffness is detected (Witham et al. 2013). In the current study, vitamin D supplementation significantly improved chosen measures of physical performance including distance cycled on bike ergometer and Borg scale as an increase in distance and a reduction in Borg scale of exertion were noted. The Borg scale is considered to be the best indicator of degree of physical strain and a reduction indicated improved performance (Borg 1982). Despite the small sample size, 2000IU vitamin D significantly improved some markers of performance, suggesting the potential of vitamin D to reduce fatigue and improve endurance and aerobic capacity, thus a role of vitamin D as ergogenic aid might be considered. Vitamin D deficiency was associated with increased rate of perceived exertion assessed by Borg scale in heart failure patients (Boxer et al. 2011). Similarly, Wyon et al. (2014) reported beneficial effects of 2000IU vitamin D supplementation for 4 months on performance parameters including muscle strength and vertical jump height in 24 ballet dancers, but lacked proper randomisation of participants into groups and consideration of confounding variables. Confounding variables including caffeine and alcohol intake which are consumed by some athletes as ergogenic aid to reduce fatigue before exercise were all taken into consideration (Mohr et al. 2013; Koundouakis et al. 2014). No significant change between these variable were found proving beneficial effects on exercise performance were due to vitamin D supplementation. However, larger scale studies investigating effects of different doses of vitamin D and the
mechanisms by which vitamin D exerts its effects on exercise performance are warranted.

**Limitations and strengths of this study**
This study was limited by the reduced sample size, short intervention period and the single blinded approach. Additionally, recommended dietary intake of vitamin D is 10-20μg/day and subject’s dietary intake was 1.87±0.41, since plasma levels of 25(OH)D were not measured, it can be assumed that subjects were vitamin D deficient (Hollis 2005). Thus, the vitamin D supplementation may have only restored body’s vitamin D levels but full biological effects were not observed (Heaney et al. 2005). More females than males were recruited in this study and sex could be a confounding factor as BP is associated with gender (Caro et al. 2012).

Furthermore, diet diaries were used as an indicator of dietary vitamin D intake and self-reporting can lead to errors such as under reporting of food consumption (Hughes et al. 2005). Of note, food frequency questionnaires focusing on food and supplements rich in vitamin D and sun exposure questionnaires to quantify UVB exposure have been reported to give accurate indication of total vitamin D intake (Caro et al. 2012). Despite the limitations this study has strengths including the analysis of confounding variables such as exercise, alcohol and caffeine to ensure beneficial effects were due to vitamin D supplementation and the fact that the study was performed during the winter and early spring months and no subjects had been on holidays 3 months prior to the study. In addition, the current study gives merit to the fact that healthy subjects who were not on medication were included, thus no interruption in the biological actions of vitamin D, and measurement of BP at three separate occasions taking the average of 3 readings at each occasion allowed the detection of small significant changes in BP post vitamin D supplementation.

**Conclusions**
We have shown that vitamin D supplementation improved some CVD risk factors in healthy volunteers. Blood pressure reduction suggests that vitamin D has antihypertensive effects possibly due to its inhibitory effects on the renin angiotensin system and 11β-HSD1 activity. Arterial stiffness was slightly reduced during the short duration of the study suggesting that long term vitamin D intake may improve arterial stiffness. Urinary cortisol and cortisol/cortisone ratio reduction suggests a decrease in the stress hormone levels, cortisol that is may be due to the reduction of 11β-HSD1 activity (the enzyme responsible for the activation of cortisone to its active form, cortisol). Exercise performance was markedly improved due to vitamin D supplementation. Our pilot study showed a reduction in the exercise-induced rise in blood pressure which may improve tolerance and reduce fatigue thus improving exercise performance. It is worth mentioning that vitamin D deficiency could be dormant and difficult to detect, as vitamin D can affect muscle work in various ways.
and there may be no visible symptoms of the deficiency (Ward et al, 2009). Future studies should assess plasma baseline and post intervention 25(OH)D levels, include a larger sample and perform a double-blinded clinical trial to verify these findings.

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