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The association of circulating 25-hydroxyvitamin D concentration with peripheral arterial disease: A meta-analysis of observational studies.

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ABSTRACT

Background and Aims: The association of vitamin D deficiency with cardiovascular disease is controversial. The present meta-analysis was performed to examine if circulating levels of 25-hydroxyvitamin D [25(OH)D] were lower in patients with peripheral artery disease (PAD) when compared to non-PAD controls.

Methods: A comprehensive database search was conducted in Web of science, Scopus, PubMed, EMBASE and The Cochrane Library to identify observational studies reporting 25(OH)D concentrations in PAD patients and non-PAD participants. Data extraction and study quality assessments were conducted independently. A random-effects model was used to meta-analyse extracted data and generate standardized mean differences (SMDs) in circulating 25(OH)D levels between PAD patients and non-PAD controls. Subgroup analyses were conducted focusing on patients presenting with intermittent claudication (IC) and critical limb ischaemia (CLI).

Results: Six case-control studies assessing 6418 individuals fulfilled the inclusion criteria. Two studies were considered to be of moderate methodological quality and four were considered to be of high quality. A meta-analysis of data from 1217 PAD patients and 5201 non-PAD participants showed that circulating 25(OH)D concentrations were lower in PAD patients compared with non-PAD participants (SMD=-0.32, 95% CI: -0.58, -0.05; P=0.02). Subgroup analyses showed that 25(OH)D levels were significantly lower among PAD patients with CLI, but not IC, when compared to non-PAD controls (SMD=-1.29, 95% CI: -1.66, -0.91; P<0.001 and SMD=-0.01, 95% CI: -0.15, 0.13; P=0.88, respectively).

Conclusions: This meta-analysis suggests that low levels of circulating 25(OH)D are associated with PAD presence, particularly in patients presenting with CLI. These data suggest

the possibility that vitamin D insufficiency may contribute to the development of more advanced PAD although this remains to be confirmed.

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INTRODUCTION

Peripheral arterial disease (PAD) is an athero-occlusive condition that leads to lower extremity ischaemia. Some patients with PAD experience pain in the muscles of the leg on walking, known as intermittent claudication (IC) [1]. Patients may develop symptoms of more advanced lower extremity ischaemia, including rest pain, lower limb ischaemic ulceration and gangrene, known collectively as critical limb ischaemia (CLI) [1]. PAD patients are prone to functional disability and amputation, and have an increased risk of cardiovascular events [1-4]. Approximately 30% of people aged over 70 years (~202 million individuals worldwide) are affected by PAD [3]. Older age, diabetes, cigarette smoking, dyslipidaemia and hypertension are well-characterized risk factors for PAD [1-5]. However, some of the risk factors for PAD development and complications remain poorly defined.

Vitamin D deficiency has been implicated in the aetiology of a number of chronic diseases including cardiovascular disease (CVD) [5-8]. Current guidelines suggest that circulating 25-hydroxyvitamin D [25(OH)D] concentration <50 nmol/L and 52.5–72.5 nmol/L are indicative of vitamin D deficiency and insufficiency, respectively [9]. Whether vitamin D deficiency or insufficiency is a risk factor for PAD is currently unclear. Some studies have reported that low levels of circulating 25(OH)D are associated with PAD [10-13] while others have not [2, 14, 15]. Furthermore, a recent study that followed up 1568 community-dwelling elderly participants for 4.4 years concluded that baseline vitamin D did not predict the onset of PAD among the 371 subjects who eventually developed the disease [16]. Currently, there is thus limited consensus regarding the association of 25(OH)D concentration with PAD. The aim of this study was to perform a meta-analysis of observational case-control studies in order to examine the association between circulating 25(OH)D concentration and PAD.

MATERIALS AND METHODS

Search strategy

The present meta-analysis was prepared in line with the preferred reporting items for systematic review and meta-analysis (PRISMA) statement [17] and meta-analysis of observational studies in epidemiology (MOOSE) guidelines [18]. A detailed protocol was published in the PROSPERO database before commencing the study, in accordance with the PRISMA-Protocols (PRISMA-P) 2015 statement [19] (PROSPERO registration number CRD42015016060).

A systematic literature search was conducted using Web of Science (via ISI Web of Knowledge; 1965), Scopus (1966), Medline (via OvidSP, 1966), PubMed and The Cochrane Library and Embase (1980). Two authors independently conducted the searches on two separate occasions (November, 2014 and March, 2015) to ensure that potentially eligible papers which were published whilst this manuscript was under preparation were also included. The search algorithm involved combinations of free keywords, specific MeSH (medical subject headings) and Emtree terms (EMBASE only). The following search string was devised: ("vitamin D OR vitamin D deficiency OR hypovitaminosis D OR 25-hydroxyvitamin D OR 1,25 dihydroxyvitamin D OR hydroxycholecalciferols OR calcifediol OR calcitriol OR cholecalciferol OR ergocalciferol" AND "peripheral arterial disease OR intermittent claudication OR peripheral arterial occlusive disease OR peripheral vascular disease OR limb ischaemia OR limb ischemia OR peripheral artery disease").

Study selection

Studies including participants with PAD and control subjects (non-PAD) were selected for the current review. PAD cases were defined based on positive clinical diagnosis by physician

examination, by ankle brachial pressure index (ABPI) < 0.9, or angiography or a combination of one or more of these.

A study was included if the following criteria were met:

1. An observational study;
2. Included PAD cases and controls as defined above;
3. A validated method of diagnosing PAD was used, including one or more of the following: physician examination; ABPI; or angiography;
4. Reported circulating 25(OH)D concentration in specific units (i.e. ng/L; nmol/L) for both PAD cases and non-PAD controls.

Studies were excluded if:

1. They had reported findings from animal studies;
2. 25(OH)D concentration was measured after a planned vitamin D supplementation intervention;
3. The study was interventional and had not documented circulating 25(OH)D concentrations at baseline (e.g. the study was investigating the effects of a previously administered drug or therapy on vitamin D concentrations);
4. The cases had self-reported PAD without clinical diagnosis;
5. Data extraction was not possible even after contacting the corresponding authors;
6. Data could not be differentiated between the two groups (i.e. cases and controls).

Relevant articles were identified and systematically screened against inclusion and exclusion criteria by two authors (VN and MF). Full texts of eligible studies were obtained and independently assessed for eligibility by one author (VN), under the expert guidance of JG.

Reference lists of all included studies were scrutinized against inclusion and exclusion criteria for potential relevance.

Data extraction

Data extraction was independently performed by three authors (VN, SO and JP) using a pre-defined form. This was cross-checked for errors and omissions in a consensus discussion. Extracted data included details related to study design, participants' characteristics, and results and statistical analyses that were reported in each study. Descriptive parameters such as participants' age, gender, body mass index (BMI), smoking, hypertension and diabetes status were recorded. A numerical value for 25(OH)D concentration was recorded in specific units (i.e. ng/ml or nmol/L). Authors of studies whose data could not be extracted were contacted by email for further information. One author responded (McDermott *et al* [15]). Since McDermott *et al* [15] had previously reported patient characteristics in relation to 25(OH)D quartiles, we requested data for all PAD cases and non-PAD controls. We also requested information on whether patients were asymptomatic, had IC or CLI.

Study quality assessment

Recognizing the limitations of generic quality assessment tools, a set of 21 content-specific questions that were relevant to PAD and vitamin D research were developed (Supplementary Table 1) [20]. The quality assessment tool was developed and scored based on the Evidence Based Library and Information Practice [21] and the Newcastle Ottawa scale [22]. The tool assessed the reporting of inclusion and exclusion criteria, definition of cases and controls, quality of 25(OH)D assays and confounders that were accounted for in analyses. The questions were first trialled on excluded studies [2, 10, 13] to evaluate the tool's suitability and consistency. Two authors who were blinded from the manuscript identifiers (i.e. authors and

journal's names, publishing institution and references) independently scored the studies. A difference of more than two points warranted a consensus meeting in which the studies concerned were discussed and inconsistencies resolved. All 21 questions were equally weighted. Individual scores were calculated based on the number of 'Yes' answers which indicated that the study satisfied the elements assessed by the quality question. The levels of agreement were calculated as a percentage of consistency between two assessors. All answers that differed between the two assessors were reassessed in a consensus meeting by reviewing the article in question. Answers were only changed if elements were unequivocally reported within the article. The average quality assessment score was calculated as a percentage of positively reported items averaged between assessors. Studies that scored <50%, 50 to 75% and > 75% were deemed of low, moderate, and high quality, respectively (Supplementary Table 1).

Quantitative data synthesis

The main outcome for the current meta-analysis was the standardized mean difference (SMD) of circulating 25(OH)D concentration between PAD cases and non-PAD controls. Statistical analysis was performed using Review Manager (Version 5.3) software package. Data were meta-analysed using random-effects models to account for anticipated inter-study heterogeneity. Where papers reported data as mean $\pm$ standard error of the mean (SEM), the standard deviation (SD) was computed as SEM x $\sqrt{n}$, where n denotes the number of participants. For studies that reported 25(OH)D concentration in ng/ml, units were converted to nmol/L by multiplying both the mean and SD by 2.5 [23]. Effect sizes were expressed as SMD and 95% confidence interval (CI).

Two studies classified their cases into Fontaine groups II and IV prior to comparing to a control group. In both studies [23, 24] Fontaine stage II PAD was characterized by pain in the calves

and/or thighs during exercise, which was relieved by rest. Based on these clinical criteria, these patients were considered to have IC for the purposes of the present review. In contrast, patients with Fontaine stage IV PAD had a history of ischaemic ulcers, and were considered to have CLI in the current meta-analysis [23, 24]. As we deemed that findings could be different for patients with IC and CLI, sub-analyses were performed to examine these patients separately. To avoid counting the control groups twice in these analyses, half of the control group from the relevant studies was allocated to each subgroup, assuming each half had the same mean and SD in line with recommendations in the Cochrane Handbook [25]. For all analyses, a leave-one-out approach was used to assess the effect of individual studies on the overall SMD score (Table 2). All these subgroup analyses were planned when designing the study as outlined in the study protocol (PROSPSERO; CRD42015016060). Inconsistency across included studies was assessed using the I^2 value. I^2 values of 25%, 50%, and 75% were acknowledged as low, moderate and high heterogeneity, respectively [26]. Publication bias was assessed by Begg's funnel plot, in which the SMD is logarithmically compared with the study's SEM (Supplementary Figure 1) [27].

RESULTS

Selection of included studies

A systematic database search yielded 498 titles and abstracts of potentially eligible studies after removal of duplicates. Overall, 481 studies were excluded following screening of the titles and abstracts. Studies were excluded mainly because they were not original clinical investigations (Figure 1). Seventeen full text articles were assessed and carefully reviewed. Five of these studies were excluded because PAD cases and/or non-PAD controls were not defined or 25(OH)D concentrations were not reported. One study was excluded as it was not a case-control investigation [16]. One study included patients with abdominal aortic aneurysm (AAA) as controls and since these individuals cannot be assumed to be healthy or not have concomitant PAD [28], this study was excluded [12]. Another study was excluded as it focused on AAA not lower limb PAD [29]. Three publications were excluded as they reported on duplicate populations [2, 10, 13]. The remaining six case-control studies which reported circulating 25(OH)D concentration in PAD cases and non-PAD controls were included in this meta-analysis (Table 1) [11, 14, 15, 23, 24, 30].

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Table 1: Demographic and clinical characteristics of participants within the studies included in this meta-analysis.

Study (Year)	Country	25(OHD) nmol/L		PAD Cases			Non-PAD controls			% Hypertension		% Smoking		% Diabetes	
		Cases	controls	n	% Males	Age	n	% Males	Age	Cases	Controls	Cases	Controls	Cases	Controls
Fahrleitner, 2002 [23]*															
IC patients	Austria	58.5±32.0	51.3±14.4	84	65.0	69.0±18.3	23	52.0	66.0±7.4	ND	ND	ND	ND	ND	ND
CLI patients	Austria	23.5±21.9	51.3±14.4	77	55.0	66.0±8.8	22	52.0	66.0±7.4	ND	ND	ND	ND	ND	ND
Fahrleitner-Pammer, 2005 [24]*															
IC patients	Austria	47.5±23.7	47.7±22.7	46	63.0	61.0±6.8	22	55.0	66.0±8.6	ND	ND	ND	ND	ND	ND
CLI patients	Austria	24.0±17.5	47.7±22.7	49	53.0	65.0±7.0	22	55.0	66.0±8.6	ND	ND	ND	ND	ND	ND
Melamed, 2008 [11]†	USA	53.7±30.2	61.5±79.9	406	42.8	67.1±16.1	4433	48.7	55.2±19.9	71.7	41.1	67.2	52.9	17.5	7.5
McDermott, 2012 [15]‡	USA	53.7±24.9	54.6±23.7	402	53.7	ND	305	48.5	71.8±7.7	71.4	64.9	15.9	6.9	32.0	23.6
Zagura, 2011 [30]†	Estonia	37.7±13.5	47.5±14.7	78	100.0	63.0±7.0	74	100.0	61.0±10.0	38.5	0.0	100.0	24.0	0.0	0.0
Liew, 2015 [14]†	Australia	72.3±32.0	67.8±27.5	75	ND	ND	300	ND	ND	ND	ND	ND	ND	ND	ND

Results are presented as mean ± SD. *These studies investigated groups of PAD patients with IC and CLI which were considered as separate groups in our analyses. Controls for these studies were divided into two and separately compared with patients that had IC or CLI, respectively. †These studies included PAD patients presenting with a mix of symptoms or symptoms that were not defined. ‡ McDermott *el al* [15] included patients presenting with no or atypical symptoms as well as those who had IC. Abbreviations: 25(OH)D: 25-hydroxyvitamin D; PAD peripheral arterial diseases; IC: intermittent claudication; CLI: critical limb ischemia; ND: not defined.

Description of included studies

Table 1 shows the characteristics of the participants of the included studies. Two studies were conducted in the USA [11, 15], two in Austria [23, 24], one in Estonia [30] and one study in Australia [14]. Two studies included only Caucasian participants [23, 24], one study [11] included mixed populations comprising non-Hispanic Caucasians and Black Americans as well as Mexican Americans and three studies [14, 15, 30] did not specify the participants races. A total of 6418 subjects were investigated across all six studies, comprising 1217 PAD patients and 5201 non-PAD participants. Melamed *et al* [11] contributed ~75% of the overall sample size (n = 4839), comprising 406 PAD patients and 4433 non-PAD controls. The weighted average percentage of males was 54.3% and 49.6% within PAD cases and non-PAD participants, respectively (Table 1). Three studies investigated PAD patients with IC [15, 23, 24]. In three studies [11, 14, 30], PAD symptoms were not explicitly defined amongst cases. These studies were considered to represent a mixed group of PAD presentations. In all six studies, ABPI < 0.9 was used as the primary means of diagnosing PAD. Three studies [23, 24, 30] indicated that angiography was used to confirm PAD. Participants with ABPIs ≥ 1.50 and ≥ 1.30 were excluded by Melamed *et al* [11] and McDermott *et al* [15], respectively, as this was suggestive of arterial rigidity. The prevalence of cigarette smoking, hypertension and diabetes were reported in three studies [11, 15, 30]. In one study [14], the majority of participants had coronary artery disease.

Five studies measured total circulating 25(OH)D (i.e. a combination of the $25(OH)D_3$ and $25(OH)D_2$ metabolites), whereas Fahrleitner *et al* [23] specifically assessed $25(OH)D_3$. In all six studies, 25(OH)D measurement was performed on blood serum. Two studies reported the levels of intact parathyroid hormone (iPTH) [23, 24], one study reported whole PTH levels; one study [15] measured whole PTH concentrations but data was not reported and two studies

[14, 30] did not report the measurement of PTH. Consequently, we were unable to adjust the current meta-analysis for this potential confounder. Assay methods used to determine 25(OH)D concentration varied across studies. Five studies used a radioimmunoassay (DiaSorin) and one study used ultra-performance liquid chromatography-tandem mass spectrometer (UPLC-MS/MS) [14]. Four studies [11, 23, 24, 30] reported circulating 25(OH)D concentrations in ng/ml and two studies [14, 15] reported this in nmol/L. Four studies [11, 14, 23, 24] reported the SEM and two studies reported the SD [15, 30]. In one publication [24] the same variance values accompanying the mean 25(OH)D concentration were alternatively reported as SD and SEM at different points within the manuscript. The authors were contacted for clarification without response. The reported variance value appeared impossibly large to be SEM and was therefore assumed to be the SD and used in this meta-analysis (Table 1).

Quality assessment of the included studies

Inter-observer agreement of the quality of included studies ranged from 95-100% (Supplementary Table 1). Most studies reported clear aims, detailed sampling methods, inclusion and exclusion criteria and population characteristics. All studies reported the method of PAD diagnosis in the cases and of PAD exclusion within the controls. Three studies adjusted their analyses for a number of CVD confounders; including BMI, hypertension, smoking and diabetes [11, 15, 30] while two studies did not [23, 24]. Four studies reported matching cases and controls for age and sex [15, 23, 24, 30]. However, one study [30] included only males in the study. Overall, two studies [14, 24] scored < 75% and were deemed of moderate quality. The remaining four studies [11, 15, 23, 30] scored > 75% and were considered of high quality. Two studies performed blood sampling during the winter season [23, 24]; two studies reported adjustment for seasonal variations [15, 30] and two studies [11, 14] did not report the season during which blood collection occurred.

Reported 25(OH)D concentrations in PAD cases and controls

Reported 25(OH)D concentrations from individual studies are presented in Table 1. Melamed *et al* [11] divided their participants by quartiles and reported that PAD prevalence was 8.1%, 5.4%, 4.9%, and 3.7%, for participants with 25(OH)D concentrations within the first, second, third and fourth quartiles, respectively ($P<0.001$). Zagura *et al* [30] reported lower circulating 25(OH)D concentration amongst PAD patients compared with non-PAD controls ($P<0.01$). Two studies [23, 24] reported that serum 25(OH)D concentrations were lower in PAD patients with CLI compared to PAD patients with IC and healthy controls ($P<0.001$). One study [24] also reported that PAD patients with IC had slightly lower 25(OH)D concentration compared with non-PAD participants (*P value* not reported). In contrast, McDermott *et al* [15] and Fahrleitner *et al* [23] found no significant difference in the concentrations of 25(OH)D between IC patients and non-PAD participants ($P=0.63$). Liew *et al* [14] reported a slightly higher concentration of 25(OH)D among PAD patients compared with non-PAD controls, although the difference was not statistically significant.

Quantitative data synthesis

A meta-analysis of all six case-control studies comprising 1217 cases and 5201 controls showed that PAD patients had lower circulating 25(OH)D concentration compared with non-PAD controls (SMD=-0.32; 95%CI:-058, -0.05; $P=0.02$; $I^2=88$) (Figure 2). A subgroup analysis of 532 patients with IC and 350 non-PAD participants showed no significant difference in the concentrations of circulating 25(OH)D (SMD=-0.01; 95% CI: -0.15, 0.13; $P=0.88$; $I^2=0$) (Figure 2, 1.1.1). In contrast, a subgroup analysis comprising 126 PAD patients with CLI and 44 non-PAD controls demonstrated that serum concentrations of 25(OH)D were markedly lower in CLI patients (SMD=-1.29; 95% CI: -1.66, -0.91; $P<0.001$; $I^2=0$) (Figure 2, 1.1.2). Sensitivity analysis by removal of either of the two studies [23, 24] that investigated

CLI patients rendered the overall effect non-significant (SMD=-0.19; 95% CI:-0.42, 0.04; *P*=0.11 and SMD=-0.22; 95% CI: -0.46, 0.03; *P*=0.09, respectively). Similarly when the studies by Melamed *et al* [11] or Zagura *et al* [30] were removed, the overall statistical significance was lost (SMD=-0.38; 95% CI: -0.77, 0.00; *P*=0.05 and SMD=-0.26; 95% CI: -0.53, 0.01; *P*=0.06, respectively) (Table 2).

Table 2. Leave-one-out sensitivity analysis for the association between 25(OH) D concentration and PAD.

Study Removed	SMD, 95% CI	*P*-value
Fahrleitner, 2002 [23]*		
IC patients	-0.39 (-0.67, -0.11)	0.01§
CLI patients	-0.19 (-0.42, 0.04)	0.11
Fahrleitner-Pammer, 2005 [24]*		
IC patients	-0.36 (-0.65, -0.07)	0.01§
CLI patients	-0.22 (-0.46, 0.03)	0.09
Melamed, 2008 [11]†	-0.38 (-0.77, 0.00)	0.05
Zagura, 2011 [30]†	-0.26 (-0.53, 0.01)	0.06
McDermott, 2012 [15]‡	-0.39 (-0.75, -0.03)	0.03§
Liew, 2015 [14]†	-0.40 [-0.70, -0.10]	0.01§
None	-0.32 (-0.59, -0.05)	0.02§

*These studies investigated groups of PAD patients with IC and CLI which were considered as separate groups in our analyses. Controls for these studies were divided into two and separately compared with patients that had IC or CLI, respectively. †These studies included PAD patients presenting with a mix of symptoms or symptoms that were not defined. ‡ McDermott *el al* [15] included patients presenting with no or atypical symptoms as well as those who had IC. §Differences are considered statistically significant ($P<0.05$). Abbreviation: PAD: peripheral artery disease; SMD: standardized mean difference; CI: confidence interval. Abbreviations: SMD: standardized mean difference; CI: confidence interval.

Assessment of publication bias

Visual examination of Begg's funnel plot suggested potential publication bias (Supplemental figure 1). However, accurate interpretation of the funnel plot was limited by the small number of included studies in the meta-analysis [27].

DISCUSSION

25(OH)D is believed to be the best circulating marker of vitamin D status [8, 9]. Observational studies comprising varying population sample sizes have previously published conflicting data in relation to the association of 25(OH)D and PAD presence. The current meta-analysis appraises data from previous case-control studies examining the association of circulating 25(OH)D concentration with PAD. Overall, the analysis suggests that PAD patients have significantly lower circulating 25(OH)D concentrations than non-PAD controls. However, sub-analyses suggested that this association is principally attributable to low 25(OH)D concentrations in patients with more severe PAD (i.e. CLI). Notably, 25(OH)D concentrations in patients with mild PAD (i.e. IC) were similar to non-PAD controls.

There are a number of possible explanations for the inverse association between 25(OH)D and severe PAD symptoms. Firstly, it is possible that vitamin D deficiency promotes the development of atherosclerosis within peripheral arteries and therefore at the time of presentation markers of vitamin D status are lower in PAD patients. This seems less likely from the data presented in this analysis since 25(OH)D concentrations were not significantly lower than controls in patients with an earlier stage of PAD, i.e. IC. Secondly, it is possible that vitamin D deficiency promotes complications in patients with established PAD; for example by promoting atherosclerosis plaque instability or thrombosis, leading to disease progression and ultimately CLI. This theory is supported by the finding that a number of included studies reported lower 25(OH)D concentrations in patients with more severe PAD symptoms, i.e. CLI, than both controls and patients with IC [23, 24]. Thirdly, it is also possible that low 25(OH)D concentrations are a consequence of PAD rather than a cause. It has for example been proposed that low 25(OH)D may be indicative of PAD patients' restricted mobility and resultant limitation in sun exposure [5, 23]. This theory is supported by a recent epidemiological study which assessed the development of PAD in a cohort of 1568 community-dwelling older adults

over a mean follow-up of 4.4 years [16]. In the latter study participants with 25(OH)D concentrations <25 nmol/L had no increased risk of developing PAD as defined by an ABPI of <0.9. Further larger epidemiology studies are however needed since the number of participants in each 25(OH)D concentration strata that developed PAD was small. The latter study was also not designed to assess whether vitamin D deficiency is important in predisposing to ischaemic complications in patients with established PAD.

Finally, the observed association could be attributable to confounding risk factors that are shared between PAD and low vitamin D levels. Observational association studies have previously linked both PAD and low 25(OH)D concentrations with smoking, hypertension, diabetes, obesity and renal disease [7, 8, 10]. An approach used to examine genetic variants, known as Mendelian randomisation, might be used to further examine the causal link between PAD and 25(OH)D [31]. A similar approach has recently suggested that vitamin D deficiency plays no causal role in type II diabetes [32]. Findings from one large genome-wide association study showed that genetic variants at three loci (4p12 in GC; 11q12 near DHCR7; and 11p15 near CYP2R1; 20q13 in CYP24A1) were significantly associated with low levels of circulating 25(OH)D (defined as <75 nmol/L) in 33996 individuals of European descent [33]. These genetic variants could be investigated in PAD patients with different presentations in order to further examine the link between vitamin D deficiency and PAD.

There are some plausible biological explanations for how vitamin D deficiency could promote PAD development and complications. Vitamin D receptors are expressed by cells present within arteries and also those implicated in the development and complications of atherosclerosis, including endothelial cells, vascular smooth muscle cells and immune cells [6, 34]. *In vitro* studies suggest that vitamin D deficiency alters a range of pathways that are implicated in PAD pathogenesis, including oxidative stress, inflammation, endothelial dysfunction and vascular smooth muscle cells proliferation, migration and apoptosis [6, 34].

Vitamin D has been shown to mediate vascular protective effects, including upregulation of nitric oxide and inhibition of platelet aggregation [6, 34]. In addition, vitamin D may protect against vascular remodelling and athero-thrombosis by upregulating thrombomodulin expression [35], and by downregulating expression of plasminogen activator inhibitor-1 [36] and matrix metalloproteinases-2 and 9 [34, 37]. Moreover, vitamin D deficiency has been shown to upregulate the renin-angiotensin-aldosterone-system and PTH levels, leading to arterial hypertension and adverse vascular remodelling [34, 38, 39]. As suggested by Brostow and colleagues [5], it is possible that deficiency of vitamin D leads to physiological elevation of PTH and hyperparathyroidism, resulting in osteomalacia and bone weakness. This may then result in vascular wall calcification, leading to PAD development and progression as reported in some of included studies [23, 24, 30].

The findings of this meta-analysis should be interpreted in the context of several limitations. Firstly, there was high qualitative heterogeneity across studies as well as differences owing to 25(OH)D assay methodology, seasonality of blood collection, geographical location and other demographic factors within included studies. Moreover, potential differences in vitamin D status owing to racial and gender factors could not be assessed here as insufficient data were available to permit sub-analyses [13, 40]. Among the six included studies, one study used UPLC-MS/MS [14] while other studies used radioimmunoassay. Although all these assays are accepted by the Vitamin D External Quality Assessment Scheme, analysis by UPLC-MS/MS is generally favoured [41]. Secondly, the number of participants included in this meta-analysis is just over 6000 which is considered relatively small. The number of participants included in the sub-analyses is much smaller. Therefore, larger studies are needed to provide more confidence in the findings presented. Finally, we were unable to adjust for many potential confounding factors such as lifestyle factors and socioeconomic status which are relevant to vitamin D status [7].

In conclusion, this meta-analysis suggests that circulating 25(OH)D concentrations are lower in PAD patients with CLI than controls. Whether this finding is because vitamin D is important in limiting complications of PAD or a consequence of PAD is not currently clear. Based on the currently available evidence, no firm recommendation can be made regarding the therapeutic benefits or harm of vitamin D supplementation, or optimal circulating vitamin D concentrations in patients that have PAD. Well-designed trials to directly examine this are needed.

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Figure 1. PRISMA flow chart illustrating study screening

PRISMA flow chart illustrating identification, screening, inclusion and exclusion of the studies for the meta-analysis. Database searches identified 939 entries, after removal of duplicates 498 studies were left. Titles and abstracts of these were screened leading to exclusion of 481 studies that did not satisfy the inclusion criteria. Full texts of the remaining 17 articles were carefully inspected by two authors, resulting in further removal of 11 articles. The remaining 6 studies were included in the meta-analysis. Abbreviations: PAD: peripheral arterial disease; 25(OH)D: 25-hydroxyvitamin D; n: number.

Figure 2. Forest plot showing the overall and subgroup concentrations of 25(OH)D (nmol/L) among PAD and non-PAD participants.

Forest plot of weighted mean difference (SMD) and 95% confidence interval of 25(OH)D concentrations in patients with peripheral arterial disease (PAD) compared with non-PAD participants. 1.1.1 Mean (SD) 25(OH)D were compared between PAD patients with intermittent claudication and non-PAD controls. 1.1.2 Mean (SD) 25(OH)D were compared between PAD patients with critical limb ischaemia and non-PAD controls. 1.1.3 Mean (SD) 25(OH)D were compared between PAD patients with mixed presentation and non-PAD controls. Standard mean difference (SMDs) and 95% confidence interval were computed in a random effects model using Review Manager (Version 5.3).

ELECTRONIC SUPPLEMENTARY MATERIAL (e-components)

Supplemental Figure 1: Begg's funnel plot assessing publication bias

Begg's funnel plot assessing publication bias. Meta-analysis of observational cross-sectional studies reporting 25-hydroxyvitamin D concentrations in patients with peripheral arterial disease (PAD) and non-PAD participants (controls). Abbreviations: PAD: peripheral artery disease; SMD: standardized mean difference.

Figure 1

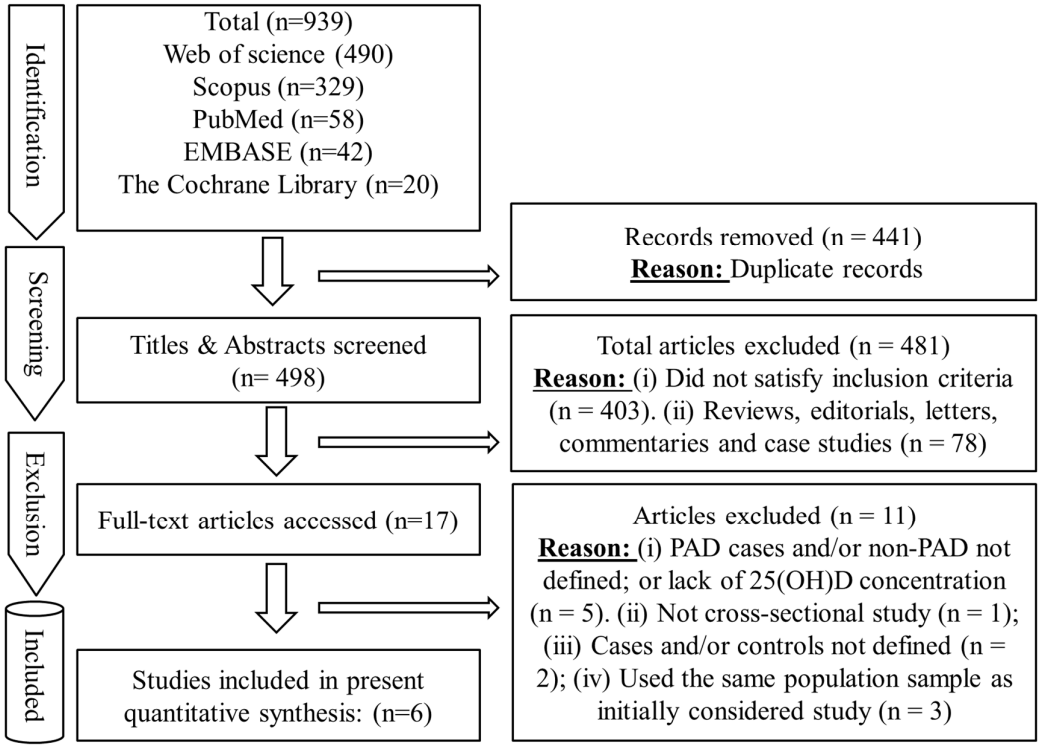
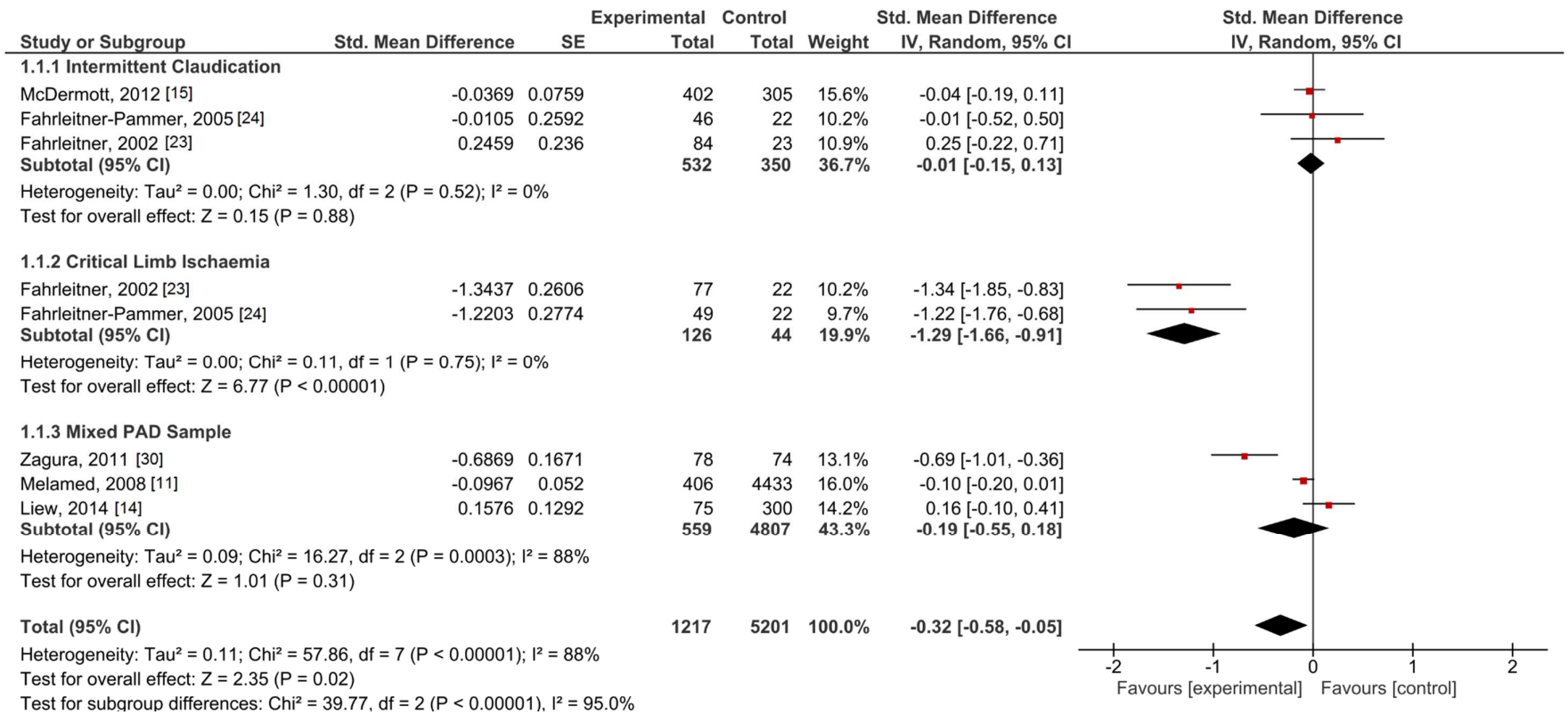

Identification
Total (n=939)
Web of science (490)
Scopus (n=329)
PubMed (n=58)
EMBASE (n=42)
The Cochrane Library (n=20)
Records removed (n = 441)
Reason: Duplicate records
Screening
Titles & Abstracts screened (n= 498)
Total articles excluded (n = 481)
Reason: (i) Did not satisfy inclusion criteria (n = 403). (ii) Reviews, editorials, letters, commentaries and case studies (n = 78)
Exclusion
Full-text articles accessed (n=17)
Articles excluded (n = 11)
Reason: (i) PAD cases and/or non-PAD not defined; or lack of 25(OH)D concentration (n = 5). (ii) Not cross-sectional study (n = 1); (iii) Cases and/or controls not defined (n = 2); (iv) Used the same population sample as initially considered study (n = 3)
Included
Studies included in present quantitative synthesis: (n=6)

Figure 2


Experimental
Control
Std. Mean Difference
Std. Mean Difference
Study or Subgroup
Std. Mean Difference
SE
Total
Total
Weight
IV, Random, 95% CI
IV, Random, 95% CI
1.1.1 Intermittent Claudication
McDermott, 2012 [15] -0.0369 0.0759 402 305 15.6% -0.04 [-0.19, 0.11]
Fahrleitner-Pammer, 2005 [24] -0.0105 0.2592 46 22 10.2% -0.01 [-0.52, 0.50]
Fahrleitner, 2002 [23] 0.2459 0.236 84 23 10.9% 0.25 [-0.22, 0.71]
Subtotal (95% CI) 532 350 36.7% -0.01 [-0.15, 0.13]
Heterogeneity: Tau² = 0.00; Chi² = 1.30, df = 2 (P = 0.52); I² = 0%
Test for overall effect: Z = 0.15 (P = 0.88)
1.1.2 Critical Limb Ischaemia
Fahrleitner, 2002 [23] -1.3437 0.2606 77 22 10.2% -1.34 [-1.85, -0.83]
Fahrleitner-Pammer, 2005 [24] -1.2203 0.2774 49 22 9.7% -1.22 [-1.76, -0.68]
Subtotal (95% CI) 126 44 19.9% -1.29 [-1.66, -0.91]
Heterogeneity: Tau² = 0.00; Chi² = 0.11, df = 1 (P = 0.75); I² = 0%
Test for overall effect: Z = 6.77 (P < 0.00001)
1.1.3 Mixed PAD Sample
Zagura, 2011 [30] -0.6869 0.1671 78 74 13.1% -0.69 [-1.01, -0.36]
Melamed, 2008 [11] -0.0967 0.052 406 4433 16.0% -0.10 [-0.20, 0.01]
Liew, 2014 [14] 0.1576 0.1292 75 300 14.2% 0.16 [-0.10, 0.41]
Subtotal (95% CI) 559 4807 43.3% -0.19 [-0.55, 0.18]
Heterogeneity: Tau² = 0.09; Chi² = 16.27, df = 2 (P = 0.0003); I² = 88%
Test for overall effect: Z = 1.01 (P = 0.31)
Total (95% CI) 1217 5201 100.0% -0.32 [-0.58, -0.05]
Heterogeneity: Tau² = 0.11; Chi² = 57.86, df = 7 (P < 0.00001); I² = 88%
Test for overall effect: Z = 2.35 (P = 0.02)
Test for subgroup differences: Chi² = 39.77, df = 2 (P < 0.00001), I² = 95.0%
-2
-1
0
1
2
Favours [experimental]
Favours [control]

Supplementary Figure 1