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Original Article

# Effects of Vitamin D3 on asymmetric- and symmetric dimethylarginine in arterial hypertension

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ABSTRACT

*Background and aims:* Accumulating evidence has proposed a correlation between vitamin D (25(OH)D) insufficiency and cardiovascular (CV) disease. Vitamin D associated effects on endothelial function have been suggested to be a possible culprit. The present study investigated the association of vitamin D3 treatment on markers of endothelial dysfunction in patients with arterial hypertension.
*Methods and results:* The Styrian Vitamin D Hypertension Trial is a double-blind, placebo-controlled, single-centre study conducted at the Medical University of Graz, Austria. A total of 200 study participants with arterial hypertension and 25(OH)D levels below 30 ng/mL were enrolled. The study participants were randomized to receive 2800 IU of vitamin D3 per day as oily drops (n = 100) or placebo (n = 100) for a duration of eight weeks. The present study uses an analysis of covariance (ANCOVA) to investigate the effect of vitamin D3 treatment on symmetric (SDMA) and asymmetric dimethylarginine (ADMA). A total of 187 participants (mean [SD] age 60.0 [11.3] years; 47% women; 25(OH)D 21.2 [5.6] ng/mL; mean systolic blood pressure of 131.4 [8.9] mmHg on a median of 2 antihypertensive drugs) completed the trial. Mean treatment effect was −0.004 (95%CI [−0.03 to 0.04]; P = 0.819) on ADMA and 0.001 (95%CI [−0.05 to 0.05]; P = 0.850) on SDMA. In the subgroup analysis patients with a 25(OH)D concentration <20 ng/mL had a significant increase in their log L-arginine/ADMA ratio (mean treatment effect 18.4 95%CI [1.84–34.9]μmol/L/μmol/L; P = 0.030). ClinicalTrials.gov Identifier: NCT02136771 EudraCT number: 2009-018125-70
*Conclusions:* Vitamin D3 supplementation in hypertensive patients with low 25-hydroxyvitamin D has no significant effect on ADMA and SDMA.



**Contents**



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## 1. Introduction

Vitamin D is a steroid hormone classically known to be responsible for bone mineralization [1,2]. It's deficiency is known to cause a childhood diseases called rickets, which is characterized by skeletal deformities [2]. Definition of normal vitamin D levels is based on the concentration of 25-hydroxyvitamin D (25[OH]D) – in this article referred to as vitamin D – which is the pro-hormone of the active metabolite, 1,25-dihydroxyvitamin D that subsequently activates the vitamin D receptor (VDR) [1,3]. Intriguingly, the VDR has also been found in extra-skeletal tissues, like myocardium and vasculature [4,5]. So the question raised whether there are non-bone related health effects of vitamin D insufficiency. Beside reports of children with heart failure and rickets which both improved with vitamin D supplementation [6], vitamin D insufficiency was broadly described to be a risk factor for cardiovascular (CV) diseases [2,4,7–10]. Low 25(OH)D concentrations have been even proposed to actually cause or mediate CV disease, but might also be only an epiphenomenon of poor health and low physical activity [2,9–12]. Nevertheless, in animal models the vitamin D receptor (VDR) activation led to improved endothelial function [2,7–10]. As endothelial dysfunction is a major component of CV disease[13] an interaction with vitamin D could explain – at least partially – the increased CV mortality seen in 25(OH)D insufficient patients [7,8,13–17]. Asymmetrical dimethylarginine (ADMA) is a marker of endothelial derangement and has been validated previously in cell based and clinical models. ADMA is a competitive inhibitor of NO-synthase which catalyses the production of nitric oxide, one of the most potent endogenous vasodilators. [18–22] Previous studies on vitamin D and ADMA reported cross sectional associations between them [23–25]. *Ngo* et al. observed an inverse association between 25(OH)D concentration and ADMA [23]. This was further supported by similar findings in patients with hypogonadism [24], phenylketonuria [25], Polycystic ovary syndrome (PCOS) [26], and in individuals on long-term haemodialysis (HD)[27]. The effect also seems to be associated with aging [28]. In line with this, *Syal* and colleagues observed that patients with lower 25(OH)D levels had significantly reduced flow-mediated brachial artery dilation, what strengthens hypothesis of a vitamin D associated effect on endothelial function [29]. Some authors further proposed the L-arginine to ADMA ratio as a more sensitive marker for endothelial function [18–20,30–32]. Similar results are reported in regard to symmetrical dimethylarginine (SDMA), a sensitive marker for renal function [33], which may also have indirect effects on NO synthesis [34]. Interventional data in humans on the effects of vitamin D supplementation on ADMA and SDMA are however, missing. We report results from our randomized double blind clinical trial supplementing vitamin D or

**Table 1**
Baseline Characteristics of the Placebo and the Vitamin D group before randomization.

| | Placebo<br>n = 99<br>Mean ± SD<br>Median (IQR) | Vitamin D<br>n = 99<br>Mean ± SD<br>Median (IQR) |
|---|---|---|
| Age (years) | 59.5 ± 11.4 | 60.7 ± 10.8 |
| Females (yes) | 48% | 46% |
| Mean 24 h systolic blood pressure | 131.8 ± 9.7 | 132.0 ± 8.4 |
| Asymmetric dimethylaginine (μmol/L) | 0.73 ± 0.09 | 0.70 ± 0.15 |
| Symmetric dimethylarginine (μmol/L) | 0.71 ± 0.10 | 0.69 ± 0.16 |
| L-arginine/ADMA ratio μmol/L/μmol/L | 183.3 ± 58.7 | 183.1 ± 49.7 |
| L-arginine (μmol/L) | 131.0 ± 35.5 | 128.6 ± 31.6 |
| Parathyroid hormone (pg/mL) | 51.5 (39.5–65.8) | 48.9 (40.0–61.7) |
| 25-hydroxyvitamin D (ng/mL) | 20.4 ± 5.7 | 21.8 ± 5.5 |
| 25-hydroxyvitamin D (nmol/L) | 50.9 ± 14.2 | 54.4 ± 13.7 |
| Serum total calcium (mmol/L) | 2.37 ± 0.11 | 2.37 ± 0.10 |
| Estimated glomerular filtration rate MDRD6 (mL/min/1.73m$^2$) | 77.0 ± 17.9 | 79.9 ± 17.9 |
| Number of different blood pressure lowering drugs | 2 (1–3) | 2 (1–3) |
| ACE inhibitor (yes) | 38% | 25% |
| AT1 receptor blocker (yes) | 31% | 33% |
| Calcium channel blocker (yes) | 25% | 27% |
| Thiazide diuretics (yes) | 45% | 39% |
| Loop diuretics (yes) | 5% | 5% |
| Beta blockers (yes) | 49% | 44% |
| Minteralocorticoid receptor blockers (yes) | 4% | 2% |
| Smoking (yes) | 16.1% | 7.4% |

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placebo in hypertensive patients with 25(OH)D insufficiency to address the question whether oral vitamin D treatment for 8 weeks has an effect on ADMA, L-arginine to ADMA ratio and SDMA serum concentrations.

## 2. Methods

### 2.1. Study design

The present study is a post-hoc analysis adhering to a stringent protocol and investigates the treatment effect of oral vitamin D3 on ADMA and SDMA in patients with arterial hypertension and vitamin D insufficiency. The methods of the Styrian Vitamin D Hypertension Trial have been already reported [35–38]. Briefly: It is a double blind, placebo-controlled study comparing the effect of 2800 IU vitamin D3 versus placebo on clinical and laboratory biomarkers in patients with arterial hypertension. The Medical University of Graz, Austria funded the study. The publication of this trial complies to the Consolidated Standards of Reporting Trials (CONSORT) 2010 statement [39]. The RCT was registered at the www.clinicaltrialsregister.eu (EudraCT Number 2009-018125-70) as well as at clinicaltrials.gov (ClinicalTrials.gov Identifier NCT02136771). The difference between previous reports and this study is a) the outcomes, i.e. ADMA and SDMA, have not been reported previously b) baseline characteristics (Table 1) restricted to those participants who had measurements of ADMA and SDMA available and are thus unique c) a discussion of vitamin D effects on endothelial function were not part of previous publications.

### 2.2. Participants

All participants included in the present study were over 18 years old. Main inclusion criteria was a diagnosis of arterial hypertension and a 25(OH)D level ≤30 ng/mL (74.9 nmol/L multiply by 2.496 to convert ng/mL into nmol/L). Arterial hypertension was defined as an office BP of systolic ≥140 mmHg or diastolic ≥90 mmHg, a mean 24-h ABPM of systolic ≥125 mm Hg or diastolic ≥80 mmHg, a home BP of systolic ≥130 mm Hg or diastolic ≥85 mmHg, or ongoing antihypertensive treatment [40]. The exclusion criteria were published previously [35]. The study was approved by the ethics committee at the Medical University of Graz, Graz, Austria. All study participants provided written

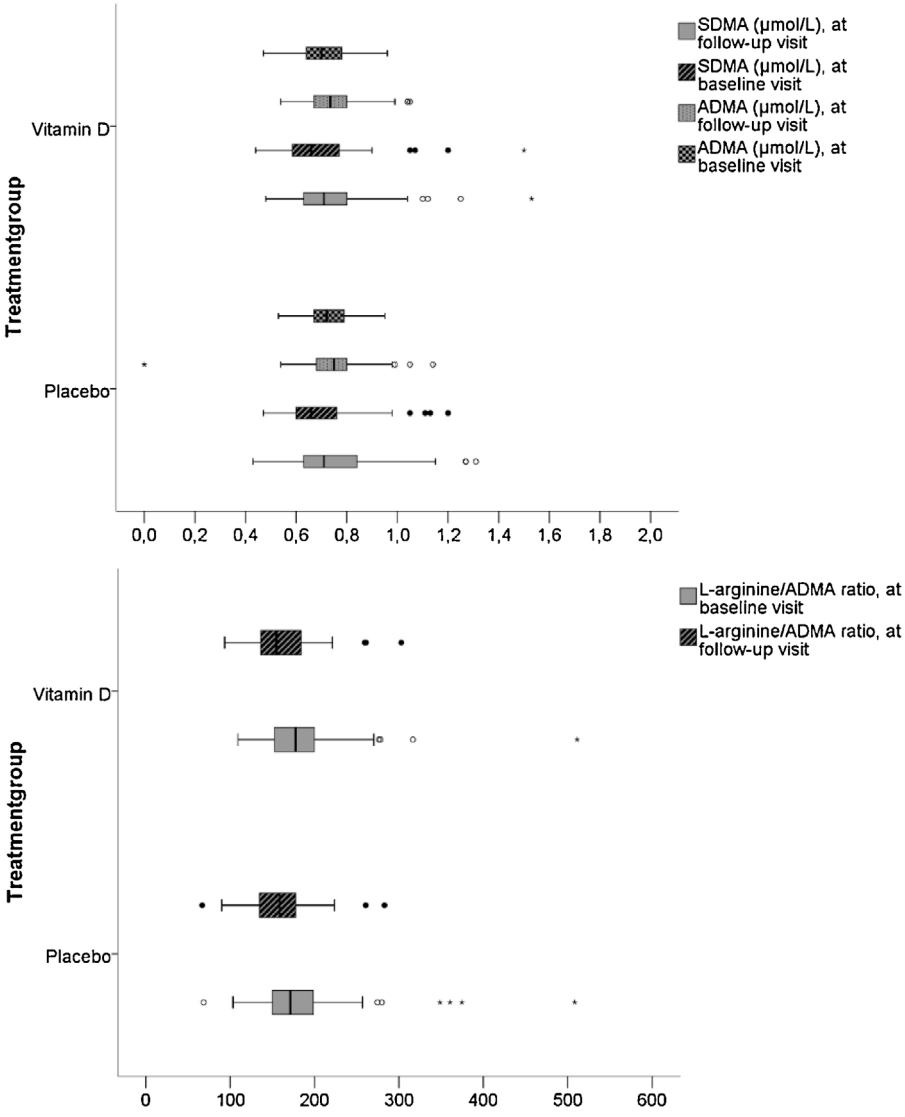


**Fig 1.** ANCOVA revealed no significant mean difference between placebo and control arm in log SDMA, ADMA or log L-arginine/ADMA ratio at the final visit (−0.004 95%CI [−0.03 to 0.04]ng/dL; P = 0.819 and 0.001 95%CI [−0.05 to 0.05] ng/dL; P = 0.850, respectively)..

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**Table 2**
Baseline, Follow-Up and Changes From Baseline of ADMA, SDMA and L-arginine/ADMA ratio.

| Characterisitics | Placebo | | Vitamin D | | mean change from baseline | | Treatment Effect (95% CI) | P-Value |
|---|---|---|---|---|---|---|---|---|
| | Baseline | Follow-up | Baseline | Follow-up | Placebo | Vitamin D | | |
| All patients randomized (Intention to treat) with follow-up n = 187 | | | | | | | | |
| ADMA (μmol/L) | 0.73 ± 0.09 | 0.75 ± 0.13 | 0.71 ± 0.10 | 0.74 ± 0.11 | 0.02 | 0.03 | −0.004 (−0.03 − 0.04) | 0.819 |
| L-arginine/ADMA ratio (μmol/L/μmol/L) | 183.9 ± 58.8 | 157.3 ± 35.1 | 183.3 ± 49.9 | 161.1 ± 39.5 | −26.63 | −22.26 | 2.2 (−7.9 − 12.3) | 0.668 |
| SDMA (μmol/L) | 0.70 ± 0.15 | 0.74 ± 0.18 | 0.69 ± 0.16 | 0.74 ± 0.17 | 0.04 | 0.06 | 0.001 (−0.05 − 0.05) | 0.850 |
| 25(OH)D <20 ng/mL/ <49.9nmol/L n = 73 | | | | | | | | |
| ADMA (μmol/L) | 0.72 ± 0.09 | 0.76 ± 0.17 | 0.69 ± 0.08 | 0.72 ± 0.09 | 0.04 | 0.02 | −0.04 (−0.11 − 0.03) | 0.256 |
| L-arginine/ADMA ratio (μmol/L/μmol/L) | 184.9 ± 57.7 | 146.6 ± 31.8 | 188.2 ± 68.6 | 165.2 ± 38.5 | −38.32 | −22.96 | 18.4 (1.3 − 35.5) | **.030** |
| SDMA (μmol/L) | 0.66 ± 0.14 | 0.75 ± 0.20 | 0.66 ± 0.16 | 0.70 ± 0.14 | 0.09 | 0.05 | −0.05 (−0.13 − 0.04) | 0.347 |
| 25(OH)D <12 ng/mL/ < 30.0 nmol/L n = 15 | | | | | | | | |
| ADMA (μmol/L) | 0.76 ± 0.10 | 0.76 ± 0.15 | 0.69 ± 0.04 | 0.68 ± 0.07 | 0.00 | −0.01 | −0.09 (−0.31 − 0.26) | 0.210 |
| L-arginine/ADMA ratio (μmol/L/μmol/L) | 156.0 ± 28.8 | 153.5 ± 30.6 | 181.0 ± 70.5 | 179.6 ± 52.8 | −2.48 | −1.40 | 23.1 (−29.4 − 75.6) | 0.353 |
| SDMA (μmol/L) | 0.70 ± 0.18 | 0.74 ± 0.28 | 0.65 ± 0.11 | 0.71 ± 0.19 | 0.04 | 0.07 | −0.02 (−0.23 − 0.06) | 0.997 |
| Treatment naive patients* n = 38 | | | | | | | | |
| ADMA (μmol/L) | 0.72 ± 0.07 | 0.72 ± 0.10 | 0.71 ± 0.10 | 0.71 ± 0.08 | −0.02 | −0.01 | 0.01 (−0.04 − 0.06) | 0.797 |
| L-arginine/ADMA ratio (μmol/L/μmol/L) | 200.8 ± 67.8 | 160.9 ± 24.4 | 169.3 ± 20.8 | 160.8 ± 36.9 | −39.90 | −8.50 | −3.9 (−20.8 − 13.0) | 0.643 |
| SDMA (μmol/L) | 0.64 ± 0.11 | 0.72 ± 0.15 | 0.72 ± 0.15 | 0.74 ± 0.17 | 0.06 | 0.03 | −0.02 (−0.08 − 0.15) | 0.516 |

ADMA (μmol/L), asymetric dimethylarginine; SDMA (μmol/L), symetric dimethylarginin; 25(OH)D, 25-hydroxyvitamin D; 95% CI, 95% confidence intervall. A P-value ≤0.050 is considered stastically significant. Values represent participants with baseline and follow-up visit. Multiple data imputation for missing values is not included in this table.

informed consent. The study complies with the Declaration of Helsinki. The study took place at the outpatient clinic at the Division of Endocrinology and Diabetology from 2011 to 2014 [35].

### 2.3. Intervention

Study medication was randomly filled into numbered bottles according to a computer generated randomization list. The randomization was conducted using web-based tool (http://www.randomizer.at/), with good clinical practice compliance. All eligible study patients were randomly assigned in a 1:1 ratio to receive 2800 IU vitamin D3 as oily drops per day (Oleovit D3, producer: Fresenius Kabi Austria, Austria) or a matching placebo. We performed a permuted block randomization (size of ten) and stratification according to gender. All investigators who enrolled patients were blinded during enrolment [35].

### 2.4. Outcome measure

The primary outcome (main endpoint) was 24-h systolic blood pressure as already published [35]. Sample size calculations were based assuming an effect size of −6 mmHg (E) systolic blood pressure with a SD of 12 mmHg (S). Based on that we calculated a standardized effect size (E/S) of 0.5. For a 2-sided α of 0.05 and a power (1−β) of 90%, we would have need at least a group size of 86 study participants [35]. The rationale for the present investigation was based on previous studies on vitamin D and ADMA reporting on inverse cross sectional associations for a wide range of 25(OH)D concentrations [23–27].

### 2.5. Measurements

Physical examinations, patient interviews and blood samplings were performed at study visits during workdays between 7 and 11 AM. After that, the patients left the hospital for 24-h BP measurements and 24-h urine collections. They were scheduled to return on the next (second) day, when the eligible study participants were randomized and started intake of the study medication. We measured serum symmetrical dimethylarginine (SDMA) in frozen serum (−80 °C) with a modified reversed-phase HPLC method. Within-day coefficients of variation (CVs) for SDMA were 4.6% (0.60 μmol/L) and 1.9% (1.0 μmol/L), and between-day CVs were 9.8% (0.60 μmol/L) and 6.1% (1.0 μmol/L). Limit of detection was 0.05 μmol/L. Serum asymmetric dimethylarginine (ADMA) was as well measured in frozen serum (−80 °C) with the reversed-phase HPLC method with slight modifications [41]. Within-day CVs for ADMA were 3.1% (0.62 μmol/L) and 1.0% (2.00 μmol/L), and between-day CVs were 9% (0.62 μmol/L) and 1.5% (2.00 μmol/L). Limit of detection was 0.05 μmol/L. Measurement of 25-hydroxyvitamin D [25(OH)D] was performed by means of a chemiluminescence assay (IDS-iSYS 25-hydroxyvitamin D S assay; Immunodiagnostic Systems Ltd., Boldon, UK) on an IDS-iSYS multidiscipline automated analyser with intra- and inter-assay CV of 6.2% and 11.6%, respectively [42]. Measurements of L-arginine have been conducted according to previously published methods [43,44]. Briefly, after precipitation of serum with perchloric acid following neutralization of the supernatant with sodium carbonate, the extracted amino acids were derivatized with o-phtalaldehyde and separated on a reversed phase column with gradient elution. Quantification were performed with ratios of fluorescence signals of the interesting amino acids to the internal standard norvaline in comparison to the appropriated calibration curves. Intra-assay and interassay CVs were all below 10%. More details on the laboratory methods used have been published previously [36,45–47].

### 2.6. Analysis

Continuous data following a normal distribution are reported as means with standard deviation and variables with a skewed distribution are shown as medians with interquartile range. Categorical data are presented as percentages. Where appropriate, skewed variables were log(e) transformed before use in parametric statistical analyses. Group comparisons at baseline were done by unpaired student's *t*-, Chi Square- or Man-Whitney *U* test. Analyses of outcome variables (ADMA, L-arginine to ADMA ratio, SDMA) were performed according to the intention-to-treat concept without data imputation. Analyses of Covariance (ANCOVA) with adjustments for baseline values were used to test for differences in the outcome parameters between the placebo and the treatment

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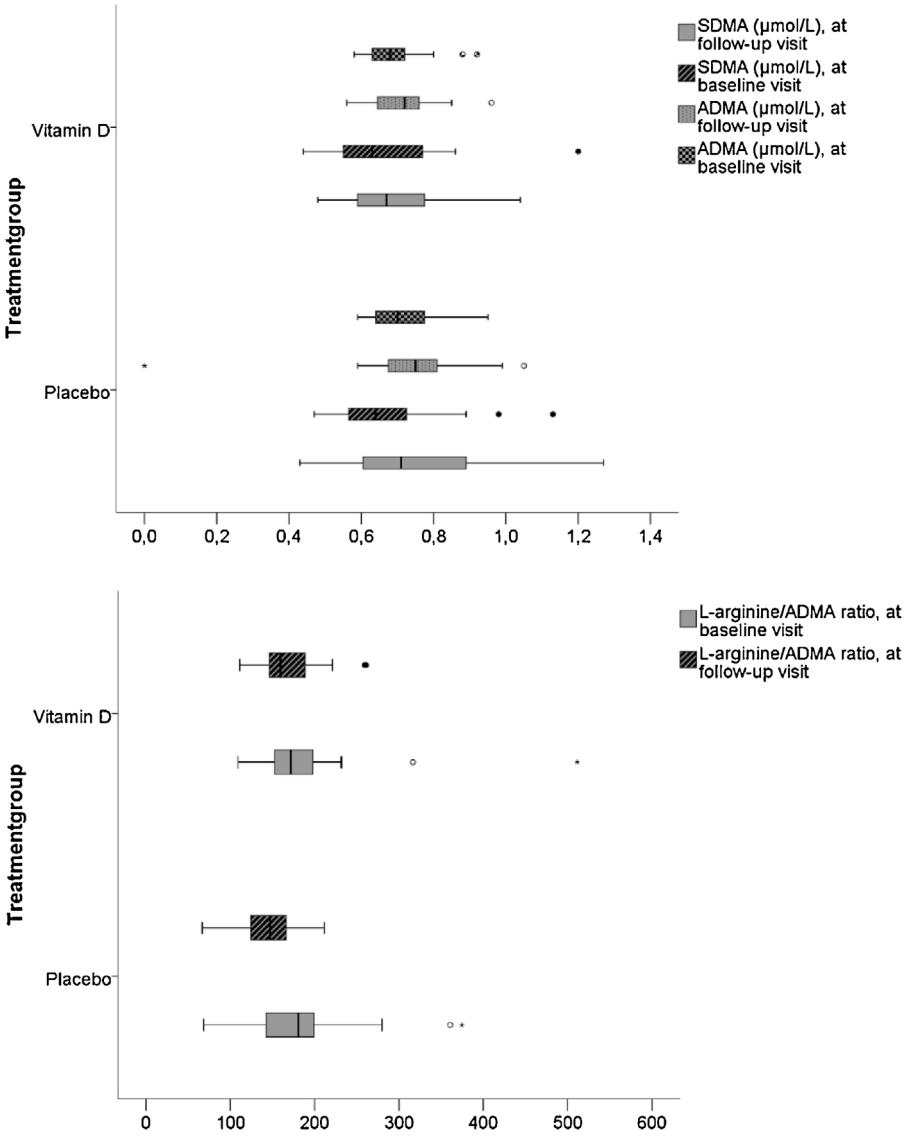


**Fig. 2.** In the subgroup of patients with a baseline 25(OH)D level below ≤20.0 ng/mL (n = 73) ANCOVA revealed no significant mean difference between placebo and control arm in log SDMA or ADMA at the final visit (−0.04 95%CI [−0.11 to 0.03]ng/dL; P = 0.256 and −0.05 95%CI [−0.13 to 0.04]ng/dL; P = 0.347, respectively). There was a statistically significant increase in log L-arginine/ADMA ratio (mean treatment effect 18.4 95%CI[1.84–34.9] µmol/L/µmol/L; P = 0.030).

arm at the second visit. In sensitivity analysis we used multiple (data) imputation for missing values [48]. A P-value below 0.05 was considered statistically significant. All statistical analyses were performed using SPSS version 21.0 software (SPSS, Chicago, IL, USA) and Stata version 13 (StataCorp LP, College Station, Texas, USA).

## 3. Results

A total of 518 invited study participants gave written informed consent and were assessed for eligibility. The entire trial was performed between 2011 and August 2014. Baseline characteristics of all randomized study participants are shown in Table 1 (restricted to participants with baseline and follow-up values of ADMA, Arginin and SDMA). All parameters were available in at least 94% of the study participants. A total of 187 study participants (mean ± SD age: 60.0 ± 11.3 years; 47% females; baseline 25(OH)D: 21.2 ± 5.6 ng/mL; 52.9 ± 14.0nmol/L) completed the trial. There was a significant increase in 25(OH)D and a significant decrease in parathyroid hormone (PTH) levels with no effect on total calcium, as already reported [35]. ANCOVA revealed no significant treatment effect on ADMA −0.004 (95%CI [−0.03 to 0.04]; P = 0.819), log L-arginine/ADMA ratio 2.201 (95%CI [−7.89 to 12.30]; P = 0.668) or SDMA 0.001 (95%CI [−0.05 to 0.05]; P = 0.850). (Fig. 1 and Table 2) Including adjustments for active smoking and ACE inhibitor intake left the results materially unchanged. There was neither an effect seen on ADMA nor SDMA when including only participants with 25(OH)D levels ≤20.0 ng/mL/49.9 nmol/L (Fig. 2), ≤12.0 ng/mL/30.0 nmol/L and in treatment naïve patients (Table 2). In the subgroup analysis restricted to patients with 25(OH)D below 20 ng/mL, vitamin D 3 supplementation as compared to placebo significantly increased the L-arginine/ADMA ratio with a mean treatment effect of 18.4 (95%CI [1.3–35.5]; P = 0.030). No patient deceased during the study period and to the best of our knowledge there was no increased rate of adverse events (i.e. hypercalcemia or hospitalisations) in the active treatment arm of the trial.

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## 4. Discussion

The present randomized controlled trial is the first to describe an effect of vitamin D3 on endothelial function in humans. In the subgroup analysis with patients below 20 ng/mL 25(OH)D there was a statistically significant increase in the L-arginine/ADMA ratio under vitamin D3 supplementation. As the effect is seen in a randomized controlled trial, the present study provides high quality clinical evidence for a possible link between vitamin D supplementation and CV events. Though we were unable to observe a statistically significant reduction of ADMA or SDMA by oral vitamin D supplementation in patients with arterial hypertension it raises the question of appropriate cut-offs for the definition of vitamin D insufficiency. Though subgroup analysis can – in general – be only hypothesis generating[49], the present findings strengthen the assumption of a possible causal link between 25(OH)D and endothelial function. Therefore, this finding should be confirmed in future trials focussing on patients with vitamin D deficiency. The answer to this question is important because it would help us understand the (potential) mechanism mediating vitamin D deficiency and CV health. More so, from a clinical point of view trials are needed to define the role of treatment with vitamin D in cardiology and vascular medicine [50–52]. Nevertheless, the main findings of the present study are "negative", as we were unable to show an effect of vitamin D on ADMA, L-arginine/ADMA ratio or SDMA. Previous studies who described an association of vitamin D on ADMA and SDMA were cross sectional investigations and were therefore limited by possible unknown confounding or they may have been subject to reverse causality [23–25]. As the present investigation was randomized and both physicians and patients were blinded to the study medication unknown confounding is less likely [53]. Furthermore, the significant reduction of PTH indicates a good therapeutic effect of vitamin D3. Nevertheless, as this study is a post-hoc analysis someone has to be cautious interpreting its results [39]. Furthermore, the cross sectional evidence so far was in quite specific patient cohorts, amongst others hypogonadism[24], phenylketonuria[25], PCOS[26] and on long-term haemodialysis (HD)[27]. In line with our results, *Abu el Maaty* and colleagues described in 69 patients suffering from coronary artery disease no difference in ADMA or SDMA levels when comparing patients above vs. below 30 ng/mL of 25(OH)D [40].

### 4.1. Strengths and limitations

As the primary outcome of our RCT, namely the reduction in systolic blood pressure, could not be achieved, the question arises whether there was any vitamin D effect on the vasculature in the present trial. Still it should be noted that there was a statistical and clinical significant reduction in PTH, which by itself has been linked to lower ADMA levels [54]. Furthermore, it has been already pointed out that – most likely – only patients who are truly deficient would benefit from vitamin D treatment warranting further trials on this topic such as the D-Cor study, an RCT restricted to individuals with 25(OH)D below 12 ng/mL (ClinicalTrials.gov Identifier: NCT02750293) [4,52]. Another potential limitation of our study may be the concurrent treatment with anti-hypertensive medication with potential impact on our outcome measures. Nevertheless we did see a significant increase in the L-arginine to ADMA ratio in patient who were vitamin D deficient (25(OH)D $\leq$20 ng/mL). Future trials should therefore focus on patients with vitamin D deficient patients, ideally also treatment naive. More so, future trials should aim to recruit larger samples as especially our subgroup analysis in participants with 25(OH)D $\leq$20.0 ng/mL is too small to draw final conclusions. The strengths of the present investigation clearly are the randomization and the blinding of both patients and treating physicians to the placebo-controlled intervention.

## 5. Conclusion

In summary, we did not observe an effect of vitamin D supplementation on ADMA or SDMA in patients with arterial hypertension and vitamin D insufficiency. In patients with a baseline 25(OH)D concentration below 20 ng/mL, we observed a statistically significant increase in the L-arginine to ADMA ratio under vitamin D supplementation. This finding warrants additional investigations and should be addressed in future trials focussing on patients with 25(OH) concentrations below 20 ng/mL.

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## Disclosures

None.

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