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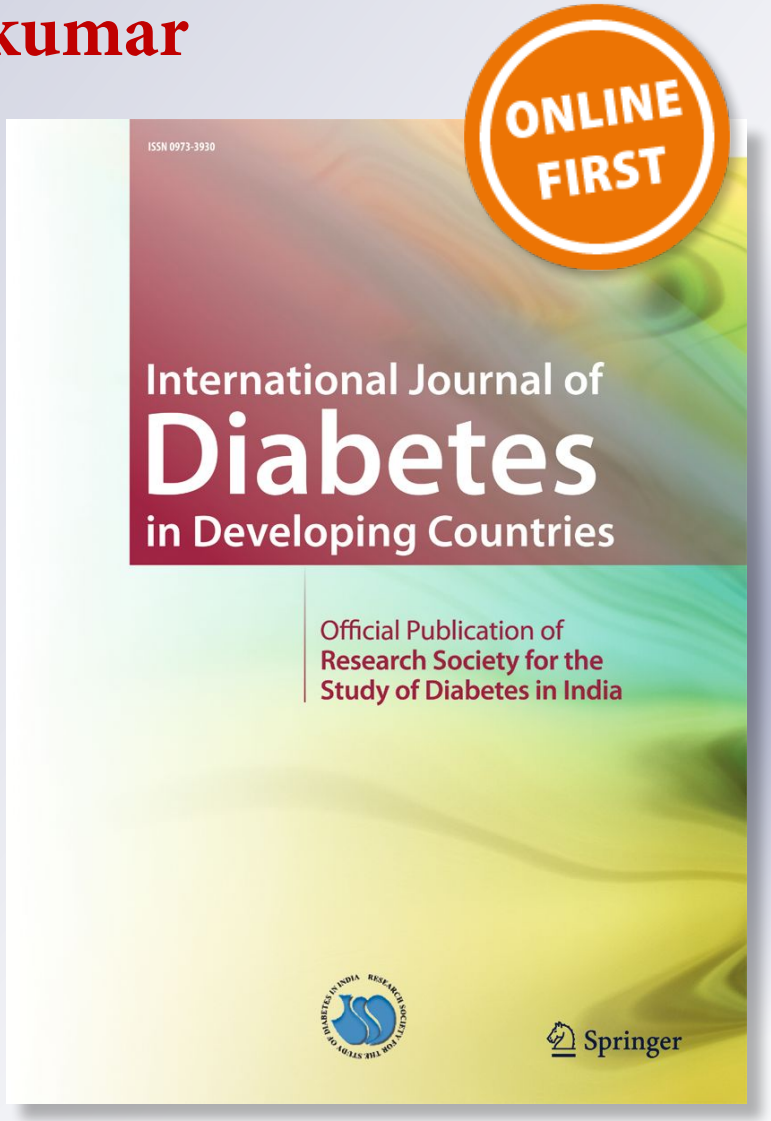


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ORIGINAL ARTICLE

# Effect of vitamin D supplementation on reduction of cardiometabolic risk in patients with type 2 diabetes mellitus and dyslipidemia

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**Abstract** Endothelial progenitor cells (EPCs) participate in endothelial regeneration. Previous studies link vitamin D deficiency, inflammatory cytokines, and cardiovascular disease (CVD) risk. This study evaluates the impact of vitamin D supplementation on EPCs, inflammatory markers, and glycemia in type 2 diabetes. This is prospective open-label randomized controlled study. Sixty-five patients with type 2 diabetes, dyslipidemia, $HbA_{1c}$ below 9%, and vitamin D deficiency (below 30 ng/ml) attending the outpatient clinic between April and December 2015 were randomized to active vitamin D (60,000 IU of vitamin D orally once a week for 8 weeks, followed by once a month for 4 months) or control for 6 months. Data was analyzed with STATA 14. Demographics include median age 54 (range 48.5–60) years, median duration of diabetes 7 (4–12.5) years, mean BMI 26.86 ± 3.8 kg/m$^2$, mean $HbA_{1c}$ 7.22±0.8%, and median vitamin D 13.42 (range 10.24–17.23) ng/ml; 50% were men. Vitamin D supplementation increased vitamin D levels in the active group compared to control ($p < 0.01$). EPCs decreased in both groups from baseline. There was no difference in change in EPCs, hsCRP, IL-6, IL-10, TNF-$\alpha$, and $HbA_{1c}$ or insulin resistance (HOMA-IR) between the active- and control-groups at the end of the study. Vitamin D supplementation did not alter EPCs or inflammatory markers, or improve glycemic control at the dose and duration investigated. Further studies are needed to study the long-term effects on markers of endothelial repair.

**Keywords** Vitamin D · EPC · Endothelial progenitor cell · Endothelial dysfunction · Type 2 diabetes · Cardiovascular disease · Inflammatory cytokines

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## Introduction

Type 2 diabetes is an independent risk factor for cardiovascular disease (CVD), and CVD is the prime cause of mortality in patients with diabetes [1]. Despite evidence that multifactorial risk management offers cardiovascular benefits, patients with diabetes continue to have residual excess cardiovascular risk. Approaches beyond conventional risk management are needed. Recent studies support inflammation [2–7] and endothelial dysfunction participates in hyperglycemia and CVD risk. Endothelial progenitor cells (EPCs) are bone marrow-derived cells in the peripheral blood, which migrate to areas of ischemia, promote angiogenesis and endothelial repair. An inverse relationship exists between cardiovascular risk factors, and the number and migratory capacity of EPCs [8]. Quantification of CD34 and CD133 positive EPCs by flow cytometry is a novel approach to identify patients with defects in endothelial repair [8–10].

Several studies report conflicting evidence on the role of vitamin D in inflammation, cardiovascular risk reduction and glycemic control [11–19]. The effect of vitamin D on inflammatory markers such as hsCRP, IL-6, Il-10 and TNF-$\alpha$, and

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EPCs in patients with low levels of vitamin D and type 2 diabetes is uncertain. Increase in inflammation and a decrease in the number of EPCs are associated with increased cardiovascular risk. A novel approach achieved by treating vitamin D deficiency, a highly prevalent, unrecognized and untreated conditions may be a cost effective alternate to the measurement of EPCs and can have paramount implications in cardiovascular residual risk reduction.

The aim of this study was to evaluate the impact of vitamin D repletion on the levels of inflammatory markers, EPC numbers, and glycemic control in patients with type 2 diabetes and dyslipidemia.

## Methods

### Design and drug administration

This study evaluated the effects of vitamin D supplementation on EPC, inflammatory cytokines, glycemic control, and insulin resistance in patients with type 2 diabetes. This randomized, parallel group, open-label 24-week study enrolled patients attending the outpatient at Diacon Hospital, a tertiary, university recognized hospital for diabetes care, research, and postgraduate studies, in Bangalore, India, between April 2015 and January 2016.

Patients who met the eligibility criteria at screening were randomized in a 1:1 allocation ratio alternatively by the trial site personnel to the intervention arm which received vitamin D supplementation and a control arm which did not receive vitamin D. Patients, trial site personnel, and investigators were aware of the randomization group.

Participants randomized to active intervention received 60,000 IU of vitamin D orally once a week for 8 weeks, followed by once a month for 4 months. Patients in both arms received rosuvastatin 10 mg once a day (or 20 mg once a day for patients with known cardiovascular disease) during the course of the study. Rosuvastatin was initiated in four patients (three in the active arm and one in the control arm) at the time of randomization. All other patients had been on the stable doses of statin for at least 3 months prior to enrollment. There was only one patient in the control arm with cardiovascular disease receiving 20 mg of rosuvastatin. Vitamin D and rosuvastatin were dispensed by the hospital at each scheduled study visit. Patients were to maintain their background medication for diabetes throughout the trial. Patients on sulfonylureas were allowed to reduce the dose if hypoglycemic episodes occurred. A treat to target approach was followed to allow patients on insulin to titrate their insulin dose as per standards of care.

Patients with baseline hypertension were allowed to continue all anti-hypertensive medications throughout the study. These medications included thiazide diuretics, angiotensin receptor antagonists, calcium channel blockers, and beta blockers. No changes were made in the dose of anti-hypertensive medications during the study. Low-dose aspirin therapy (75 to 150 mg per day) was continued for patients receiving aspirin prior to enrollment; no subsequent changes were made during the course of the study.

## Trial population

### Inclusion criteria

Eligible trial patients were men and women, aged 25–65 years (inclusive), previously diagnosed with type 2 diabetes and dyslipidemia, had A1c below 9%, low vitamin D levels (< 30 ng/ml) and were on stable doses of oral/injectable anti-diabetic medications and rosuvastatin for at least 90 days prior to screening. The following background anti-diabetic medications were allowed as monotherapy or in combination: metformin, sulfonylureas, DPP4 inhibitors, pioglitazone, alpha glucosidase inhibitors, and insulin (regular insulin, NPH, rapid acting analogues, premixed insulin, and basal insulin based on the prescribed insulin regimen).

### Exclusion criteria

Exclusion criteria included prior use of vitamin D supplementation, acute infections, sepsis, any malignancy, hyperparathyroidism, chronic renal insufficiency or failure (eGFR < 60 ml/min/1.73 $m^2$ MDRD formula), nephrocalcinosis, statin intolerance, women in reproductive age group planning pregnancy, pregnancy and lactation, patients with type 1 diabetes or chronic fibrocalculous pancreatic diabetes, malabsorption, chronic inflammatory autoimmune disorders e.g., rheumatoid arthritis, patients on steroids, patients on immunosuppressive drugs, or medications used in the treatment of autoimmune and rheumatological disorders (e.g., hydroqxychloroquine, methotrexate), patients on drugs which may impair hydroxylation of vitamin D ,such as isoniazid, and patients on drugs which induce cytochrome P450 and cause accelerated loss of vitamin D, such as rifampicin.

### Study end points and assessments

The primary efficacy end points were the changes from baseline in levels of EPCs 24 weeks after treatment. Secondary efficacy end points included changes in inflammatory marks: hsCRP, IL-6, IL-10, and TNF-$\alpha$ from changes in HbA1c levels and insulin resistance 24 weeks after treatment.

Vitamin D, hsCRP, IL-6, IL-10, TNF-$\alpha$, fasting insulin levels, $HbA_{1c}$, and lipid profile were measured at baseline, 12 and 24 weeks. Patients were categorized based on insulin resistance (IR) into normal IR (< 3), moderate IR

(3–5) and severe IR (> 5) using homeostatic model assessment (HOMA) [20].

### Insulin resistance—fasting insulin in mU/l × fasting glucose in mg/dl / 405

Endothelial progenitor cells were isolated by flow cytometry using PE-CD34, PE-CD45, and FITC-CD133 antibodies at baseline and 24 weeks [using mouse anti-human CD34 PE (555822; BD Biosciences, CA, USA), mouse anti-human CD 45 PE (555484; BD Biosciences, CA, USA), and mouse anti-human CD 133 VioBright FITC (130-105-225/226; Miltenyi Biotec GmbH, Bergisch Gladbach, Germany)]. Quantification of serum levels of cytokines was by ELISA (BD Biosciences, USA).

Patients were evaluated for micro and macrovascular complications of diabetes as per the American Diabetes Association Standards of Care clinical practice recommendations [1, 21]. Safety assessments included adverse events (AEs), physical examinations, vital signs (pulse, blood pressure) electrocardiogram, and laboratory assessments at the study site, (hemoglobin, RBC count, WBC count, differential count, urea, creatinine, and urinalysis). A plasma glucose < 70 mg/dl was used as cut-off to define hypoglycemia.

### Compliance with ethics guidelines

The study protocol was approved by the institutional ethics committee and the Research Society for the Study of Diabetes in India (RSSDI). All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1964, as revised in 2013. Informed consent was obtained from all patients for being included in the study.

### Statistical analysis

This pilot study planned to recruit at least 50 patients (25 in each arm) and a sample size of 65 was considered to include loss to follow-up rates. As there was a lack of preliminary data on the effect of vitamin D supplementation on EPC, the results of this pilot will help provide estimates to aid in sample size calculations for similar studies in the future.

Descriptive statistics summarized the data using mean, standard deviation, median, and interquartile range (25th to 75th percentiles). Baseline differences between groups were evaluated using the *t* test, Wilcoxon rank-sum test or the $\chi^2$ test based on the distribution and type of variable appropriately. The primary and secondary end points were assessed using analysis of covariance (ANCOVA) model for continuous

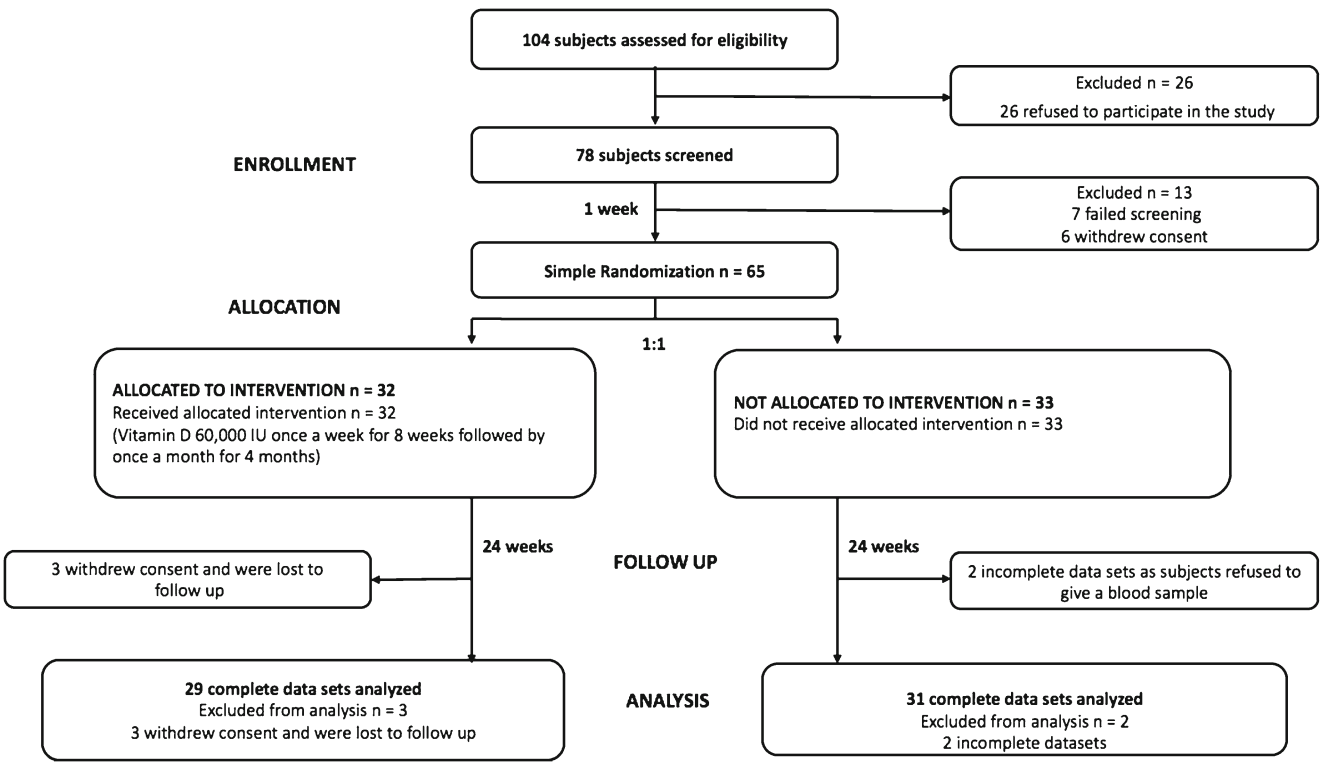


**Fig. 1** Flow diagram of efficacy of vitamin D supplementation on reduction of cardio-metabolic risk in patients with type 2 diabetes mellitus and dyslipidemia: enrollment, randomization, follow-up, and data analysis

outcomes with treatment group, age, and sex as fixed effects and baseline value as covariate; and the $\chi^2$ test for categorical outcomes. A two-sided $p$ value at 5% alpha was considered statistically significant. The primary analysis was a complete case analysis; patients with missing data sets and loss to follow-up were excluded from the analysis. Data was analyzed using Stata/IC version 14.2 (StataCorp LP, college Station, TX).

## Results

Eligible participants were recruited from April 2015 to August 2015. Study participants attended clinic visits at the time of randomization (baseline), 12 and 24 weeks. Figure 1 shows the flow of participants throughout the study. Characteristics of the 60 subjects who completed the study are as follows: median age 54 (48.5–60) years, 50% male, mean BMI 26.86 ± 3.8 kg/m$^2$, mean $HbA_{1c}$ 7.22 ± 0.8%, duration of diabetes 7 (4–12.5) years, and median vitamin D levels 13.42 (10.24–17.23) ng/ml. At baseline, eight patients in the active and nine patients in the control arm were receiving aspirin (most patients were on 75 mg aspirin per day, only one patient in the control arm with cardiovascular disease was on 150 mg aspirin per day). None of the patients received anti-inflammatory doses of salicylates (3 to 6 g per day).

The primary analysis was a complete case analysis. Baseline subject demographics are summarized in Table 1. Subjects in the active group had higher levels of total serum cholesterol and LDL at baseline; and a higher percentage were women. All patients with baseline hypertension were well controlled with blood pressure < 140/90 mmHg throughout the study. All other measures, clinical and laboratory were distributed equally across both groups.

Evidence of adherence to vitamin D supplementation was observed by the increase in levels of vitamin D in the intervention compared to the control arm [20.48 (95% CI 16.35 24.61) versus − 3.49 (95% CI − 5.42 − 1.56); $p < 0.01$].

### Effect on endothelial progenitor cells and inflammatory markers

There were no differences in EPCs or the inflammatory cytokines between groups at 24 weeks when adjusted for baseline covariate, age, and sex. There were no interactions between sex and the dependent variable (Table 2). A reduction in EPCs, hsCRP, and TNF-$\alpha$ and an increase in IL-6 were observed in both groups at 24 weeks compared to baseline. IL-10 levels remained below detectable limits in both groups.

**Table 1** Comparison of baseline demographic data between active and control group

| | Intervention, $n = 29$ | Control, $n = 31$ |
|---|---|---|
| Age years | 53 (48–60) | 55 (50–61) |
| Duration of diabetes years | 7.98 ± 6.14 | 8.89 ± 5.72 |
| Male | 10 (34.48) | 20 (64.52)* |
| Female | 19 (65.52) | 11 (35.48) |
| Hypertension | 17 (58.62) | 18 (58.06) |
| Neuropathy | 3 (10.34) | 4 (12.90) |
| Retinopathy | 5 (17.24) | 5 (16.13) |
| Ischemic heart disease | 0 | 1 (3.23) |
| Peripheral arterial disease | 0 | 0 |
| Cerebrovascular accident | 0 | 0 |
| Body mass index (kg/m$^2$) | 25.9 (24–30) | 26.5 (23.52–28.78) |
| HbA1c % | 7.21 ± 0.88 | 7.24 ± 0.73 |
| Total cholesterol (mg/dl) | 149 (124–170) | 131 (114–148)* |
| Triglycerides (md/dl) | 125 (100–193) | 127 (95–162) |
| HDL (mg/dl) | 39 (35–47) | 35 (33–41) |
| LDL (mg/dl) | 83.02 ± 33.81 | 67.03 ± 18.86* |
| VLDL (mg/dl) | 25 (20–38.6) | 25 (19–30.4) |
| Vitamin D (ng/ml) | 13.09 (9.07–19.84) | 13.51 (10.73–16.22) |
| hsCRP (mg/l) | 2 (0.82–3.24) | 1.25 (0.54–1.82) |
| IL-6 (pg/ml) | 0.01 (0–1.96) | 0 (0–0.83) |
| IL-10 (pg/ml) | 0 (0–0.009) | 0 (0–0) |
| TNF-$\alpha$ (pg/ml) | 9.16 (7.41–9.63) | 8.53 (7.69–9.36) |
| Insulin resistance | | |
| Normal < 3 | 18 (62.07) | 15 (48.39) |
| Mild–moderate 3–5 | 4 (13.79) | 15 (48.39) |
| Severe > 5 | 7 (24.14) | 8 (25.81) |
| EPC cells/μl | 5 (2–8) | 4 (3–6) |

Continuous variables are shown as mean ± SD or as median (interquartile range). Categorical variables are presented as $n$ (percentages). $T$ test for normally distributed continuous variables, Wilcoxon rank-sum test for non-normally distributed continuous variables, and $\chi^2$ test for categorical variables

*EPC* endothelial progenitor cells

*$p$ values < 0.05.

### Effect on glycemic control and insulin resistance

There was no significant difference in $HbA_{1c}$ between groups at the 24 weeks when adjusted for baseline covariate, age, and sex. There were no interactions between sex and the dependent variable No differences were observed in the proportion of patients with no insulin resistance, mild to moderate insulin resistance, and severe insulin resistance from baseline within groups or between groups. However, a higher proportion i.e., 41.94% of patients in the control arm had severe insulin resistance compared to 20.69% of patients in the intervention arm at 24 weeks (Table 2).

**Table 2** Summary of results: Differences in outcomes in intervention and control arm at 24 weeks

| | Intervention, $n = 29$ | Control, $n = 31$ | $p$ value |
|---|---|---|---|
| Vitamin D (ng/ml) | 20.48 (16.35 24.61) | − 3.49 (− 5.42 − 1.56) | < 0.01* |
| hsCRP (mg/l) | − 1.2 (− 2.66 0.25) | − 0.42 (− 0.85 0.01) | 0.4 |
| IL-6 (pg/ml) | 1.73 (− 0.35 3.82) | 2.48 (0.28 4.69) | 0.93 |
| IL-10 (pg/ml) | 0.04 (− 0.36 0.46) | − 0.09 (− 0.2 0.01) | 0.63 |
| TNF-$\alpha$ (pg/ml) | − 3.68 (− 4.37 − 2.99) | − 4.1 (− 4.56 − 3.64) | 0.63 |
| EPC cells/μl | − 1.65 (− 3.09 − 0.21) | − 0.06 (− 2.17 − 2.04) | 0.74 |
| HbA1c % | − 0.003 (− 2.34 0.22) | − 0.22 (− 0.5 0.04) | 0.15 |
| Insulin resistance | | | |
| Normal < 3 | 12 (41.38) | 12 (38.71) | 0.13 |
| Mild–moderate 3–5 | 11 (37.93) | 6 (19.35) | |
| Severe > 5 | 6 (20.69) | 13 (41.94) | |
| Body mass index (kg/m$^2$) | − 0.12 (− 0.45 0.21) | − 0.22 (− 0.5 0.45) | 0.42 |
| Total cholesterol (mg/dl) | − 20.51 (− 33.72 − 7.3) | − 7.48 (− 21.04 6.08) | 0.62 |
| Triglycerides (mg/dl) | − 6.72 (− 27.84 14.39) | − 18.74 (− 49.03 11.55) | 0.46 |
| HDL (mg/dl) | 6.27 (4.23 8.31) | 8.75 (6.60 10.90) | 0.07 |
| LDL (mg/dl) | − 25.42 (− 36.94 − 13.89) | − 12.89 (− 23.7 − 2.07) | 0.61 |
| VLDL (mg/dl) | − 1.33 (− 5.56 2.88) | − 3.19 (− 9.3 2.91) | 0.43 |

Continuous variables are shown as change in means from baseline (95% CI). Categorical variables are presented as $n$ (percentages). For continuous outcomes: ANCOVA adjusted for baseline covariate, age, and sex

*EPC* endothelial progenitor cells

*significant $p$ value

*Safety*

The study showed no differences in BMI, total cholesterol, serum triglycerides, HDL, LDL, and VLDL between groups at 24 weeks when adjusted for baseline covariate, age, and sex (Table 2). No adverse symptoms related to vitamin D toxicity necessitating investigation for hypercalciuria or hypercalemia, or hypoglycemia (defined as plasma glucose < 70 mg/dl) occurred during the study.

## Discussion

In the past decade, inflammatory markers have come to light as independent risk factors for CVD[2, 4, 22–25]; and EPCs [7–10] have emerged as regenerative cells, offering a novel target of untapped therapeutic potential. To our knowledge, this is the first randomized clinical trial investigating the effect of vitamin D supplementation on both inflammatory cytokines and EPCs in patients with type 2 diabetes.

### Vitamin D: Inflammatory cytokines and EPCs

Inflammation plays a complex, intricate, and yet incompletely understood role in diabetes and CVD. The inflammatory response is initiated when IL-1 and TNF-$\alpha$ are released from the site of inflammation, resulting in a cascade of changes including release of IL-6 and acute-phase reactants like fibrinogen and hsCRP among others [22]. IL-6, TNF-$\alpha$, and hsCRP have been implicated in the progression of atherosclerosis and plaque rupture [23–28]. IL-10 on the other hand is considered to be an anti-inflammatory cytokine that reduces the production of other inflammatory cytokines, and is associated with better acute coronary syndrome outcomes and inversely with stroke mortality [29]. In large prospective cohort studies like the Framingham Offspring Study, vitamin D has been associated with an increase in cardiovascular risk over and above traditional risk factors [30]. In a few smaller clinical trials, vitamin D supplementation has reduced the levels of inflammatory cytokines [16]. In this study, a reduction in hsCRP, TNF-$\alpha$, and IL-10, and an increase in IL-6 were observed in both groups compared to baseline. The lack of an unidirectional change in inflammatory cytokines in the group which received vitamin D, compared to the control arm, demonstrates that vitamin D supplementation had no consistent or significant effect on levels of inflammatory cytokines.

Endothelial injury is now recognized as a pathophysiological process in patients with diabetes, hypertension, acute myocardial infarction, heart failure, stroke, and peripheral vascular disease [31–35]. Assessment of endothelial function includes flow-mediated dilatation (FMD) and quantification of circulating endothelial cells (CECs) and EPCs [8–10]. CECs have been identified as markers of endothelial damage and EPCs as biomarkers of vascular repair. EPCs are bone

marrow-derived immature cells that home in, differentiate, and maintain the endothelium by reendothelialization and neovascularisation at sites of trauma and ischemia [9, 10, 36, 37]. Increased levels of EPCs are inversely associated with cardiovascular outcomes [8]. The impact of vitamin D on EPCs has not been previously studied. In this study, a reduction in number of EPCs was observed in both groups compared to baseline. No quantitative differences were observed between the vitamin D and the control group at the end of the study.

### Vitamin D: Glycemic control and insulin resistance

Conflicting reports on the role of vitamin D and glycemic control are available in medical literature. Initial studies showcased a promise of improvement in hyperglycemia and reduction in insulin resistance; more recent evidence has highlighted contrasting outcomes [17, 19]. In this study, vitamin D supplementation did not improve glycemic control or insulin resistance measured by HOMA-IR. Vitamin D supplementation did not result in changes in BMI and levels of total cholesterol, triglycerides, LDL, HDL, or VLDL.

The limitations of this study include small sample size and relatively short study duration. The study methods did not include measurement of migratory and functional ability of EPCs. In retrospect, all patients in the study had normal hsCRP at baseline. It is well known that hsCRP > 10 mg/l confers high cardiovascular risk to individuals. The inability to detect anti-inflammatory effects or changes in EPCs could perhaps be attributed to this low risk study population.

Patients in this study were on other potentially confounding background medications like aspirin and rosuvastatin, known to reduce inflammation and CV risk. However, only low-dose aspirin was used in this study and none of the patients received anti-inflammatory doses of aspirin. The use of both aspirin and statin was also similar across groups, thus highlighting the importance of randomization in balancing known and other unknown confounders.

Even though there appears to be a lack of benefit of vitamin D on EPCs in this study, based on previous evidence from studies of acute coronary syndrome and EPCs [9, 38–41], it is possible that a biologically meaningful benefit or a trend towards benefit could be seen if the baseline risk is high. A better approach for future studies would be to augment identification of high risk individuals based on hsCRP levels and to study newer targets of intervention in the high risk population. Individuals with high baseline risk perhaps would be a more suitable study population, in whom new markers and therapeutic targets for intervention would also provide additional benefit. This would also facilitate a better use of resources while offering the greatest benefits to the high risk individuals.

A greater understanding of inflammation and the role of EPCs are necessary to further explore the implications of additional markers and therapeutic targets. Larger studies of longer duration are required to validate our findings. In this study, vitamin D supplementation failed to demonstrate a reduction in inflammation and improvement in endothelial repair.

#### Compliance with ethical standards

**Ethical approval** The study protocol was approved by the institutional ethics committee and the Research Society for the Study of Diabetes in India (RSSDI). All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1964, as revised in 2013.

**Statement of informed consent** Informed consent was obtained from all patients for being included in the study.

**Conflict of interest** Authors declare they have no conflict of interest.

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