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Randomized control trials

Effect of cholecalciferol on vitamin D-regulatory proteins in monocytes and on inflammatory markers in dialysis patients: A randomized controlled trial

Marion Schneider Meireles [a], Maria Ayako Kamimura [a], Maria Aparecida Dalboni [b], José Tarcísio Giffoni de Carvalho [b], Danilo Takashi Aoike [b], Lilian Cuppari [a, b, *]

[a] The Nutrition Graduation Program, Federal University of São Paulo, Brazil
[b] Department of Medicine, Division of Nephrology, Federal University of São Paulo, Brazil

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SUMMARY

Background & aims: Hypovitaminosis D and inflammation are highly prevalent among patients undergoing dialysis, and the association of both conditions with worse survival has been well recognized. Although a potential role for vitamin D in the immune system has been suggested, the effect of the treatment of hypovitaminosis D on the modulation of the inflammatory response remains unclear. The aim of this study was to investigate the effect of the restoration of the vitamin D status on the expression of vitamin D-regulatory proteins in monocytes and on circulating inflammatory markers in dialysis patients.

Methods: In this randomized double-blind placebo-controlled 12-week trial, 38 patients on dialysis with serum 25-hydroxyvitamin D [25(OH)D] <20 ng/mL were randomized either to the cholecalciferol group ($n = 20$; 50,000 IU of cholecalciferol twice weekly) or to the control group ($n = 18$; 50 drops of a placebo solution twice weekly). The expression of vitamin D receptor (VDR), CYP27B1, CYP24A1 and interleukin-6 (IL-6) in monocytes was determined by flow cytometry. Serum concentrations of 25(OH)D, interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α) and C-reactive protein (CRP) were measured.
The trial is registered at ClinicalTrials.gov #NCT01974245.

Results: After 12 weeks, the serum 25(OH)D increased from 14.3 ± 4.7 ng/mL to 43.1 ± 11.0 ng/mL ($p < 0.05$) in the cholecalciferol group and did not change in the control group (13.9 ± 4.2 ng/mL to 13.5 ± 4.3 ng/mL; $p = 0.56$). In monocytes, while CYP27B1 expression and VDR expression increased in the cholecalciferol group ($p < 0.05$), CYP27B1 expression did not change, and VDR expression decreased in the control group ($p < 0.05$). There were no changes in IL-6 and CYP24A1 expression in both groups. Serum concentration of IL-6 and CRP decreased from 8.1 ± 6.6 pg/mL to 4.6 ± 4.1 pg/mL ($p < 0.05$) and from 0.50 (0.10–1.27) mg/dL to 0.28 (0.09–0.62) mg/dL ($p < 0.05$), respectively only in the cholecalciferol group. Assessed overtime, the treatment group differences in 25(OH) D, PTH, CRP and IL-6, CYP27B1 and VDR remained significant.

Conclusions: Restoration of vitamin D status of patients undergoing dialysis promoted upregulation of CYP27B1 and VDR expression in monocytes and a decrease in circulating inflammatory markers.

1. Introduction

Hypovitaminosis D and disorders of vitamin D metabolism are common among patients with chronic kidney disease (CKD) [1,2]. The impaired conversion of 25-hydroxyvitamin D [25(OH)D] to 1,25-dihydroxyvitamin D [1,25$(OH)_2$D], due to decreased renal 1-α hydroxylase enzyme (CYP27B1) activity, contributes to the development of bone and mineral disorders highly prevalent in this

* Corresponding author. Federal University of São Paulo, Rua Pedro de Toledo, 282, Vila Clementino, São Paulo, SP 04039-000, Brazil. Tel.: +55 1159048499; fax: +55 1155721862.
E-mail addresses: mariondschneider@gmail.com (M.S. Meireles), m.kamimura@uol.com.br (M.A. Kamimura), dalboni@nefro.epm.br (M.A. Dalboni), tarcisiogiffoni@yahoo.com.br (J.T. Giffoni de Carvalho), aoike@ig.com.br (D.T. Aoike), lcuppari@uol.com.br (L. Cuppari).

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population of patients [3]. More recently, it has been demonstrated that in addition to tubular renal cells, CYP27B1 is present in numerous extrarenal sites, allowing the local production of $1,25(OH)_2D$, which binds to its receptor (VDR) triggering autocrine and paracrine responses [4]. It has been estimated that approximately 85% of serum 25(OH)D is used as a substrate for the extrarenal synthesis of $1,25(OH)_2D$ [5]. Therefore, the availability of 25(OH)D is of paramount importance to produce effective responses. The pleiotropic effects of vitamin D have been demonstrated in numerous organs and cells, including prostate, mammary, colon, pancreatic β-cells, keratinocytes and immune cells [6]. Particularly in the immune system, the $1,25(OH)_2D$ synthesized in monocytes has been proposed to have immunomodulatory properties through the inhibition of the proinflammatory cytokine production [7].

A chronic inflammatory state and a low 25(OH)D serum concentration are highly prevalent among patients with CKD, and both conditions are associated with poor outcomes especially due to cardiovascular events [8–10]. Although multiple causes involve an elevated inflammatory response in these patients, low serum concentration of 25(OH)D has been associated with elevated markers of inflammation [11]. Therefore, the repletion of nutritional vitamin D might represent a safe and low-cost therapeutic approach to minimize inflammation in patients with CKD. Currently, randomized clinical trials investigating the impact of the correction of hypovitaminosis D on inflammation in dialysis patients are scarce.

Therefore, in the present study we tested the hypothesis that restoration of vitamin D status would modulate the expression of vitamin D regulatory proteins in monocytes and decrease circulating markers of inflammation of patients undergoing dialysis.

2. Materials and methods

2.1. Subjects

Patients undergoing hemodialysis (HD) and peritoneal dialysis (PD) from single dialysis unit of the Oswaldo Ramos Foundation (São Paulo, Brazil), aged between 18 and 80 years, with a dialysis vintage of at least 3 months and serum 25(OH)D <20 ng/mL, were invited to participate in the present study. The exclusion criteria were the use of any vitamin D compound, glucocorticoids, or immunosuppressors or history of liver failure, intestinal malabsorption, malignancy, autoimmune disease, active infection, positive HIV, peritonitis in the last month or elevated serum ionized calcium (>1.40 mmol/L).

As shown in Fig. 1, from September 2012 to May 2014, 348 patients were screened (194 HD/154 PD). Among these, 112 patients were excluded due to the use of vitamin D compounds, 77 were excluded because the serum 25(OH)D was greater than 20 ng/mL, and 104 patients did not meet other inclusion criteria. Therefore, 55 patients were randomized. Seventeen patients were lost during follow-up due to hospitalization (n = 8), death (n = 2) or lack of compliance with the study protocol (n = 7). Therefore, the present study was completed with a total of 38 patients (23 HD/15 DP). Patients undergoing HD were on conventional HD for 4 h, 3 times a week, using bicarbonate-buffered dialysate and polysulfone dialyzer membranes. The majority of PD patients (87%) were treated by automated PD using a glucose-based solution. The baseline demographic, clinical, laboratory and nutritional characteristics of the patients lost during follow-up were similar to those of the patients in the group that completed the study (data not shown).

The study was approved by the Human Investigation Review Committee of the Federal University of São Paulo and was conducted in accordance with the Declaration of Helsinki. Written consent was obtained from each participant. The trial is registered at ClinicalTrials.gov #NCT01974245.

2.2. Study design and protocol

The present study was a 12-week randomized, double blind, placebo-controlled clinical trial. Participants and researchers were blinded. The patients were assigned to cholecalciferol or control groups after a blocked randomization procedure by using a random block of 4 participants. An independent researcher generated a computerized random list and the allocation sequence was concealed in closed box. A pharmacist was responsible for label and number all containers according to the random schedule. The patients in the cholecalciferol group received 50,000 IU of cholecalciferol (1000 IU/drop; Magister Pharmacy, São Paulo, SP, Brazil) twice a week, while the control group received a placebo solution and were instructed to take 50 drops twice a week. The pharmaceutical presentations of the placebo and cholecalciferol were identical. To minimize the effects of vitamin D synthesis in the skin, all patients received and were instructed to wear sunscreen (SPF 30) during the study period. To evaluate compliance, the patients were requested to bring the containers to the follow-up visits. It was expected that the supplied volume would be finished in 6 weeks, when the patient received another container to complete the study. In addition, during the monthly visits and through phone calls, the patients were encouraged to follow the treatment.

2.3. Data collection

Demographic and clinical data were collected from medical records. Body weight and height were measured, and the body mass index (BMI) was calculated. Patients underwent nutritional evaluation using the 7-point Subjective Global Assessment (SGA) and were classified as malnourished when the SGA score was below five. Blood samples were collected after eight hours of fasting from all participants at baseline and after 12 weeks. Serum levels of 25(OH)D (chemiluminescence immunoassay, Abbott Architect assay, Germany), high-sensitivity C-reactive protein (immunoturbidimetric assay, Beckman Coulter Biomedical, Ireland), intact parathyroid hormone (chemiluminescence immunoassay, reference values: 10–69 pg/mL), alkaline phosphatase (calorimetric method, reference values: 35–104 U/L), phosphorus (colorimetric method, reference values: 2.3–4.3 mg/dL), ionized calcium (ion-selective electrode *method*, reference values: 1.11–1.40 mmol/L), albumin (bromocresol green), urea (enzymatic method) and creatinine (kinetic Jaffe colorimetric method) were measured. The serum concentrations of high-sensitivity interleukin-6 (IL-6), high-sensitivity tumor necrosis factor-α (TNF-α) and intact fibroblast growth factor 23 (FGF-23) were measured using the enzyme linked immune sorbent assay technique (R&D Systems, Minneapolis, MN, USA). Respective inter- and intra-assay coefficients of variation were 8.4% and 5.3% (TNF-α), 7.7% and 7.4% (IL-6) and 4.1% and 5.9% (FGF-23).

2.4. Flow cytometry

The expression of vitamin D receptor (VDR), 1-α hydroxylase enzyme (CYP27B1), 24-hydroxylase enzyme (CYP24A1) and interleukin-6 (IL-6) in monocytes was determined by flow cytometry.

An aliquot of 100 μL of heparinized whole blood was incubated with 10 μL of PE-conjugated anti-human CD14 antibody (BD Biosciences, San Diego, CA, USA) for 15 min in the dark at room temperature (according to the manufacturer's instructions) to characterize the monocytes. Subsequently, the cells were further

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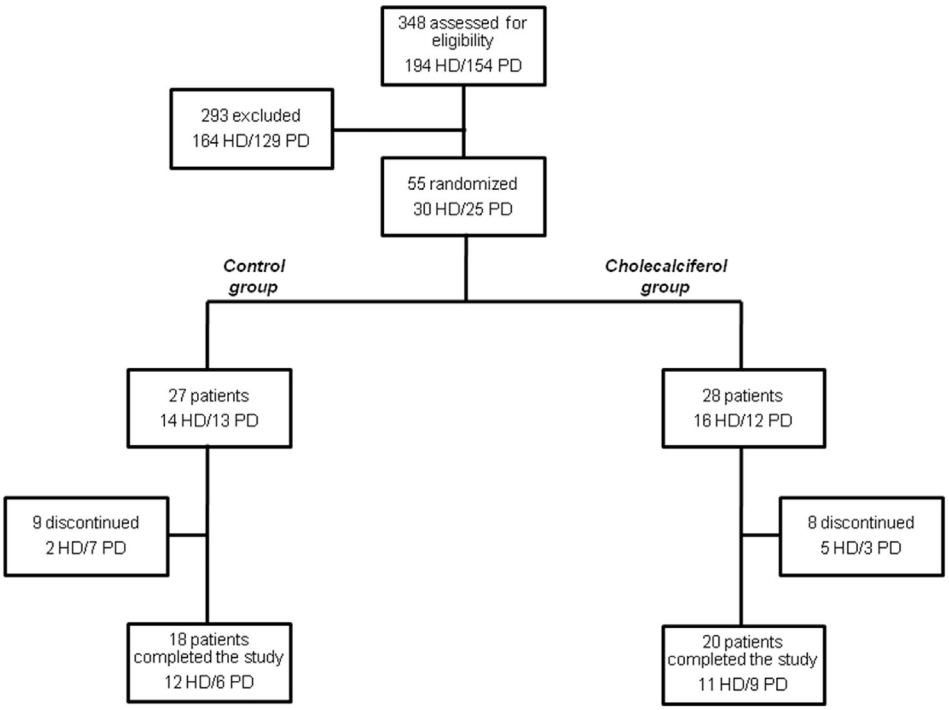

Fig. 1. Flow chart of participants through the study.

incubated with monensin for 30 min at 37 °C and 5% CO_2, followed by incubation with 3 μL of FITC-conjugated anti-human IL-6 antibody (BD Biosciences, San Diego, CA, USA) according to manufacturer instructions, for intracellular IL-6 detection.

Another aliquot of 100 μL heparinized whole blood was incubated with 1 μg/mL APC-conjugated anti-human VDR antibody, 1 μg/mL Alexa-Fluor 647-conjugated anti-human CYP27B1 antibody and 1 μg/mL Alexa-Fluor 488-conjugated anti-human CYP24A1 antibody according to manufacturer's instructions (primary antibodies: VDR, CYP27B1 and CYP23A1 – Santa Cruz Biotechnology, Santa Cruz, CA, USA and secondary antibodies Alexa-Fluor 647 and Alexa-Fluor 488 – Invitrogen, Carlsbad, CA, USA).

The acquisition of samples was performed by a flow cytometer (FacsCanto I, BD Biosciences, San Diego, CA, USA). For each sample, the data from 10,000 cells were collected and analyzed. Forward and side scatters were used to gate and exclude cellular debris, and the values are presented as the mean fluorescence intensity peak (MFI) in gated CD14+ monocytes expressing IL-6, VDR, CYP27B1 and CYP24A1. Figure 2 shows the monocytes characterized by CD-14 and the expression of VDR as an example.

2.5. Statistical analysis

The sample size calculation was based on a report by Bucharles et al. [12]. With a power of 95%, significance level of 5% and assuming a dropout rate of 25%, the sample size was estimated in 20 patients in each arm to detect a decrease of 50% in IL-6 concentration after cholecalciferol supplementation. The sample size calculation was performed using the GPower program, version 3.1.2 (Franz Faul, University of Kiel, Germany).

The data are expressed as the mean ± standard deviation for variables with normal distribution, as median and interquartile range for skewed distribution, and as frequencies for categorical

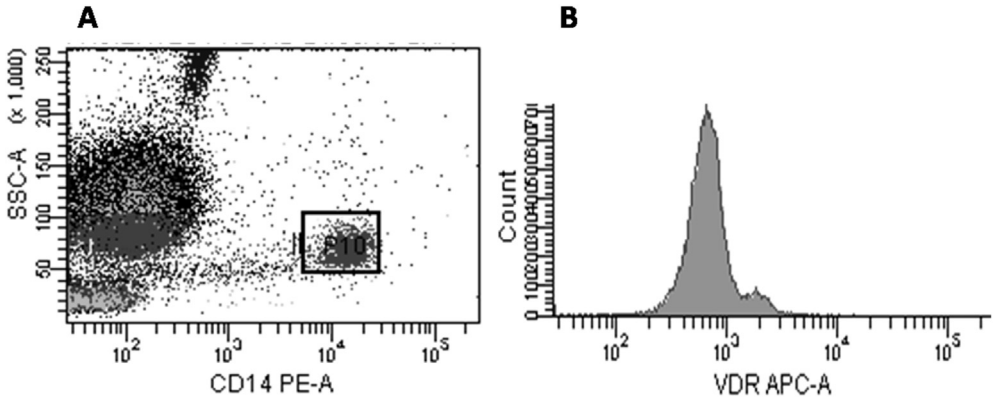

Fig. 2. Identification of monocytes by CD14 (A). Expression of VDR in monocytes (B).

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variables. Variables with skewed distribution were Z-score transformed. Student's *t* test or Pearson's Chi-square test were used to compare the baseline characteristics between the two groups, as appropriate.

The effect of the intervention on the studied parameters was analyzed by generalized estimating equations using time as repeated factor, and group and residual renal function as fixed factors. Because the interaction of time*group*residual renal function was not significant for the variables of interest, the reported analyses were performed only with time and group as factors. Contrast analyses were used to assess between- and within-group differences. Statistical significance was defined as $p < 0.05$. All statistical analyses were conducted using the Statistical Package for Social Sciences for Windows version 18.0 (SPSS Inc., Chicago, IL, USA).

3. Results

In the cohort examined, the majority of patients were men (53%), and 42% of these individuals had diabetes, 90% had hypertension and 26% were elderly patients (age > 65 years). According to SGA, 21% of the patients were classified as malnourished (score ≤ 5), and the mean BMI was 25.4 ± 5.6 kg/m^2. The median dialysis vintage was 30.5 months (interquartile range 12.5–54.5 months). The patients received antihypertensive medication (79%), erythropoietin-stimulating agents (74%), statins (37%), sevelamer hydrochloride (61%) and calcium-based binders (13%). The main baseline characteristics of the studied patients according to groups are shown in Table 1. No differences were observed between the groups in terms of demographic, clinical and nutritional parameters.

Table 1
Baseline characteristics of patients according to the groups.

	Control group (n = 18)	Cholecalciferol group (n = 20)	p
Age, years	56.5 ± 12.9	55.5 ± 14.2	0.83
Gender, male/female	9/9	11/9	0.76
Black race [n (%)]	3 (16.7)	5 (25.0)	0.70
Etiology of CKD [n (%)]			0.28
Diabetes Mellitus	6 (33.3)	6 (30.0)	
Hypertension	1 (5.6)	2 (10.0)	
Polycystic kidney disease	6 (33.3)	2 (10.0)	
Undetermined	2 (11.1)	7 (35.0)	
Other	3 (16.7)	3 (15.0)	
Comorbidities [n (%)]			
Diabetes Mellitus	8 (44.4)	8 (40.0)	0.78
Hypertension	16 (88.9)	18 (90.0)	1.00
Phosphate binders (%)			
Sevelamer hydrochloride	66.7	55.0	0.46
Calcium carbonate	11.1	15.0	1.00
Dialysis vintage, months	32.5 (15.2–62.2)	28 (8.7–49.7)	0.53
HD/PD	12/6	11/9	0.46
Kt/V			
HD	1.46 ± 0.25	1.59 ± 0.38	0.38
PD	2.33 ± 0.37	2.60 ± 0.52	0.31
Residual renal function [n (%)][a]	5 (27.8)	15 (75.0)	<0.01
Body mass index, kg/m^2	25.3 ± 6.4	25.5 ± 5.0	0.90
Malnourished [n (%)]	5 (27.8)	3 (15.0)	0.48
Season at enrollment [n (%)]			0.09
Summer	3 (12.5)	1 (3.7)	
Autumn	11 (45.8)	6 (22.2)	
Winter	7 (29.2)	11 (40.7)	
Spring	3 (12.5)	9 (33.3)	

Values are expressed as mean ± SD, median and (interquartile range), or n (%).
CKD, chronic kidney disease; HD, hemodialysis; PD, peritoneal dialysis.
[a] The presence of residual renal function was considered when the referred urinary output was greater than 200 mL per 24 h.

The laboratory data collected overtime are shown in Table 2. At baseline, the parameters did not differ between the groups. The serum 25(OH)D concentration increased after 12 weeks of treatment in the cholecalciferol group and did not change in the control group. In the cholecalciferol group, all patients achieved a serum 25(OH)D concentration greater than 30 ng/mL. Fifteen patients achieved a serum 25(OH)D concentration between 30 and 50 ng/mL, and in five patients the serum concentration of 25(OH)D was between 50 and 70 ng/mL. There was no change in the serum concentration of phosphorus, ionized calcium, alkaline phosphatase and FGF-23 in both groups during the follow-up. PTH decreased in the cholecalciferol group and did not change in the control group. No patient developed hypercalcemia or hyperphosphatemia.

A decrease in CRP and serum IL-6 was observed only in the cholecalciferol group, while TNF-α did not change in both groups. Figure 3 depicts the changes in serum CRP and IL-6 according to the groups. The expression of CYP27B1, VDR, CYP24A1 and IL-6 in monocytes is demonstrated in Fig. 4. No change was observed in IL-6 and CYP24A1 expression in both groups. In the cholecalciferol group, the VDR and CYP27B1 expression increased, while in the control group, the VDR expression decreased. Assessed overtime, the treatment group differences in 25(OH) D, PTH, CRP and IL-6, CYP27B1 and VDR remained significant.

Figure 5 depicts an example of VDR and CYP27B1 expression at baseline and after 12 weeks of one patient in the cholecalciferol group and one patient of the control group.

For the variables of interest, such as IL-6, TNF-α, CRP, CYP27B1, VDR, CYP274A1 and IL-6, the results remained the same when analyzed according to the dialysis modality (data not shown).

4. Discussion

In the present clinical trial, we showed that a high-dose of cholecalciferol supplementation for 12 weeks was effective in restoring the vitamin D status of patients with hypovitaminosis D on maintenance dialysis. The repletion of 25(OH)D upregulated the expression of CYP27B1 and VDR in monocytes and promoted a decreased in the serum concentration of circulating markers of inflammation.

Over recent years, there has been growing interest in the potential benefits of maintaining an adequate vitamin D [25(OH)D] status in individuals with all stages of CKD, not only for minimizing the mineral and bone disturbances but also to potentially provide benefits in other associated complications, including inflammation. Although a guideline of clinical practice has proposed a protocol for treatment of hypovitaminosis [13], studies on vitamin D supplementation have been greatly heterogeneous with respect to dose, frequency and duration of intervention. Although the serum 25(OH)D concentration typically increases with both cholecalciferol and ergocalciferol, regardless of the protocol employed, several studies have reported that the complete restoration of vitamin D status is often not achieved [14–16]. In a previous study, we showed that a weekly dose of 50,000 IU of cholecalciferol for 3 months restored the vitamin D status of 78% of non-dialysis-dependent CKD patients, while a monthly dose of 50,000 IU was not sufficient to maintain the levels of 25(OH)D in the subsequent three months [17]. It seems that higher doses of vitamin D are necessary for effectively correct hypovitaminosis D in CKD patients. Indeed, studies show that a weekly dose of 100,000 IU of cholecalciferol for eight weeks [18] and 200,000 IU for three weeks [16] normalized the vitamin D status in majority patients on hemodialysis with no adverse effects. Therefore, in the present study, we used a high dose of 100,000 IU/week of cholecalciferol to ensure

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Table 2
Laboratory parameters at baseline and after 12 weeks of intervention.

Parameters	Control group (n = 18)		Cholecalciferol group (n = 20)	
	Baseline	12-week	Baseline	12-week
25(OH)D (ng/mL)	13.9 ± 4.2	13.5 ± 4.3	14.3 ± 4.7	43.1 ± 11.0[a,b,c]
CRP (mg/dL)	0.57 (0.19–1.73)	0.48 (0.21–1.71)	0.50 (0.10–1.27)	0.28 (0.09–0.62)[a,b,c]
IL-6 (pg/mL)	9.0 ± 5.2	9.6 ± 5.6	8.1 ± 6.6	4.6 ± 4.1[a,b,c]
TNF-α (pg/mL)	5.5 (4.3–5.8)	4.7 (3.8–5.7)	6.0 (4.0–6.7)	5.1 (3.7–7.1)[b]
PTH (pg/mL)	276.0 (168.5–392.5)	278.5 (126.2–521.7)	432.0 (225.5–558.5)	331.5 (243.2–504.0)[a,c]
FGF-23 (pg/mL)	2220 (696–9475)	1620 (980–7895)	930 (522–3800)	980 (347–3260)[b]
Phosphorus (mg/dL)	5.3 ± 1.4	5.6 ± 1.7	5.1 ± 1.5	5.2 ± 1.4
Ionized calcium (mmol/L)	1.24 (1.18–1.28)	1.23 (1.19–1.28)	1.24 (1.19–1.28)	1.27 (1.21–1.31)
Alkaline phosphatase (U/L)	83.5 (59.5–142.7)	83.5 (58.5–123.0)	84.5 (71.7–130.7)	85.0 (70.2–126.0)

Values are expressed as mean ± SD or median and (interquartile range).
25(OH)D, 25-hydroxyvitamin D; CRP, C-reactive protein; IL-6, interleukin-6; TNF-α, tumor necrosis factor-α, PTH, parathyroid hormone.
FGF-23, fibroblast growth factor 23.
[a] $p < 0.05$ versus baseline, [b] $p < 0.05$ cholecalciferol group versus control group, [c] $p < 0.05$ group*time interaction.

that all supplemented patients would achieve a serum concentration of 25(OH)D greater than 30 ng/mL.

The correction of vitamin D deficiency in patients treated with cholecalciferol resulted in changes in the vitamin D-regulatory proteins in monocytes. The cells from the immune system, including monocytes, express both CYP27B1, the enzyme that catalyzes the conversion of 25(OH)D to $1,25(OH)_2D$, and the nuclear receptor for $1,25(OH)_2D$, VDR [19]. It has been shown in an *in vitro* study that when the monocytes from vitamin D deficient individuals were treated with 25(OH)D, the synthesis of $1,25(OH)_2D$ increased indicating the activity of CYP27B1 in these cells [20]. In the present study we did not measure intracellular $1,25(OH)_2D$, but the increased expression of CYP27B1 in the monocytes of the supplemented patients strongly suggests a local production of $1,25(OH)_2D$ as a result of greater supply of 25(OH)D. Furthermore, the increase in the VDR expression may be another indirect indication of the potential intracellular synthesis of $1,25(OH)_2D$. However, since we did not measure the serum concentration of $1,25(OH)_2D$ we cannot exclude the possibility that the increase in the VDR expression in the monocytes may also be the result of increased circulating levels of $1,25(OH)_2D$ due to its synthesis in the renal cells. Increase in the circulating levels of $1,25(OH)_2D$ have been shown after cholecalciferol administration, even in patients submitted to dialysis with low or no residual renal function [15,16], providing evidence for some renal CYP27B1 activity.

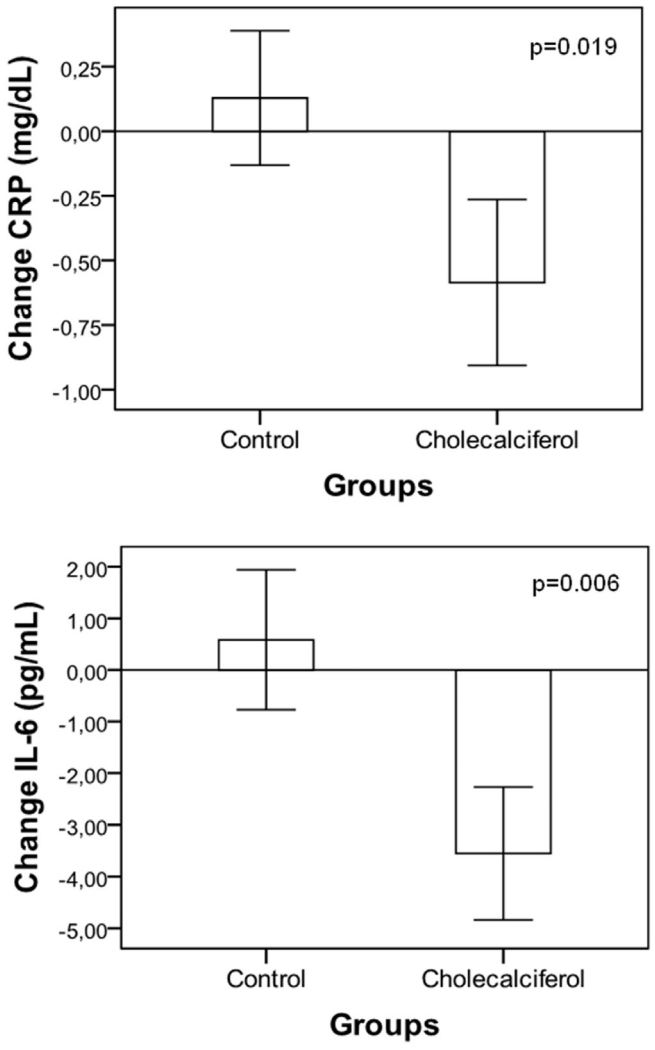

Fig. 3. Change in serum C-reactive protein (CRP) and interleukin-6 (IL-6) according to the treatment group. Data presented as the mean ± standard error.

The lack of change in the CYP24A1 in the current study suggests that the potential rise in the $1,25(OH)_2D$ concentration was not sufficient to upregulate the expression of this catabolic enzyme and consequently to stimulate the negative feedback mechanisms. The mechanisms regulating the CYP24A1 in immune cells are not completely understood. It has been demonstrated that in addition to $1,25(OH)_2D$ itself, FGF-23 and PTH also regulate the activity of CYP24A1 in renal tubule cells [21]. However, the participation of these hormones in the regulation of CYP24A1 in monocytes remains unclear. Viaene L et al. did not demonstrate any effect of FGF-23 on this enzyme in healthy monocytes exposed to FGF-23 at a concentration that mimics uremic levels [22]. In contrast, in a recent report, the monocytes expression of CYP24A1was surprisingly suppressed by FGF-23 [23].

To our knowledge, only one study has investigated the *in vivo* effect of 25(OH)D repletion on vitamin D-responsive pathways in the monocytes of patients on HD. Stubbs et al. examined seven patients with vitamin D insufficiency treated with a weekly dose of 100,000 IU of cholecalciferol for eight weeks. These authors also observed an increase in VDR expression in monocytes in response to vitamin D repletion. However, in contrast with our findings, CYP27B1 expression decreased, while CYP24A1 expression increased [18]. The reasons for the discrepancy between Stubbs et al. results and ours cannot be elucidated.

Systemic inflammation is invariably present in patients with CKD and plays a major role in the associated complications [24].

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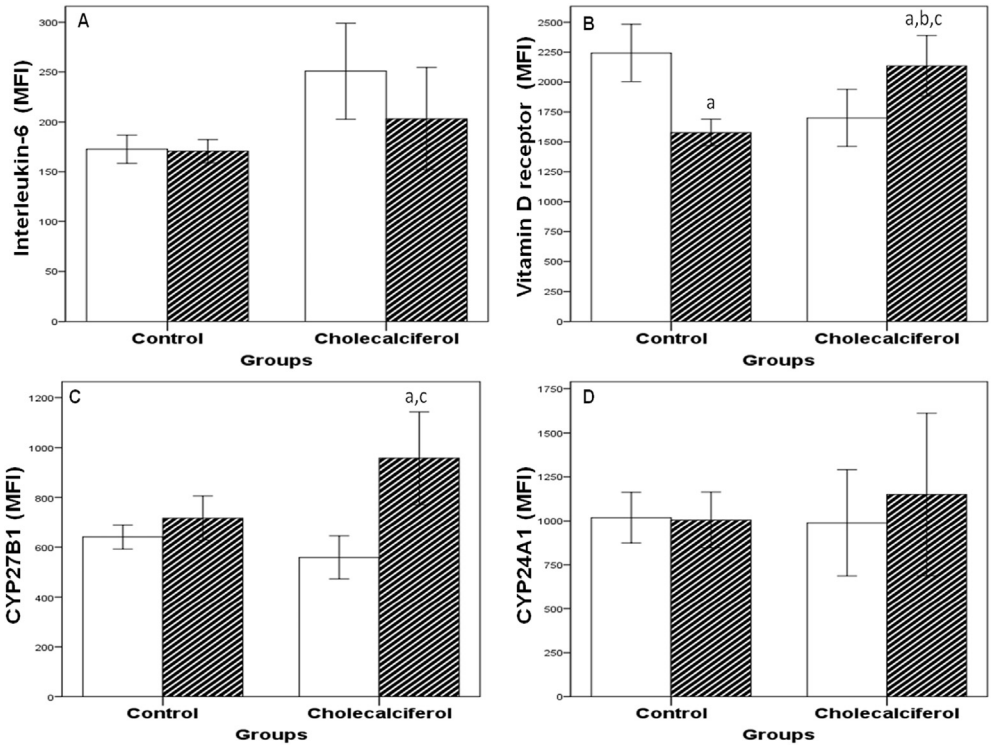

Fig. 4. Monocytes expression of interleukin-6 (A), vitamin D receptor (B), CYP27B1 (C), and CYP24A1 (D) at baseline and after 12 weeks of intervention according to the treatment group. □ Baseline; ▩ 12 weeks. MFI, mean fluorescence intensity. Data presented as the mean ± standard error. [a] $p < 0.05$ versus baseline, [b] $p < 0.05$ cholecalciferol group versus control group, [c] $p < 0.05$ group*time interaction.

There is evidence showing an inverse association between inflammatory markers and 25(OH)D serum concentration [25] and that inflammation is linked to vitamin D deficiency [11,26] in CKD, but the impact of replenishing vitamin D on inflammatory markers has been scarcely investigated in this population of patients [12,18,27]. Although the molecular mechanisms involved in this relationship are not fully understood, there is compelling evidence that the anti-inflammatory action of $1,25(OH)_2D$ occurs through the downregulation and suppression of mediators and gene transcription factors associated with the inflammatory response [28].

In vitro studies have demonstrated in mononuclear cells of healthy subjects that $1,25(OH)_2D$ [29] or a vitamin D analog (paricalcitol) [30] inhibited the production of cytokines. Given that potential anti-inflammatory effect of vitamin D a decrease in IL-6 expression in monocytes was expected in the current study after cholecalciferol treatment. However, we did not demonstrate this local effect, likely reflecting the fact that the cytokines are rapidly secreted after synthesis from the monocytes, a condition that makes it difficult to reliably determine whether the synthesis of IL-6 was decreased. In addition, although the increase in the expression of CYP27B1 was indicative of $1,25(OH)_2D$ production in the monocytes, we cannot guarantee that $1,25(OH)_2D$ was locally synthesized since its expression was not determined. However, a nearly two-fold decrease in the serum concentration of circulating of IL-6 was observed in the patients supplemented with vitamin D probably reflecting the vitamin D inhibition effect in many cell types that produce IL-6. The concomitant decrease in CRP supports the potential beneficial effect of vitamin D replenishment on inflammation. In uncontrolled studies, reductions in IL-6 and CRP levels were also demonstrated after treating with cholecalciferol patients with hypovitaminosis D on hemodialysis [12,18,27]. In contrast, in randomized clinical trials the cholecalciferol supplementation did not provide any favorable effect on markers of inflammation [31,32]. The reasons for these discrepant findings are unclear.

Another beneficial effect of cholecalciferol supplementation observed in the current study was the decrease in the serum concentration of PTH. Although it is not possible to clearly identify the mechanisms involved in such effect, we can speculate that the greater availability of 25(OH)D provided substrate for the conversion of 25(OH)D to$1,25\ (OH)_2D$ through the action of CYP27B1 expressed in the parathyroid cells. The interaction of $1,25(OH)_2D$ locally synthesized or produced by renal cells with the VDR may have suppressed PTH secretion. This result has been observed in some previous trials [17,27] but not in other studies [12,18,31–34]. Although it is difficult to explain such inconsistent findings, methodological differences such as sample size, level of vitamin D deficiency, the dose and duration of supplementation and the concomitant use of other vitamin D analogs and phosphate binders are among the potential factors.

The limitations of the present study include the relatively small cohort of patients and the significant number of loss of follow-up. The strengths include the study design and concomitant analysis of vitamin D replenishment in systemic and intracellular environments.

In conclusion, we demonstrated that the protocol for supplementation employed in the present study was safe and appropriate for the complete restoration of vitamin D status. The increased expression of CYP27B1 and VDR indicated that cholecalciferol supplementation positively regulated vitamin D-regulatory proteins in monocytes. The decrease in circulating IL-6 and CRP highlights a potential pleiotropic role of vitamin D in patients undergoing dialysis. Nevertheless, the maintenance dose of cholecalciferol to ensure the benefits found in the present study needs to be established. Moreover, long-term studies and the investigation of the impact of the vitamin D replenishment on hard outcomes are still needed.

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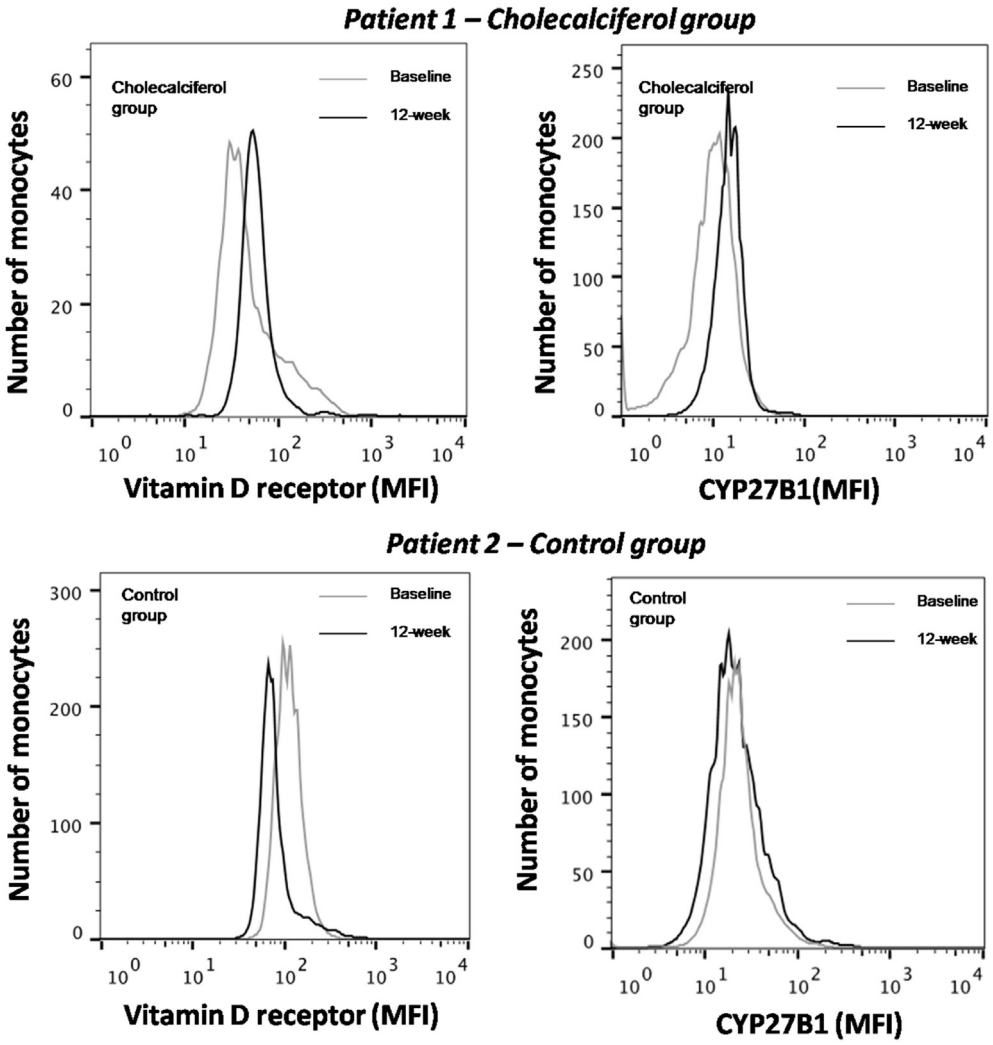

Fig. 5. Expression of vitamin D receptor and CYP27B1 in monocytes by flow cytometry analysis from patient 1 (cholecalciferol group) and patient 2 (control group) at baseline and after 12 weeks of intervention. MFI, mean fluorescence intensity.

Statement of authorship

The authors made the following contributions: M.S.M., M.A.K., M.A.D. and L.C. conceived, designed, and implemented the project; M.A.D. and J.T.C. performed the analytical procedures; M.S.M. collected the data; M.S.M. and D.T.A. performed the statistical analysis; M.S.M. and L.C. interpreted the data, drafted the manuscript and were responsible for the final revisions; and M.A.D. and M.A.K. reviewed the manuscript.

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Conflict of interest

The authors report no conflict of interest.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.clnu.2016.04.014.

References

[1] González EA, Sachdeva A, Oliver DA, Martin KJ. Vitamin D insufficiency and deficiency in chronic kidney disease. A single center observational study. Am J Nephrol 2004;24(5):503–10.
[2] Figuiredo-Dias V, Cuppari L, Garcia-Lopes MG, de Carvalho AB, Draibe SA, Kamimura MA. Risk factors for hypovitaminosis D in nondialyzed chronic kidney disease patients. J Ren Nutr 2012;22(1):4–11.
[3] Andress DL. Vitamin D in chronic kidney disease: a systemic role for selective vitamin D receptor activation. Kidney Int 2006;69(1):33–43.
[4] Adams JS, Rafison B, Witzel S, Reyes RE, Shieh A, Chun R, et al. Regulation of the extrarenal CYP27B1-hydroxylase. J Steroid Biochem Mol Biol 2014;144(Pt A):22–7.

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[5] Cunningham J, Zehnder D. New vitamin D analogs and changing therapeutic paradigms. Kidney Int 2011;79(7):702–7.
[6] Christakos S, DeLuca HF. Minireview: vitamin D: is there a role in extraskeletal health? Endocrinology 2011;152(8):2930–6.
[7] Zhang Y, Leung DY, Richers BN, Liu Y, Remigio LK, Riches DW, et al. Vitamin D inhibits monocyte/macrophage proinflammatory cytokine production by targeting MAPK phosphatase-1. J Immunol 2012;188(5):2127–35.
[8] Drechsler C, Verduijn M, Pilz S, Dekker FW, Krediet RT, Ritz E, , et alNECOSAD Study Group. Vitamin D status and clinical outcomes in incident dialysis patients: results from the NECOSAD study. Nephrol Dial Transplant 2011;26(3):1024–32.
[9] Honda H, Qureshi AR, Heimbürger O, Barany P, Wang K, Pecoits-Filho R, et al. Serum albumin, C-reactive protein, interleukin 6, and fetuin a as predictors of malnutrition, cardiovascular disease, and mortality in patients with ESRD. Am J Kidney Dis 2006;47(1):139–48.
[10] Wolf M, Shah A, Gutierrez O, Ankers E, Monroy M, Tamez H, et al. Vitamin D levels and early mortality among incident hemodialysis patients. Kidney Int 2007;72:1004–13.
[11] Bucharles S, Barberato SH, Stinghen AE, Gruber B, Meister H, Mehl A, et al. Hypovitaminosis D is associated with systemic inflammation and concentric myocardial geometric pattern in hemodialysis patients with low iPTH levels. Nephron Clin Pract 2011;118(4):c384–391.
[12] Bucharles S, Barberato SH, Stinghen AE, Gruber B, Piekala L, Dambiski AC, et al. Impact of cholecalciferol treatment on biomarkers of inflammation and myocardial structure in hemodialysis patients without hyperparathyroidism. J Ren Nutr 2012;22(2):284–91.
[13] Group KDIGOKC-MW. KDIGO clinical practice guideline for the diagnosis, evaluation, prevention, and treatment of Chronic Kidney Disease-Mineral and Bone Disorder (CKD-MBD). Kidney Int Suppl 2009:S1–130.
[14] Tokmak F, Quack I, Schieren G, Sellin L, Rattensperger D, Holland-Letz T, et al. High-dose cholecalciferol to correct vitamin D deficiency in haemodialysis patients. Nephrol Dial Transplant 2008;23(12):4016–20.
[15] Jean G, Souberbielle JC, Chazot C. Monthly cholecalciferol administration in haemodialysis patients: a simple and efficient strategy for vitamin D supplementation. Nephrol Dial Transplant 2009;24(12):3799–805.
[16] Wasse H, Huang R, Long Q, Singapuri S, Raggi P, Tangpricha V. Efficacy and safety of a short course of very-high-dose cholecalciferol in hemodialysis. Am J Clin Nutr 2012;95(2):522–8.
[17] Garcia-Lopes MG, Pillar R, Kamimura MA, Rocha LA, Canziani ME, Carvalho AB, et al. Cholecalciferol supplementation in chronic kidney disease: restoration of vitamin D status and impact on parathyroid hormone. Ann Nutr Metab 2012;61(1):74–82.
[18] Stubbs JR, Idiculla A, Slusser J, Menard R, Quarles LD. Cholecalciferol supplementation alters calcitriol-responsive monocyte proteins and decreases inflammatory cytokines in ESRD. J Am Soc Nephrol 2010;21(2):353–61.
[19] Liu PT, Stenger S, Li H, Wenzel L, Tan BH, Krutzik SR, et al. Toll-like receptor triggering of a vitamin D-mediated human antimicrobial response. Science 2006;311(5768):1770–3.
[20] Adams JS, Ren S, Liu PT, Chun RF, Lagishetty V, Gombart AF, et al. Vitamin d-directed rheostatic regulation of monocyte antibacterial responses. J Immunol 2009;182(7):4289–95.
[21] Bosworth C, Boer IH. Impaired vitamin D metabolism in CKD. Semin Nephrol 2013;33(2):158–68.
[22] Viaene L, Evenepoel P, Meijers B, Vanderschueren D, Overbergh L, Mathieu C. Uremia suppresses immune signal-induced CYP27B1 expression in human monocytes. Am J Nephrol 2012;36(6):497–508.
[23] Bacchetta J, Sea JL, Chun RF, Lisse TS, Wesseling-Perry K, Gales B, et al. Fibroblast growth factor 23 inhibits extrarenal synthesis of 1,25-dihydroxyvitamin D in human monocytes. J Bone Miner Res 2013;28(1):46–55.
[24] Stenvinkel P. Inflammatory and atherosclerotic interactions in the depleted uremic patient. Blood Purif 2001;19(1):53–61.
[25] Eleftheriadis T, Antonialdi G, Liakopoulos V, Stefanidis I, Galaktidou G. Iran J Kidney Dis 2012;6:129–35.
[26] Isakova T, Gutiérrez OM, Patel NM, Andress DL, Wolf M, Levin A. Vitamin D deficiency, inflammation, and albuminuria in chronic kidney disease: complex interactions. J Ren Nutr 2011;21(4):295–302.
[27] Matias PJ, Jorge C, Ferreira C, Borges M, Aires I, Amaral T, et al. Cholecalciferol supplementation in hemodialysis patients: effects on mineral metabolism, inflammation, and cardiac dimension parameters. Clin J Am Soc Nephrol 2010;5(5):905–11.
[28] Chen Y, Liu W, Sun T, Huang Y, Wang Y, Deb DK, et al. 1,25-Dihydroxyvitamin D promotes negative feedback regulation of TLR signaling via targeting microRNA-155-SOCS1 in macrophages. J Immunol 2013;190:3687–95.
[29] Muller K, Haahr PM, Diamant M, Rieneck K, Kharazmi A, Bendtzen K. 1,25-Dihydroxyvitamin D3 inhibits cytokine production by human blood monocytes at the post-transcriptional level. Cytokine 1992;4:506–12.
[30] Eleftheriadis T, Antonialdi G, Liakopoulos V, Kartsios C, Stefanidis I, Galaktidou G. Int Urol Nephrol 2010;42:181–5.
[31] Marckmann P, Agerskov H, Thineshkumar S, Bladbjerg EM, Sidelmann JJ, Jespersen J, et al. Randomized controlled trial of cholecalciferol supplementation in chronic kidney disease patients with hypovitaminosis D. Nephrol Dial Transplant 2012;27(9):3523–31.
[32] Seibert E, Heine GH, Ulrich C, Seiler S, Köhler H, Girndt M. Influence of cholecalciferol supplementation in hemodialysis patients on monocyte subsets: a randomized, double-blind, placebo-controlled clinical trial. Nephron Clin Pract 2013;123(3–4):209–19.
[33] Bhan I, Dobens D, Tamez H, Deferio JJ, Li YC, Warren HS, et al. Nutritional vitamin D supplementation in dialysis: a randomized trial. Clin J Am Soc Nephrol 2015;10(4):611–9.
[34] Armas LAG, Andukuri R, Barger-Lux J, Heaney RP, Lund R. 25-Hydroxyvitamin D response to cholecalciferol supplementation in hemodialysis. Clin J Am Soc Nephrol 2012;7(9):1428–34.

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