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ORIGINAL ARTICLE

# Vitamin D supplementation ameliorates arthritis but does not alleviates renal injury in pristane-induced lupus model

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## ABSTRACT

Systemic lupus erythematosus (SLE) is a multifactorial and autoimmune inflammatory disease with pleomorphic clinical manifestations involving different organs and tissues. The study of different murine models has provided a better understanding of these autoimmune phenomena. Pristane-induced lupus represents a suitable model to study factors that could influence the induction and/or progression of SLE, including genetic factors. The objective of the present study was to evaluate the development and evolution of SLE after vitamin D supplementation in PIL model. Here, we evaluated the effects of vitamin D supplementation in model of pristane-induced SLE in female BALB/c mice. The animals were randomly divided into three groups: control group (CO), pristane-induced lupus group (PIL) and pristane-induced lupus group plus vitamin D (VD). Lupus was induced in PIL and VD groups using pristane. PIL group showed arthritis and kidney injury, characterized by increased proteinuria, glomerular mesangial expansion and inflammation. Moreover, PIL model showed increased levels of IL-6, TNF-α and IFN-γ in serum. We observed that treatment with vitamin D improved arthritis through reduced of incidence and arthritis clinical score and edema, but does not influenced renal injury. Treatment with vitamin D was not able to reduce proteinuria levels, decrease mesangial hypercellularity or IgG and IgM deposition in the kidney. Vitamin D supplementation did not alter IL-6, TNF-α, IL-2 and IL-4, but reduce IFN-γ. These results support that the role of vitamin D may be different depending on acting site, what could explain different responses according clinical phenotype. Therefore, further investigations of vitamin D are needed to explore the supplement dosage, timing, and the molecular basis in SLE.

## Introduction

Systemic Lupus Erythematosus (SLE) is a chronic, multisystem, inflammatory and autoimmune disease, characterized by the production of autoantibodies and tissue damage. An incidence of 1–22 cases is estimated for every 100,000 people per year, affecting more women of childbearing age [1]. Etiology of SLE is multifactorial and remains poorly understood. However, it is well known that hormonal, environmental, genetic and immunological factors contribute to the disease onset [2]. Several genes have been linked to the onset of SLE, including the *VDR* (vitamin D receptor) that synthesizes the vitamin D receptor [3].

Experimental models have shown to be advantageous when reproducing clinical features of the disease in humans.

Such models contribute significantly to the understanding of the SLE pathogenesis. Moreover, pristane-induced lupus (PIL) model is useful for examining the role of environmental triggers involved in the disease [4]. PIL model develop clinical manifestations of SLE, including arthritis, immune complex-mediated glomerulonephritis and vasculitis, as well as increases in autoantibodies, including anti-dsDNA and anti-Sm and overproduction of interferon (IFN) I, which is an important feature of SLE pathogenesis. The PIL model replicates many phenotypic and functional abnormalities of human SLE and proved to be very useful in identifying putative pathogenic mechanisms and environmental triggers of this disease. Pristane is an isoprenoid alkane found at high concentrations in mineral oil and is widely used as a sensitizer for increasing the yield of mice ascites.

Phagocytosis of pristane by macrophages induces the production of cytokines and the formation of lipogranuloma on mesenteric and other peritoneal surfaces [5].

Animal model studies have demonstrated a relationship between vitamin D supplementation/deficiency and SLE symptoms. However, there are conflicts in the literature about this association. Female MRL/lpr mice that received 0.1–0.15 μg vitamin D3 intraperitoneally showed a reduced degree of serum single stranded-DNA antibodies and proteinuria [6]. On the contrary, female NZB/W mice injected intraperitoneally 3 or 10 μg vitamin D3 demonstrated renal histopathology worsening [7]. In this context, we conducted a study to evaluate the development and evolution of SLE after vitamin D supplementation in the PIL model.

## Materials and methods

### Animals and ethics

Female BALB/c mice that were 8 weeks-old were obtained from the Universidade Federal de Pelotas (Pelotas, RS, Brazil). All mice were housed in the Animal Experimentation Unit at Hospital de Clínicas de Porto Alegre (HCPA) and maintained in standard 12 h light/dark cycle, with controlled temperature ($22 \pm 2\,^{\circ}C$) and given water and food *ad libitum*. Mice were adapted to the new environmental conditions for 2 weeks. The present study was approved by the Animal Ethics Committee of HCPA (number 17-0011) and was conducted in accordance with National Institute of Health guidelines.

### Sample size and experimental design

The sample size calculation was made considering 80% sample power, alpha error 5% and to detect a 30% reduction in the mononuclear cell infiltrate in the kidney. Since there is a chance of error in the intraperitoneal (i.p.) in the induction of the model and early death of the animals due to ascites were added 2 animals per pristane induction group. Twenty-eight female BALB/c mice were randomly divided into the following three groups: [1] Control group (CO; $n = 8$); [2] Pristane-induced lupus group (PIL; $n = 10$); and [3] Pristane-induced lupus treated for six months with

vitamin D group (VD; $n = 10$). CO group received a single i.p. injection with 500 μl 0,9% saline solution and the PIL and VD groups received a single i.p. injection with 500 μl pristane oil (2,6,10,14-tetramethylpentadecane; Sigma-Aldrich, MO, USA), according to Satoh et al [8]. During the procedures, mice were anesthetized with isoflurane 10% (Abbott Laboratório do Brasil Ltda., Brazil) and 90% of oxygen. Throughout the experiment, we had some losses of animals: 1 animal from the control group (accident), 1 animal from the PIL group (accident) and 3 animals from the vitamin d group (1 animal died in the induction of the model and 2 animals died from intestinal intussusception).

Vitamin D supplementation was administrated from the day of disease induction to the euthanasia. VD group mice received a subcutaneous injection of Calcijex (Abbott Labs, Chicago, Ill) containing 2 μg/kg/day of calcitriol (1,25-[OH]2-D3) in PBS-Tween 20 buffer every two days [9]. CO and PIL groups received subcutaneous injections with PBS-Tween 20 buffer on the same days. Six months after pristane induction, animals were killed and specimens including blood, spleen, liver, tibiotarsal joint and kidney were collected. The illustration of the experimental design is shown in Figure 1.

### Body and organ weight measurement

The animals were observed weekly for body weight measurements. At the end of the experimental period, mice were killed by cervical dislocation, and weight of organs such as spleen, liver, tibiotarsal joint and kidney was measured.

### Evaluation of articular nociception

Nociception was evaluated before induction of the experimental model and on days 60, 120 and 180 after induction of the model. Nociception was assessed as Oliveira et al [10]. The nociceptive mechanical threshold from the hinds paws were measured by the electronic Von Frey method (electronic Von Frey, Insight Equipamentos Ltda, Ribeirão Preto, SP, Brazil). Mice were placed in acrylic cages ($12 \times 20 \times 17$ cm) with wire grid floors in a quiet room 15–30 min before testing for environmental adaptation. The test consisted of evoking a hind paw flexion reflex with a
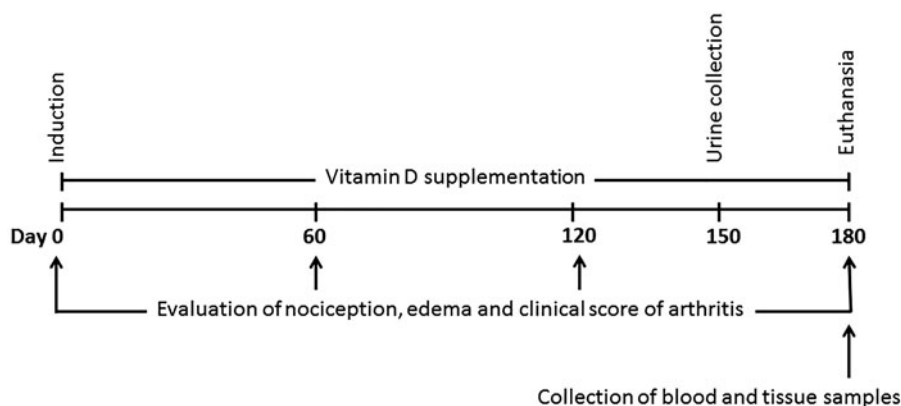


Figure 1. Illustration of experimental design.

handheld force transducer adapted with a tip. The investigator was trained to apply the tip in the plantar region with a gradual increase in pressure. The stimulus was automatically discontinued, and its intensity was recorded, in grams (g), when the paw was withdrawn.

### Measurement of articular edema

Edema was evaluated before induction of the experimental model and on days 60, 120 and 180 after induction of the model. Hind paw edema volume was measured using a plethysmometer (Insight Ltda., Ribeirão Preto, Brazil). Briefly, this equipment is a small cylinder filled with a buffer connected to a device capable to measure the total fluid volume, the hind paw of the animal was immersed into the cylinder and the total volume added was then measured. The difference between the final volume minus the initial volume results to the paw total volume.

### Determination of arthritis severity score

Mice were examined for the onset and severity of arthritis every 2 months after pristane injection. For scoring of arthritis severity, a previously published scoring system was used as follows: score scale of 0–3, where 0 = normal paw, 1 = slight swelling or erythema of the wrist/ankle joint or footpad, 2 = moderate swelling and erythema of the wrist/ankle joint or footpad, and 3 = severe swelling and erythema of the paw [11]. The scores for individual limbs were summed to obtain a total clinical arthritis severity score of 12 per animal. The incidence of arthritis was determined as the percentage of mice that had developed redness or swelling in at least 1 paw.

### Histological analysis of joint morphology

Hind paws were collected to confirm the development of arthritis by histological analysis with hematoxylin and eosin (HE) staining. The hind paws of the BALB/c animals were dissected and immersed in 10% buffered formalin for fixation for up to 3 days. Then, the joints were decalcified in 10% nitric acid for 24 h. These tissues were dehydrated and embedded in paraffin blocks. Slices 6 µm thick were arranged on microscope slides. Histopathological scoring was performed using a semi-quantitative score to evaluate individual joints and assess arthritis severity. For synovial inflammation, five high-power magnification fields were scored for the percentage of infiltrating mononuclear cells as follows: 0, absent; 1, mild (1–10%); 2, moderate (11–50%); 3, severe (51–100%); for synovial hyperplasia: 0, absent; 1, mild (5–10 layers); 2, moderate (11–50 layers); 3, severe (>20 layers); for extension of pannus formation based on the reader's impression: 0, absent; 1, mild; 2, moderate; 3, severe; for cartilage erosion, that is, the percentage of the cartilage surface that was eroded: 0, absent; 1, mild (1–10%); 2, moderate (11–50%); 3, severe (51–100%); and for bone erosion: 0, none; 1, minor erosion(s) observed only at high-power magnification fields; 2, moderate erosion(s) observed at low magnification; 3, severe transcortical erosion(s) [12].

### Quantification of urinary protein

On day 150 after pristane induction, the animals were placed in individual metabolic cages for urine collection for a period of 12 h. Urine samples were analyzed using urine test strips Sensi 10 Sensitive (Cral, Brazil) to measure total protein level. The results were expressed as mg/dL.

### Kidney histopathological evaluation

After killing, one kidney from each animal was removed and fixed for 24 h in 10% buffered formalin prior to paraffin embedding and sectioned at 3 µm thickness in the transversal plane containing the renal long axis. Slides were stained using HE, according to standard procedures.

HE stained sections were observed with a light microscopy (Olympus, Germany) at a final magnification of 400x. The glomerular cellularity was quantified by counting the total cell nuclei per glomerulus on HE-stained slides. At least 30 glomeruli/slides were assessed, and the results were expressed as number of nuclei per glomerulus. All measurements and analyses were performed in a blind fashion and using an image analyzer (Image Pro Premier 9.1, Media Cybernetics Inc., Rockville, USA).

### Detection of IgG and IgM in renal tissue by immunofluorescence

Immune complex deposition in kidneys was examined by direct immunofluorescence. The other kidney from each animal was removed and immediately frozen in dry ice and stored in -80 °C freezer. Kidney was embedded in OCT (optimal cutting temperature compound) and frozen in a cryostat at −20 °C. Kidney was cut into sections of 5 µm thickness to detect IgG and IgM by immunofluorescence analyses. Frozen sections were stained for IgG and IgM with goat polyclonal antibody anti-mouse IgG-FITC (1:100 dilution; Abcam – ab97022, USA) or goat polyclonal anti-mouse IgM-Alexa Fluor 647 (1:200 dilution; Abcam – ab150123, USA) antibody. All antibodies were incubated with PBS-tween 20 containing 2% BSA in a humid incubator for 24 hours at 4 °C. Sections were washed three times for 5 min using PBS-tween 20. Fluorescence intensity was scanned and quantified by ImageJ software.

### Quantification of serum cytokines

Blood sample was collected by cardiac puncture at the end of the experiment (180 days), prior to euthanasia of the animals. Blood was obtained in vacutainer tubes (BD Biosciences, San Diego, CA); serum was prepared within 45 minutes of collection and stored at −80 °C for later analysis. Cytokines levels Interleukin (IL) 2, IL-4, IL-6, IFN-γ and tumor necrosis factor-alpha (TNF-α) were analyzed using a ProcartaPlex High Sensitivity mouse multiplex assay,

according to the manufacturer's instructions. Events were counted on a Luminex® 200™ system (Luminex, Austin, TX, USA) and cytokine concentrations in the samples were determined using a standard curve for each cytokine. All results were obtained in duplicate.

### Statistical analysis

All group results are expressed as mean ± standard deviation (SD). Clinical scores were analyzed using the non-parametric Mann-Whitney U test and histological scores were assessed using the one-way analysis of variance (ANOVA) test. Other data were compared using the Student's t-test or one-way ANOVA. $p$ values <.05 were considered statistically significant. Statistical analysis was performed using SPSS version 17.0 (SPSS, Chicago, IL, USA).

## Results

Body weight was measured and recorded weekly. All groups showed a body weight gain over the experimental period in relation to time 0 ($p < .0001$). PIL group presented the greatest weight gain in relation to the other groups after 30 days. VD group gained weight in a similar manner to the CO group until 90 days ($3.67 \pm 1.26$g vs. $3.64 \pm 1.28$g). After this time the VD group increased significantly the body weight, equaling the PIL group at the end of the experimental period (Figure 2). Furthermore, vitamin D supplementation did not reduce the size and the number of lipogranuloma in the peritoneal cavity of mice in comparison with the PIL group (data not show).

Spleen, liver and kidney weight are summarized in Table 1. PIL animals present a higher spleen ($0.177 \pm 0.036$g vs. $0.114 \pm 0.011$g; $p < .01$) and liver ($1.306 \pm 0.110$g vs. $1.157 \pm 0.132$g; $p < .05$) weight in relation to the CO group. However, there was no difference in relation to the weight of the kidney among the groups. Vitamin D supplementation was not able to affect the spleen and liver weight.

A single injection of pristane oil into the peritoneal cavity induces an erosive arthritis in BALB/c mice [13]. The incidence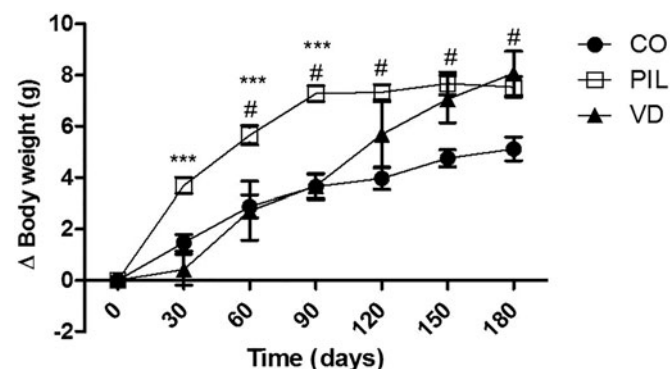 of arthritis has increased over time. We observed an incidence of 85% in the PIL group and 43% in the VD group at the end of the experiment (Figure 3(A)). After 180 days of pristane oil injection, the mean clinical arthritis severity score was significantly reduced in VD mice compared to PIL mice (Figure 3(B)). These results indicate that vitamin D supplementation protects against arthritis development in mice, and reduces the severity of arthritis in mice. However, there was no statistically significant difference in relation to joint pain (Figure 3(C)). Interestingly, PIL group had significantly higher hind paw edema volumes than CO and VD groups, observed after 180 days of immunization (Figure 3(D)). Synovial hyperplasia, severe leukocyte infiltration, pannus formation, and cartilage erosions were present in the joints of PIL, whereas the joints of VD mice were largely spared, showing marked reductions in the extent of synovitis and severity of joint erosion (Figure 3(E)). The histopathologic arthritis severity scores were significantly lower in VD mice than in PIL mice (Table 2).

After 150 days of observation, total protein in the urine of PIL mice was significantly increased compared with the CO mice ($p < .01$). However, compared to PIL mice, VD mice showed no difference in proteinuria in the same experimental period (Figure 4(A)). Therefore, vitamin D appears to be unable to prevent the development of proteinuria in this PIL model.

In HE staining, pathologic kidneys features were detected through the assessment of glomerular hypercellularity. PIL mice showed significant increase of glomerular cellularity after 6 months induction compared to CO mice. The treatment of vitamin D was not unable to affect significantly the glomerular hypercellularity. These observations are summarized in Figure 4(B).

Immune complex deposits are a primary cause of lupus nephritis. In our study, the complex deposits were detected by an immunofluorescence assay. Kidneys from the PIL mice showed the presence of immune complex deposits in the glomeruli (Figure 5). However, vitamin D supplementation did not show any effect on immune complex deposition. Further quantitative analysis using fluorescence intensity supported the results (Figure 5(B,C)).

We assessed serum cytokine levels to evaluate the role of vitamin D supplementation in immunomodulation (Figure 6). Levels of IL-6, TNF-α and IFN-γ were elevated in PIL group ($p < .05$) in response to pristane-induced inflammation when compared to the CO group. Interestingly, vitamin D supplementation was able to dramatically decrease serum IFN-γ levels. There is no significant difference in the levels of IL-2 and IL-4 in all groups.



**Figure 2.** Delta body weight of control (CO), pristane-induced lupus (PIL) and pristane-induced lupus + vitamin D (VD) animals. ***PIL vs. VD: $p < .001$ and #CO vs. PIL: $p < .05$. Data are shown as the mean ± SD.

**Table 1.** Organs weight of control (CO), pristane-induced lupus (PIL) and pristane-induced lupus + vitamin D (VD) mices.

|  | CO group ($n = 7$) | PIL group ($n = 9$) | VD group ($n = 7$) |
|---|---|---|---|
| Spleen (g) | $0.114 \pm 0.011$ | $0.177 \pm 0.036$** | $0.163 \pm 0.024$*** |
| Liver (g) | $1.157 \pm 0.132$ | $1.306 \pm 0.110$* | $1.428 \pm 0.394$ |
| Kidney (g) | $0.163 \pm 0.016$ | $0.169 \pm 0.013$ | $0.176 \pm 0.019$ |

Values are expressed in mean ± SD. *$p < .05$ vs. CO group; **$p < .01$ vs. CO group; ***$p < .001$ vs. CO group.
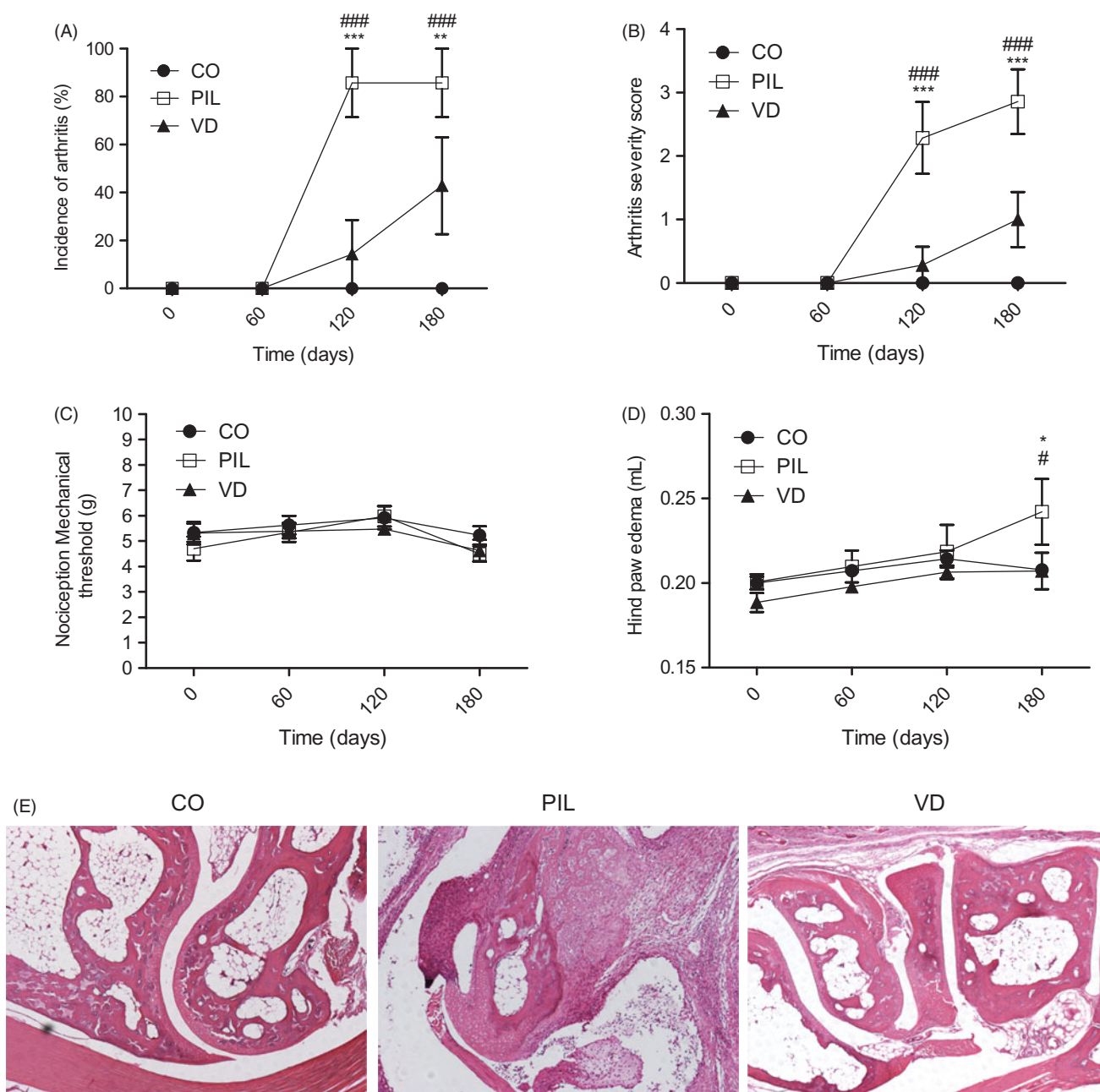
**Figure 3.** Clinical analysis evaluated at 0, 60, 120 and 180 days after induction and joint histology in HE. (A) Incidence of arthritis, calculated as the percentage of mice that developed redness or swelling in at least 1 paw among all mice in each group. (B) Mean clinical arthritis severity score. The arthritis severity score (scale of 0–3) evaluated the severity of erythema/swelling in the wrist or ankle. (C) Nociceptive mechanical threshold from the hind paw measured by the electronic Von Frey method, recorded in grams (g). (D) Hind paw edema volume measured in plethysmometer, recorded in milliliters (mL). (E) Representative histopathology of ankle joint in control (CO), pristane-induced lupus (PIL) and pristane-induced lupus treated for six months with vitamin D (VD) groups at 180 day after immunization (x400). Results are the mean ± SD. #CO vs. PIL: $p < .05$. ###CO vs. PIL: $p < .001$. *PIL vs. VD: $p < .05$. **PIL vs. VD: $p < .01$. ***PIL vs. VD: $p < .001$, by two-away analysis of variance (ANOVA) followed by Tukey's post hoc test.

**Table 2.** Histopathology parameters of ankle joints from mice in the control (CO), pristane-induced lupus (PIL) and pristane-induced lupus with vitamin D (VD) groups.

|  | CO group (n = 7) | PIL group (n = 9) | VD group (n = 7) |
|---|---|---|---|
| Inflammatory infiltration | 0 (0,0) | 3 (2,3)* | 2 (0,3) |
| Synovial hyperplasia | 0 (0,0) | 2 (2,3)* | 0 (0,1)# |
| Pannus extension | 0 (0,0) | 3 (2,3)* | 1.5 (0,3) |
| Cartilage erosion | 0 (0,0) | 3 (2,3)* | 1 (0,2)# |
| Bone erosion | 0 (0,0) | 2 (2,2)* | 0 (0,1)# |

Values are the median (25th–75th percentile). Statistical analysis between groups was performed using Chi-square analysis. *$p < .05$ vs. CO group; #$p < .05$ vs. PIL group.

## Discussion

SLE therapy includes corticosteroids and immunosuppressants, with varying success and usually several side effects [14]. For this reason, new therapeutic strategies continue to be investigated and dietary supplements and nutritional therapy may be considered as promising therapeutic strategies for SLE patients. The PIL represents a suitable model for studying the effects of the factors other than the genetic factors which could influence the induction and/or on the progression of SLE [15]. To assess the clinical symptoms and SLE disease development we demonstrated increase of
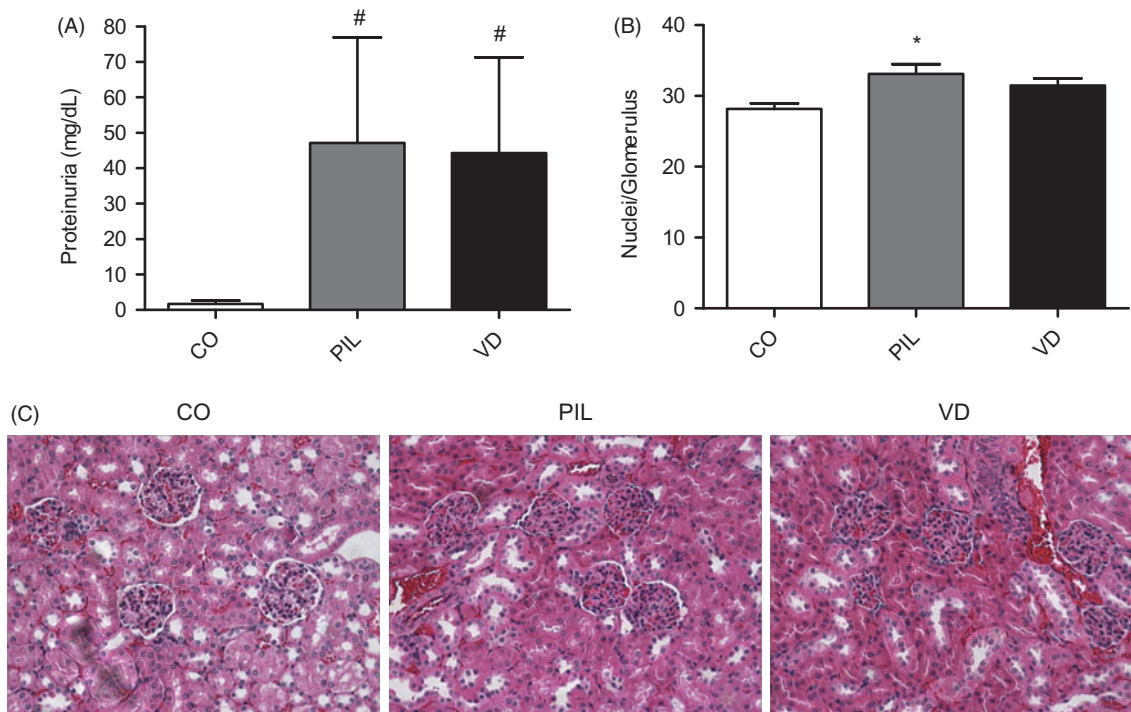
**Figure 4.** The graph shows proteinuria levels at 150 days after pristane induction (A) and summarizes the glomerular cellularity evaluation of control (CO), pristane-induced lupus (PIL) and pristane-induced lupus with vitamin D (VD) groups (B,C; x400). Results are the mean ± SD. *vs. CO: $p < .05$; #vs. CO: $p < .01$, by one-away analysis of variance (ANOVA) followed by Tukey's post hoc test.

IL-6, TNF-α and IFN-γ serum levels, arthritis, IgG and IgM deposition in kidney and proteinuria in PIL group. Vitamin D supplementation showed a significant improve in arthritis but did not influence on renal disease in this model.

Many reports suggested that cytokines are considered to have a critical role in SLE progression. Pro-inflammatory cytokines were related to immune dysregulation and tissue damage. IL-6, TNF-α and IFN-γ participates the differentiation of T helper (Th) 17 cells. In the present study, the levels of IL-6, TNF-α and IFN-γ in the serum were significantly increased in PIL group, corroborating with literature [16].

SLE model universally display high IFN levels, thus providing excellent animal models to investigate this matter [17]. In SLE, vitamin D deficiency was associated with increased expression of interferon-stimulated genes (ISGs). IFN-γ is one of the major mediators of several autoimmune disorders and administration of this cytokine accelerates disease progression. Our results show high IFN-γ serum levels in PIL model, while the vitamin D supplementation influenced the production of IFN-γ in 180 days. In fact the dominance of Th1 or Th2 cytokines in SLE has been controversial until now [16]. However, most studies have demonstrated that the Th1 response may play a major role in the onset and progression of SLE. Interestingly, vitamin D and its receptor were found in significant concentrations in the T lymphocyte and macrophage populations, suggesting that the vitamin D plays an important role involved in immunomodulation on Th1 or Th2 phenotype [18].

IL-6 has a range of biological activities on various target cells that plays an important role in immune regulation and inflammation. Data from several studies suggest that IL-6

plays a critical role in the B cell hyperactivity and immunopathology of murine models of SLE, induction of IgG production, and may have a direct role in mediating tissue damage [19]. Consistent with previous report, we have observed a marked increase in levels of IL-6 in the PIL model [20]. These results were similar to the findings of previous studies which have shown the role of increased levels of IL-6 in the pathogenesis of SLE in MRL/lpr mice [21–23].

A report demonstrated that IL-6 and TNF-α can influence the permeability of the glomerular basement membrane and alter glomerular filtration [24]. In the current study we showed that vitamin D supplementation neither alleviated the renal injury of SLE nor reduced IL-6 nor TNF-α. The vitamin D supplementation did not inhibit IL-6 secretion, and there by not suppress B-cell hyperactivity and autoantibodies production. Nevertheless, reduction of IL-6 have already been previously reported [6,25].

Proinflammatory cytokines, play a remarkable role in lipogranuloma and autoimmunity initiation in PIL model [26]. Lipogranulomas are regarded as a form of ectopic lymphoid tissue, i.e., tertiary lymphoid tissue [5]. Furthermore, pristane is found to activate the TLR7-MyD88 pathway on monocytes, thus upregulating IFN-γ production [27]. Our results showed that vitamin D supplementation not suppressed lipogranuloma development after 180 days. These data are consistent with non-decrease in serum IL-6 levels after vitamin D treatment.

Few SLE models are characterized by arthritis, and PIL represents an inducible SLE-associated arthritis in previously healthy mice. In the model studied, joint evaluation is usually not reported by the authors. The reason may be that
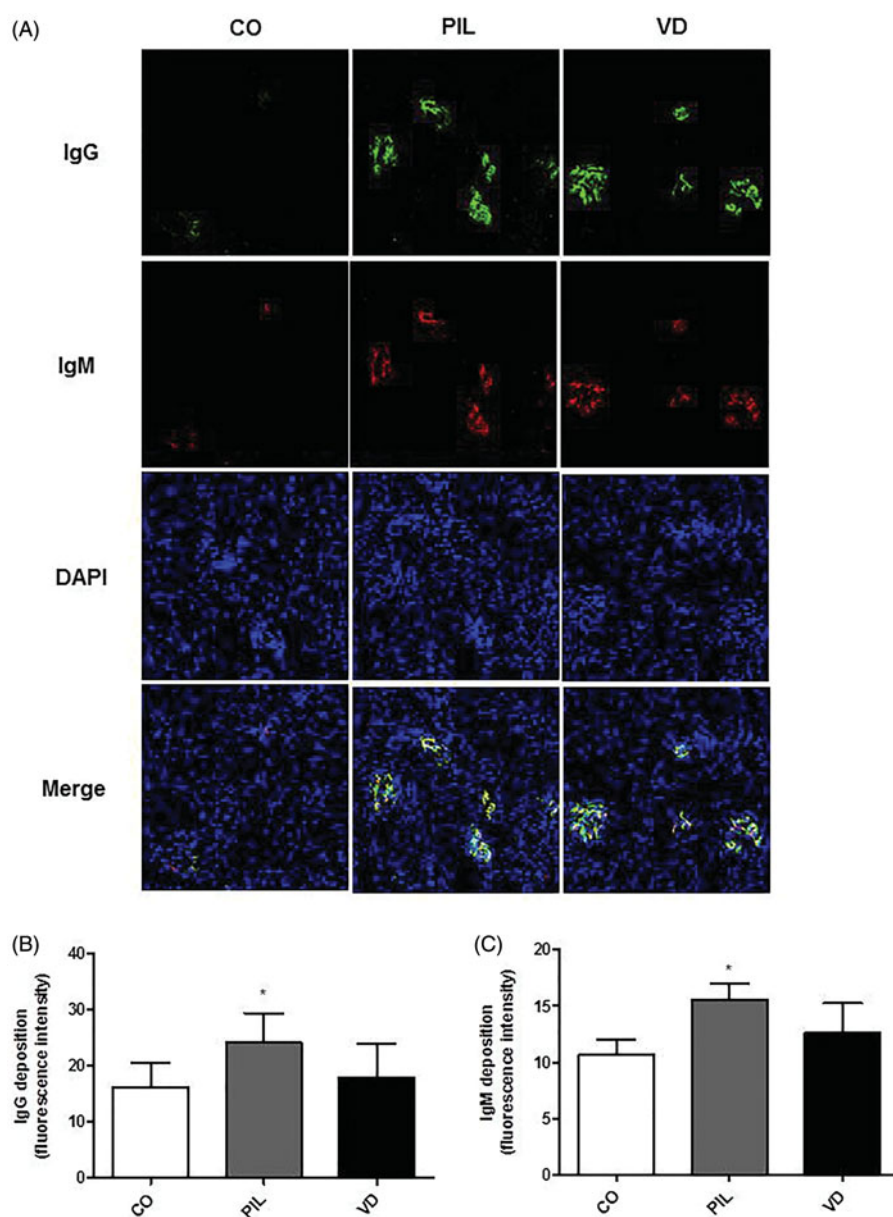
**Figure 5.** Deposition of total IgG and IgM in kidneys of BALB/c mice in each group was observed under fluorescence microscopy (x400) (A). Fluorescence intensity of total IgG (B) and IgM (C) in kidneys of BALB/c mice in each group was analyzed and calculated by ImageJ. Data represent mean ± SD. *vs. CO=$p < .05$.

arthritis is erosive, which makes it more alike to rheumatoid arthritis (RA). As a difference from RA, the inflammatory synovial infiltrate in PIL model is dominated by granulocytes; this contrasts with arthritis in CIA mice as well as in human RA, in which macrophages are the cells that prevail [28,29]. Our findings demonstrate that vitamin D treatment significantly attenuates the development of arthritis in PIL model. This was evidenced by the observed reduction in clinical scores and synovial inflammation in the VD animals. The reduction of synovial hyperplasia into the joint cavity, damage to the articular cartilage and bone erosion were observed. The ability of 1,25(OH)2D3 to suppress inflammation has been linked to its capacity to regulate DC and T cell functions [30].

Despite the results beneficial on arthritis developed by animal model, we did not observe improvement in nociceptive mechanical threshold. The analysis of nociceptive by electronic Von Frey method may have been a bias of this result. The values obtained by this technique are from the middle area of the plantar region of the hind paws and a more exacerbated edema was observed in the large joints of the animals, wrists and ankles, both PIL group and VD group.

One of the most common clinical features of SLE is glomerulonephritis. Lupus nephritis is thought to involve glomerular inflammation induced by immune complexes and complement deposition [16]. IgG autoantibodies induced by pristane injection can promote kidney injury [27]. Autoantibodies are directly targeted to a variety of nuclear components [5,8]. Our results demonstrate an accumulation of IgG and IgM deposits in the glomeruli in PIL model. In addition, renal injury involves the release of pathogenic mediators which worsen glomerular mesangial damage, such as inflammatory cytokines [20]. The
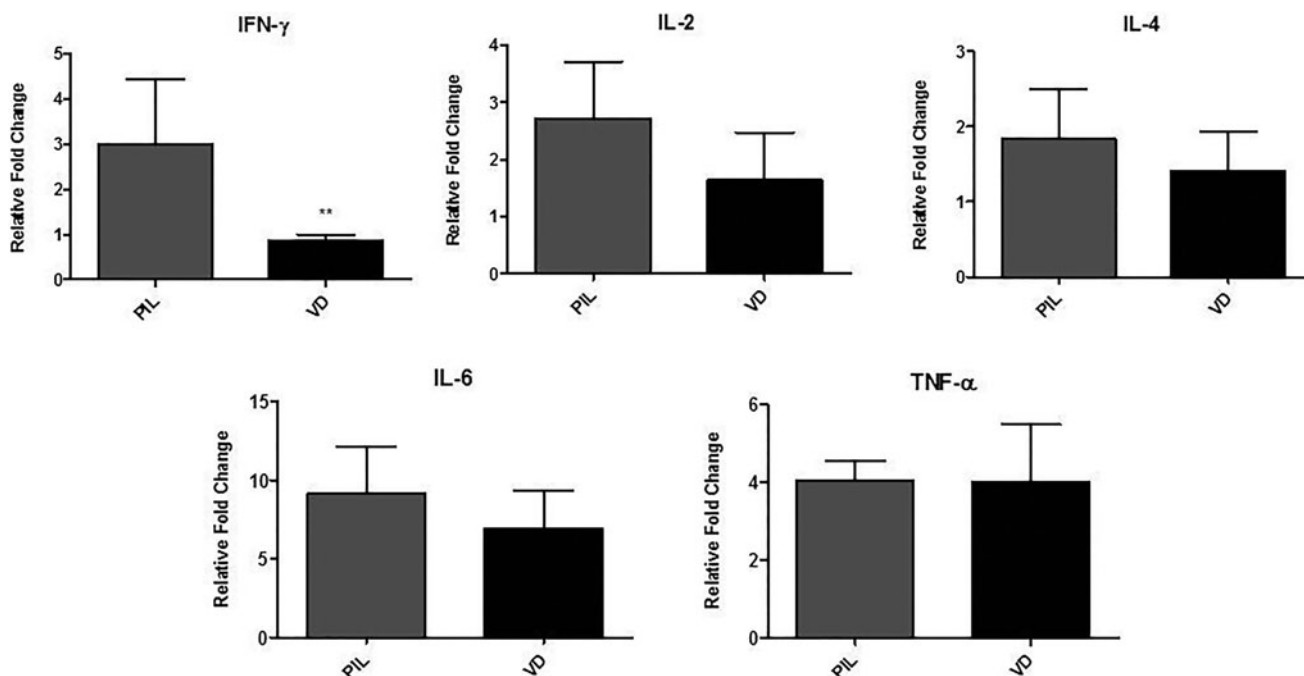
**Figure 6.** Effect of vitamin D supplementation on cytokines. Cytokine concentrations were determined using the Luminex multiplex assay and are presented as fold change relative to CO group. Control group mean values for each cytokine were set at value of 1. Values are expressed as mean ± SD; Statistical analysis between groups was performed using One-away ANOVA. **$p < .01$ vs. PIL group; (PIL) pristane-induced lupus and (VD) pristane-induced lupus with vitamin D.

inflammatory infiltration in the glomeruli, a prominent feature in mice glomerulonephritis, induces the secretion of inflammatory cytokines and chemokines that contribute to the apoptosis of both glomerular mesangial cells. The PIL model display an imbalance of cytokine network with increasing level of IL-6 that, in turn, induces B cells to secret more antibodies and promote the development and progression of SLE nephritis [31–34].

We have shown no decreased amount of proteinuria, in IgG and IgM deposition or suppression the proliferation of glomerular mesangial cells after vitamin D supplementation. In contrast, Lemire et al., showed a remarkable reduction of proteinuria after administration of 1,25-dihydroxyvitamin D3 in MRL/lpr mice [6]. In addition, Deluca et al. observed the preventive effects of 1,25-dihydroxyvitamin D3 on the proteinuria and pathologic renal disease in the same model [35].

There were some limitations in this study. First, in this study we chose vitamin D supplementation through subcutaneous injections, whereas generally vitamin D treatment occurs through the i.p. via. We chose to treat the animals subcutaneously via, because the experimental model is induced by an i.p. injection of 500ul pristane. Different routes of administration may have different absorption from treatments. Second, significant renal tissue lesions are the last to be manifested in this model and our study included an experimental period of 180 days after induction. A longer experimentation period (240 to 360 days after induction) could lead to more severe renal damage. Third, in this study we had some losses of animals throughout the experimental period, which reduced our sample. Some analyzes, such as cytokine levels, had a lot of variation, leading to a result with no statistical significance.

In summary, vitamin D supplementation had protective effect on arthritis but did not influence kidney disease in a PIL model. These results support that the role of vitamin D may be different depending on acting site, what could explain different responses according clinical phenotype. Vitamin D effect on inflammatory pathways is still under investigation and not fully elucidated. Therefore, further investigations of vitamin D are needed to explore the supplement dosage, timing, and the molecular basis in SLE.

## Ethics approval

The present study was approved by the Animal Ethics Committee of Hospital de Clínicas de Porto Alegre (HCPA, Porto Alegre, RS, Brazil) and was conducted in accordance with National Institutes of Health guidelines.

## Disclosure statement

The authors declare that they have no competing interests.

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