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Factors associated with vitamin D levels in patients with glomerulopathies

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Abstract

Introduction: Little is known about the relationship between serum levels of 25-hydroxyvitamin D [25(OH)D] and urinary excretion of proteins or renal function decline in patients with glomerulopathies.

Objectives: To evaluate the relationship between blood levels of 25(OH)D and proteinuria, demographics, clinical and laboratory renal and osteo-metabolic parameters, and chronic kidney disease (CKD) progression to advanced stages.

Patients and Methods: In this prospective observational cohort study including 175 adults with glomerulopathy, we evaluated clinical and laboratory renal and osteometabolic parameters, such as 25(OH)D, calcium, phosphorus, intact parathyroid hormone (iPTH), urinalysis, serum creatinine, and estimated glomerular filtration rate (eGFR).

Results: The mean age was 44.5 ± 14.9 years, and 62.9% of participants were females; mean body mass index (BMI) was 28 ± 5.1 kg/m²; proteinuria, 1.48 ± 2.18 g/24 h; eGFR, 75.9 ± 32.9 mL/min; and follow-up, 1102 ± 494 days. We identified that 32% and 54.3% of patients had 25(OH)D deficiency and insufficiency, respectively. Vitamin D and proteinuria levels were inversely related, regardless of the stage of CKD ($P < 0.001$). Most patients were overweight or obese. BMI was significantly associated with reduced levels of 25(OH)D ($P = 0.024$). Among patients with >1 year of follow-up, levels of 25(OH)D < 15 ng/mL were associated with a higher rate of decline in eGFR ($P = 0.017$).

Conclusion: Low levels of vitamin D were highly prevalent in chronic kidney disease patients with proteinuria, and vitamin D deficiency may have an impact on the progression of glomerulopathies.

Keywords: 25-hydroxyvitamin D, Body mass index, Chronic kidney disease, Glomerulopathy

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Introduction

Patients with proteinuric glomerulopathies, particularly with nephrotic range proteinuria, lose several proteins with critical function, including vitamin D-binding protein. Nevertheless, little is known about vitamin D influence on clinical outcomes such as renal function decline or bone metabolism in patients with glomerulopathies. There are indications that 25(OH)-vitamin D [25(OH)D] deficiency could be associated with a worse prognosis in patients with IgA nephropathy (IgAN) (1). It was reported that 25(OH)D may attenuate renal fibrosis by suppressing the renin-angiotensin-aldosterone system (RAAS) (2,3) and reducing proteinuria in patients with some types of glomerulopathies (4,5).

Objectives

Considering the scarcity of information on vitamin D profiles in glomerular diseases, the present study was designed to evaluate the relationship between blood levels

of 25(OH)D and proteinuria, demographics, clinical and laboratory renal and osteo-metabolic parameters, and clinical outcome of chronic kidney disease (CKD) progression to advanced stages.

Patients and Methods

Study design

A prospective observational cohort study was conducted in patients followed at the Glomerulopathies Section of the Division of Nephrology of the Federal University of São Paulo (UNIFESP). All participants gave their informed consent to participate. The study design was analysed and approved by the research ethics committee of UNIFESP.

We included 175 patients, over 18 years of age, who were non-diabetic. The subjects were not using calcium or vitamin D supplements at the beginning of the study and during follow-up as well. The specific blood and urine sample collection for this study was always performed from August to October.

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■ Implication for health policy/practice/research/medical education

In a prospective observational cohort study including 175 adults with glomerulopathy we found, low levels of vitamin D were highly prevalent in CKD patients.

Regarding vitamin D, participants with levels of 25(OH)D below 15 ng/mL were considered to have a deficiency, those with levels between 15 and 29.9 ng/mL were considered to have an insufficiency, and normal (or sufficient) levels were greater than or equal to 30 ng/mL (6).

When blood and urine were collected, the prescriptions of each patient were evaluated regarding the use of angiotensin-converting enzyme (ACE) inhibitors, angiotensin type 1 receptor blockers (ARBs), both (dual blockade), statins, furosemide, hydrochlorothiazide, oral corticosteroids and pulse therapy with methylprednisolone. We searched for bone fractures in medical files. Body mass index (BMI) was used for weight classification, as recommended by the World Health Organization (WHO). Patients aged between 18 and 59 years were considered adults, and those over 60 years were considered elderly. All participants collected 24-hour urine samples to measure proteinuria and to determine creatinine clearance. Proteinuria was considered abnormal when values were above 0.15 g/24 hours.

Serum creatinine was determined by the alkaline picrate method, while calcium, alkaline phosphatase and phosphorus were quantified by a colorimetric method, with AU-480 reagent and equipment (Beckman Coulter, Indianapolis, IN). The glomerular filtration rate (GFR) was estimated using the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) formula (7). The patients were divided into groups according to the stage of CKD: mild CKD (stages 1 and 2) and moderate to severe CKD (stages 3, 4 and 5).

For the 25(OH)D test, blood was collected in an aluminum-coated tube, and aliquots were stored at -20°C. Quantification of intact-parathyroid hormone (iPTH) and 25(OH)D3 was performed using a chemiluminescent microparticle immunoassay (CMIA) with reagents and Architect i4000 equipment (Abbott).

Statistical analysis

Categorical variables were presented as percentages and compared using the Chi-square test or Fisher’s exact test. Numerical variables were presented as the mean and standard deviation or median and minimum and maximum values, when appropriate, and the comparison between the groups was performed using student’s *t* test.

The statistical analysis was performed to evaluate the interactions between 25(OH)D levels and demographic, clinical and laboratory variables. Estimates of the Spearman’s correlation coefficients between 24-hour

proteinuria and 25(OH)D, iPTH, alkaline phosphatase, and corrected calcium and phosphorus were performed. The multiple linear regression analysis involved three models, one for each group of CKD (stages 1+2 and 3+4+5) and a general model. To evaluate the renal outcome, it was necessary to control potential confounding factors. Analysis of variance (ANOVA) was adjusted, using the rate of decline in GFR (% per day) as the dependent variable and vitamin D levels, use of prednisone and pulse therapy with methylprednisolone, BMI, race, use of ACEi, ARB, dual blockade, and 24-hour proteinuria levels (covariate) as independent variables.

Statistical analysis was performed with SPSS version 18.0 (SPSS Inc., Chicago, IL, USA), and a statistically significant difference corresponded to a *P* value < 0.05.

Results

This study sample was composed of 175 patients with glomerulopathies. Their demographic, clinical, histological and laboratory characteristics are presented in Table 1.

The mean age was 44.5 (18-82) years. Of the participants, 62.3% were female, 50% were Caucasians, 31.5% were of

Table 1. Demographic, clinical, laboratory and histopathological characteristics of patients with glomerulopathies

Characteristics	Total (N = 175)
Age (y), Mean ± SD	44.5±14.9
Women, No. (%)	109 (62.3)
Race, No. (%)	
Caucasians	84 (50)
Mixed	53 (31.5)
African descent	31 (18.5)
BMI (kg/m ²)	28.3±5.1
Glomerulopathies (%)	
Focal segmental glomerulosclerosis	21.7
Lupus nephritis (class IV)	15.4
Membranous nephropathy	14.2
Minimal change disease	8
Lupus nephritis (other classes)	6.3
IgA nephropathy	5.7
Membranoproliferative glomerulonephritis	5.1
Other GN	8.5
Proteinuria (g/24 h)	1.48±2.18
Baseline eGFR (mL/min/1.73 m ²)	75.9±32.9
CKD stages 1+2 (n = 111)	96.6±20.9
CKD stages 3+4+5 (n = 64)	39.9±12.7
Medications, No. (%)	
ACEi	53 (30.2)
ARB	20 (11.4)
ACEi + ARB	52 (29.7)
Furosemide	51 (29.1)
Hydrochlorothiazide	44 (25.1)
Statins	90 (51.4)
Follow-up (days)	1102±494.4

ACEi; angiotensin-converting enzyme inhibitor, ARB; angiotensin type 1 receptor blockers, BMI; body mass index, CKD; chronic kidney disease, eGFR; estimated glomerular filtration rate, GN; glomerulonephritis

mixed ancestry and 18.5% were of African ancestry. The elderly accounted for 18.8% of the patients. Regarding weight classification, patients were classified as below normal (1.3%), normal (27.9%), overweight (37.3%), obese class I (22.4%), II (8.0%) and III (3.1%). Thus, 70.8% of the individuals were overweight or obese.

Proteinuria levels were above 3 g/24 hours, between 1 and 3 g/24 hours, less than 1 g/24 hours and absent (less than 0.15 g/24 hours) in 18.3%, 19.4%, 28.6% and 33.7% of the 175 patients evaluated, respectively.

Renal biopsy was conducted in 152 individuals (86.8%), and in such cases, glomerulopathies were classified as primary in 91 (59.9%) and secondary in 61 (40.1%) patients.

Levels of 25(OH)D were <30 ng/mL in 151 of 175 patients (86.3%), so that 56 (32%) were considered deficient (<15 ng/mL) and 95 (54.3%) were insufficient (15 - 29.9 ng/mL). The distributions of gender, age, BMI, race and stage of CKD according to 25(OH)D levels are presented in Table 2.

The correlations between 24-hour proteinuria and 25(OH)D, eGFR, PTH, alkaline phosphatase, calcium and phosphorus are presented in Figure 1A-E.

The mean estimated GFR in the stages 1 + 2 CKD group was 96.6 ± 20.9 mL/min/1.73 m², and in the CKD stages 3 + 4 + 5 group, it was 39.9 ± 12.7 mL/min/1.73 m².

In the analysis of the three multiple linear regression models, for each group of CKD stages and in general, 25(OH)D levels were inversely correlated with 24-hour proteinuria for patients with CKD stages 1 + 2 ($P < 0.001$) and CKD stages 3 + 4 + 5 ($P < 0.001$) as well as for all patients together, regardless of the stage of CKD ($P <$

0.001).

No relationship was identified between 25(OH)D levels and gender, race, age, eGFR and stages of CKD. Patients with 25(OH)D levels < 30 ng/mL had higher BMI values than those with normal 25(OH)D levels ($P < 0.024$).

Five cases of bone fracture were identified, and in three of them, the vitamin D level was normal. The presence of fracture was not significantly associated with 25(OH)D levels or with previous use of prednisone or pulse therapy with methylprednisolone. Two patients had systemic lupus erythematosus (SLE): two, focal segmental glomerulosclerosis (FSGS); and one, membranous nephropathy (MN). The fractures occurred during the follow-up period, and the patients were 37, 54, 55, 62 and 65 years old. Only one of these patients did not receive pulse therapy. All five patients used prednisone for more than six months, and two of them used it for more than five years. The relationship between the serum levels of 25(OH)D, PTH, alkaline phosphatase, calcium corrected by albumin and phosphorus in the patients and the use of angiotensin-converting enzyme inhibitors (ACEis) and ARBs, double blockade (ACEi + ARB), hydrochlorothiazide, furosemide and statin were investigated. In these comparisons, 24-hour proteinuria was used as a covariate. There were statistically significant associations between the use of ACEi (without association with ARB) and low levels of PTH ($P = 0.033$) and alkaline phosphatase ($P = 0.003$); between thiazides and higher levels of calcium corrected by albumin ($P = 0.015$) and phosphorus ($P = 0.047$); and between furosemide and low-levels of 25(OH)D ($P = 0.033$), ranges of 25(OH)D ($P = 0.001$) and high levels of phosphorus ($P = 0.003$).

Table 2. Distribution of gender, race, age, BMI and stage of CKD according to the range of vitamin D levels.

Patient distribution	25(OH) vitamin D			P value
	< 15 ng/mL	15–29.9 ng/mL	≥ 30 ng/mL	
Gender				
Female, No. (%)	36 (64.30)	59 (61.5)	14 (60.9)	0.931 ^a
Male, No. (%)	20 (35.7)	37 (38.5)	9 (39.1)	
Race				
Caucasian, No. (%)	25 (47.2)	46 (48.9)	13 (61.9)	0.238 ^a
Mixed, No. (%)	20 (37.7)	26 (27.7)	7 (33.3)	
African descent, No. (%)	8 (15.1)	22 (23.4)	1 (4.8)	
Age (y)				
Mean	43.3	44.9	45.5	0.791 ^b
SD	15.5	14.4	15.7	
BMI* (kg/m ²)				
Mean	28.7	28.8	25.5	0.024 ^c
SD	5.7	5.1	2.9	
CKD Stages				
1+2, No. (%)	33 (58.9)	65 (67.7)	13 (56.5)	0.821 ^d
3+4+5, No. (%)	23 (41.1)	31 (32.3)	10 (43.5)	

BMI; body mass index, CKD; chronic kidney disease

*It was necessary to use the logarithmic mathematical transformation for the proper use of ANOVA. ^a Pearson's chi-square, ^b Kruskal-Wallis, ^c Analysis of variance (ANOVA) with a fixed factor, ^d Test of linear-by-linear association.

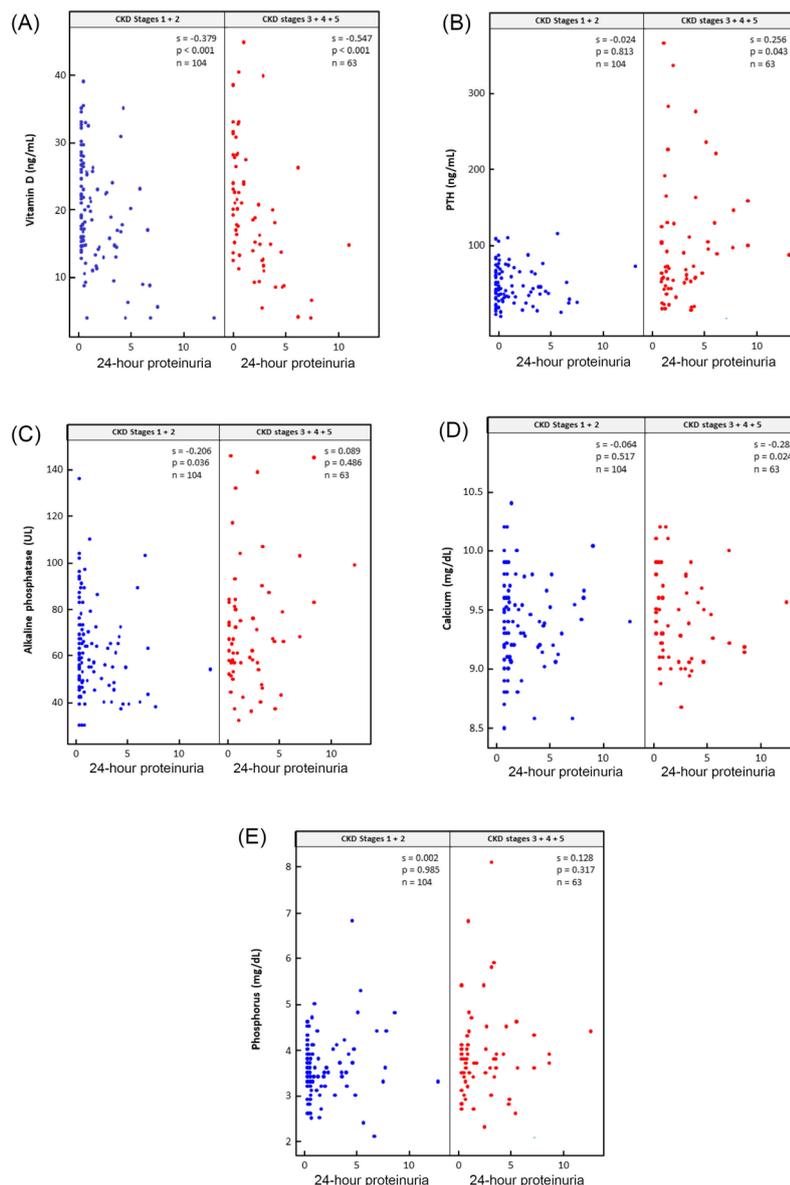


Figure 1. (A) Two-dimensional dispersion diagram between vitamin D and 24-hour proteinuria, according to chronic kidney disease (CKD) stages (1 + 2 and 3 + 4 + 5). (B) Two-dimensional dispersion diagram between PTH and 24-hour proteinuria, according to CKD stages. (C) Two-dimensional dispersion diagram between alkaline phosphatase and 24-hour proteinuria, according to CKD stages. (D) Two-dimensional dispersion diagram between corrected calcium and 24-hour proteinuria, according to CKD stages. (E) Two-dimensional dispersion diagram between phosphorus and 24-hour proteinuria, according to CKD stages

To evaluate the association between phosphorus and 25(OH)D with the use of furosemide, we evaluated correlations between PTH, eGFR (estimated glomerular filtration rate), 25(OH)D, calcium and phosphorus. Based on significant results, we conducted two models of multivariate regression using all 175 patients. In the first, we considered PTH as the dependent variable and eGFR, 25(OH)D and calcium as independent variables. In this model, eGFR ($P < 0.001$) and 25(OH)D ($P = 0.043$) were inversely related to PTH. We did not find an association between PTH and calcium ($P = 0.961$). In the second model, we used phosphorus as a dependent variable and eGFR, 25(OH)D and calcium as independent variables. In this case, the higher the phosphorus level was, the lower the eGFR ($P = 0.020$) and 25(OH)D ($P = 0.004$), and the

higher the calcium ($P = 0.024$).

The influence of low levels of 25(OH)D on the rate of renal function decline was evaluated. We used the ratio between the relative decline in the eGFR (%) and follow-up time (days). Twenty-three patients with less than 1 year of follow-up were excluded, so this analysis involved 152 individuals, and their mean follow-up time was 1256.2 ± 312.9 days.

It was found that patients with 25(OH)D levels lower than 15 ng/mL had a faster decline in eGFR than those with higher levels of 25(OH)D ($P = 0.017$).

Discussion

Low levels of vitamin D in patients with CKD and with glomerulopathies is a known occurrence (8,9). It is not

well established, however, whether it occurs due to CKD progression and/or proteinuria. In previous reports, vitamin D deficiency has been associated with worse prognosis (4,10) and normal levels with better clinical outcomes (2,5).

In the present study, we sought to evaluate levels of 25(OH)D and their relationship with other parameters indicative of mineral metabolism and renal function in patients with glomerulopathies followed in a single centre. The population consisted of 175 individuals, predominantly female (62.3%) and Caucasian (50%), with a mean age of 44 years, although 18.8% were elderly. Of all the patients, 70.8% were above the healthy weight range, with 37.3% being overweight and 33.5% obese.

It is noteworthy that the profile of the Brazilian population in relation to weight has been changing in recent years. According to data from VIGITEL/Ministry of Health, 34.9% of the Brazilian population is overweight and 18.9% is obese (11). This high frequency of overweight and obese patients is relevant, since this is a condition known to be associated with chronic diseases, increased cardiovascular risk and progression of CKD (10,12).

Patients with higher BMI values had a significantly increased frequency of 25(OH)D deficiency and insufficiency as a group. Similar results were found in another study, in which 337 individuals without CKD were evaluated, and vitamin D levels below 30 ng/mL correlated inversely with age, BMI, HDL and sun exposure (13). In contrast to the results of other studies (8,13), in our study, gender, race and age were not correlated with 25(OH)D levels. Diniz et al analysed serum 25(OH)D levels of 125 adult patients with pre-dialytic CKD living in São Paulo. Lower levels were associated with elevated PTH and waist circumference. In both studies (8,13), there was a correlation between higher BMI and lower levels of 25(OH)D. In fact, obese adults may need two to three times more vitamin D to treat and prevent the deficiency because vitamin D is fat soluble and does not become bioavailable (14-16).

Reports of 25(OH)D deficiency are becoming more common, even in the general population. Considering the Brazilian population, a study with 250 elderly people from São Paulo showed that 15.4% had vitamin D deficiency and 41.9% had an insufficiency (17). In a comprehensive evaluation of 603 healthy people in São Paulo, the mean 25(OH)D was 21.4 ng/mL, with 77.4% of the participants presenting with insufficiency or deficiency. This finding was more common in individuals of African descent and the elderly, as well as in tests performed during the winter months (18).

It is worth noting that in our study, all sample collections were carried out in the same period of the year, from August to October, to eliminate the variations due to seasonality. We evaluated the drugs used by the patients at the time of the study collection. We found a significant association between the use of ACEi and low levels of

PTH and alkaline phosphatase, as well as between the use of hydrochlorothiazide and higher levels of calcium corrected by albumin levels and phosphorus, and between the use of furosemide and low levels of 25(OH)D and high levels of phosphorus. In this population, patients with higher proteinuria tend to use ACEi (or ARB) and loop diuretics more often. As would also be expected, in our population, lower levels of 25(OH)D were associated with higher levels of proteinuria.

Regarding the association between higher levels of serum calcium and the use of hydrochlorothiazide, we attributed this to a known side effect of the drug. Thiazides inhibit distal tubule sodium-chloride cotransporter (NCC), increasing sodium elimination (19). The resulting volume contraction stimulates sodium reabsorption in the proximal tubule. In this segment, sodium and calcium absorption is connected, which leads to a reduction in calciuria and a slight elevation in calcium blood level. This effect is beneficial for patients with nephrolithiasis and hypercalciuria and has the potential to improve bone mineral density (17).

It is possible that an association which we found between the use of furosemide and hyperphosphatemia occurred due to a confounding bias, since among the nine patients with phosphorus elevation, four had an eGFR below 25 mL/min. Among the five patients with moderate to normal renal function and hyperphosphatemia, all had normal calcium levels and PTH at the lower limit of normal, but low 25(OH)D levels (ranging from 5.4 to 16.8 ng/mL). In a study by Barreto et al, low levels of vitamin D correlated with hyperphosphatemia (16). In our study, no patient was on vitamin D replacement, and no conditions associated with hyperphosphatemia, such as haemolysis, tumour lysis, rhabdomyolysis, acute leukaemia, or hyperthermia, were identified.

We verified that phosphorus and 25(OH)D were related to other variables, indicating that the association with furosemide was a confounding bias, since the medication is used more often as CKD progresses.

It is of note that in contrast to our results, another single-centre observational study identified an association between the use of RAAS blockers or allopurinol with higher 25(OH)D levels (20). RAAS blockade is commonly used in patients with glomerulopathies for its antihypertensive and antiproteinuric properties (21-27).

As CKD progresses, active vitamin D deficiency becomes more pronounced, resulting in hypocalcemia and secondary hyperparathyroidism (28). However, in the present study, we found no association between 25(OH)D levels and stage of CKD or levels of GFR, corroborating previous publications. Bosworth et al evaluated 248 patients with CKD and found that 25(OH)D levels were not decreased with the decline in GFR, which the authors attributed to the balance between less synthesis and less catabolism (29). The decrease in vitamin D catabolism is evidenced by the decrease in 24,25(OH)₂D. Low levels

of this metabolite, and not of 25(OH)D, correlated with significant hyperparathyroidism.

It is noteworthy that in our sample, there was a predominance of the initial stages of CKD. It is possible that more obvious differences would eventually arise if the population included more individuals with advanced CKD for comparison.

Clinical manifestations of bone disease usually occur in patients with advanced CKD on dialysis. However, subclinical changes in bone metabolism may already be present in the earlier stages of CKD (30). In patients with nephrotic syndrome and normal GFR, changes in bone histology, such as osteomalacia and demineralization, may be found (31).

We did not find a statistically significant association between 25(OH)D levels and the occurrence of bone fracture. However, it should be clarified that we searched for bone fractures only by medical file review. It is possible that high cumulative doses of pulse therapy and prolonged use of corticosteroids were the main factors related to these fractures. Among the 32 (18.3%) patients who submitted to the of bone densitometry examination by indication of the attending physician, 40% presented with normal results, 42% with osteopenia and 18% with osteoporosis. Among those with normal densitometry, 38.5%, 46.1% and 15.4% presented with deficiency, insufficiency and sufficiency of vitamin D, respectively; among those with osteopenia, the percentages were 20.0%, 66.7% and 13.3%; and with osteoporosis, they were 50.0%, 50.0% and 0%, respectively.

We found that 25(OH)D levels of less than 15 ng/mL were associated with a higher rate of decline in renal function. This conclusion was based on statistical analysis involving 152 (86.8%) patients with a follow-up greater than 365 days. This was a particularly relevant finding in the present study, which is reinforced by the results of others. Li et al studied 105 patients with a recent diagnosis of IgAN and demonstrated that vitamin D deficiency (<15 ng/mL) was associated with lower GFR and higher proteinuria (1). After a 13-month follow-up, 28 patients (26.7%) achieved the primary outcome (30% reduction in GFR compared to baseline); among these, 24 patients had vitamin D deficiency. Individuals with 25(OH)D levels <15 ng/mL had a higher Oxford score for tubulointerstitial involvement. In that study, 25(OH)D deficiency was considered an independent risk factor for progression of renal disease, regardless of age, gender, systolic blood pressure, eGFR, vitamin D supplementation and treatment with ACEi/ARB or immunosuppressants.

The present study has limitations. It was performed in a single centre, which may indicate a selection bias, depending on the type of disease. Diet, sun exposure or the use of sun blockers were not evaluated. In any case, all collections were carried out in the same period of the year, reducing the variability of sun exposure in the same region. On one hand, our sample is relatively small for statistical

analysis of some aspects; on the other hand, it can be said that it is quite broad when compared to publications on the subject involving patients with glomerulopathies.

Vitamin D deficiency, sun exposure and seasonality have been linked to some autoimmune diseases such as multiple sclerosis, vitiligo, Sjögren syndrome, inflammatory bowel disease, rheumatoid arthritis, and SLE (32). Vitamin D supplementation has also been described as a complementary therapeutic strategy for some of these diseases (33).

In patients with CKD, vitamin D status has been studied (33), and its supplementation is recommended by international guidelines (35-37). Proteinuria is known to be considered an independent risk factor for worsening renal function. This concept comes from experimental research (37), observational studies (38, 39) and post hoc analysis of clinical trials (40). In a randomized controlled crossover trial, paricalcitol was not superior to sodium restriction to reduce residual albuminuria (41). However, to date, there is no double-blind, placebo-controlled clinical trial designed specifically to assess the role of vitamin D in the decline in GFR or initiation of renal replacement therapy. In fact, guidelines recommend administration of vitamin D only for treatment of conditions related to disorders of bone metabolism (42), although some studies indicate that patients with CKD and decreased levels of 25(OH)D may present with higher mortality (43,44).

In the evaluation of patients with glomerulopathies, it was possible to demonstrate an inverse association between vitamin D and proteinuria levels, regardless of eGFR levels and, in specific analyses, the stage of CKD.

Among the demographic, clinical and laboratory parameters evaluated, low levels of vitamin D (below 30 ng/mL) were related to high BMI but not to age, gender and ethnicity or eGFR, emphasizing that overweight or obesity were evidenced in most patients.

In the evaluation of the "progression of CKD" outcome by analysis of the rate of decline in eGFR, in patients with more than a year of follow-up, vitamin D levels below 15 ng/mL were associated with a faster decline in renal function.

Conclusion

Our study reinforced the existence of an inverse association between proteinuria and vitamin D levels and its deficiency with loss of renal function. The role of vitamin D in the course of glomerulopathies, however, still needs to be investigated in more detail, including interventional clinical trials, to verify whether supplementation is associated with better outcomes.

Limitations of the study

This is a single centre study. Diet, sun exposure or the use of sun blockers which could possibly be interfering factors were not evaluated.

Authors' contribution

Conceptualization: GMK, ABC. Methodology: FKAU, GMK, ABC, SRM, DEF. Formal Analysis: FKAU, GMK, ABC. Writing—Original Draft Preparation: FKAU, GMK, ABC. Writing—Review and Editing: FKAU, GMK, ABC, SRM, DEF. Supervision: GMK, ABC.

Conflicts of interest

The authors declare that they have no competing interests.

Ethical issues

The research followed the tenets of the Declaration of Helsinki. The Ethics Committee of UNIFESP approved this study (Ethical code#1203/10). Accordingly, written informed consent was taken from all participants before any intervention. Moreover, ethical issues (including plagiarism and double publication) have been completely observed by the authors.

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