Chronic kidney disease (CKD), which has a high morbidity and mortality rate, negatively affects the quality of life. The incidence of the disease has increased significantly in recent years [1]. Renal replacement therapies, such as dialysis and renal transplantation, are implemented for patients with CKD. In renal transplantation patients, survival and quality of life is improved markedly compared with dialysis patients, and less cardiovascular disease is observed [2]. However, there is still a high risk for acute rejection and chronic allograft nephropathy in renal transplantation [3, 4]. Due to the increasing importance of the renal transplantation, it is very important to reduce major risk factors involved in graft failure.

Vitamin D, which plays an important role in the regulation of calcium, phosphorus, and bone metabolism, is a steroid hormone. It is obtained through nutrition and solar radiation. Two hydroxylation steps are required to convert it to the physiologically active form of vitamin D (1,25-dihydroxyvitamin D, calcitriol). The first step occurs in the liver, producing 25-hydroxyvitamin D3 (25(OH)D3), and plasma 1,25(OH)2D3 levels were measured. In addition, the urine protein/creatinine (P/C) ratio was calculated. The plasma 1,25(OH)2D3 level was determined using liquid chromatography-tandem mass spectrometry.

Objectives: Bone and mineral metabolism disorders are important potential complications after renal transplantation. The purpose of this study was to demonstrate the relationship between vitamin D, 1,25-dihydroxyvitamin D3 [1,25(OH)2D3], calcium, and phosphorus metabolism with graft function in renal transplant recipients.

Methods: This prospective longitudinal study included 30 renal transplant recipients (10 female, 20 male; mean age: 40.3±12.86 years). Blood and urine samples were collected before and 6 months after transplantation. Serum creatinine, blood urea nitrogen (BUN), calcium, phosphorus, alkaline phosphatase (ALP), glucose, albumin, parathyroid hormone (PTH), 25-hydroxyvitamin D [25(OH)D], and plasma 1,25(OH)2D3 levels were measured. In addition, the urine protein/creatinine (P/C) ratio was calculated. The plasma 1,25(OH)2D3 level was determined using liquid chromatography-tandem mass spectrometry.

Results: The posttransplant level of serum phosphorus, PTH, creatinine, BUN and ALP was found to be significantly decreased (p=0.0001; p=0.011 for ALP). Although the plasma 1,25(OH)2D3 level had significantly increased (p=0.0001) after transplantation, no significant difference in the serum 25(OH)D level was observed. The urine P/C ratio was found to be significantly decreased after transplantation (p=0.007). A deficiency of vitamin D was observed frequently both before (87%) and after (73%) transplantation.

Conclusion: Persistent vitamin D deficiency was detected in the recipients even after transplantation, although the serum PTH level decreased. Some studies published to date draw a direct link between serum vitamin D level and graft function; however, evidence for this link was not observed in the present study. Long-term monitoring may be needed to evaluate the correlation between vitamin D level and graft function.

Keywords: 1,25-dihydroxyvitamin D3, graft function, liquid chromatography-tandem mass spectrometry, protein/creatinine ratio, renal transplantation, vitamin D

Chronic kidney disease (CKD), which has a high morbidity and mortality rate, negatively affects the quality of life. The incidence of the disease has increased significantly in recent years [1]. Renal replacement therapies, such as dialysis and renal transplantation, are implemented for patients with CKD. In renal transplantation patients, survival and quality of life is improved markedly compared with dialysis patients, and less cardiovascular disease is observed [2]. However, there is still a high risk for acute rejection and chronic allograft nephropathy in renal transplantation [3, 4]. Due to the increasing importance of the renal transplantation, it is very important to reduce major risk factors involved in graft failure.

Vitamin D, which plays an important role in the regulation of calcium, phosphorus, and bone metabolism, is a steroid hormone. It is obtained through nutrition and solar radiation. Two hydroxylation steps are required to convert it to the physiologically active form of vitamin D (1,25-dihydroxyvitamin D, calcitriol). The first step occurs in the liver, producing 25-hydroxyvitamin D3...
The second hydroxylation step is mainly carried out by the 1α-hydroxylase enzyme in the kidney [5]. Calcitriol levels are usually insufficient in patients whose glomerular filtration rate (GFR) is less than 30 mL/minute, due to the 1α-hydroxylase enzyme inhibition associated with hyperphosphatemia [6]. Vitamin D deficiency and insufficiency have been reported in the majority of CKD patients [7]. The situation is similar in renal transplantation. Transplant patients must avoid sunlight due to immunosuppressive therapy and there is accelerated vitamin D catabolism as a result of using glucocorticoids. Although vitamin D supplementation is included in the guidelines, it is thought it may be a trigger in cases of hypercalcemia, hyperphosphatemia, or hypoparathyroidism, and is not generally used [8, 9]. There is still no specific guidance for vitamin D supplementation in renal transplantation patients [1].

Because of the renal protective properties of vitamin D, renal graft function is affected when it is deficient. Vitamin D regulates the renin-angiotensin-aldosterone system negatively. Previous research has demonstrated that proteinuria, a risk factor for progressive renal failure, is reduced with vitamin D supplementation [10, 11]. Vitamin D insufficiency and proteinuria are strongly correlated in renal transplantation recipients [12]. The immune regulatory effects of vitamin D also play an important role in renal transplantation. Calcitriol receptors are available in various immune cells, such as T and B cells, monocytes, and antigen presenting cells. Due to the suppression of helper T cell proliferation and dendritic cell differentiation, calcitriol may be protective in transplantation [13, 14].

Accurate measurement of vitamin D is crucial in clinical laboratory settings. Therefore, it requires standardization. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) is the gold standard method for vitamin D measurement [15]. The active form of vitamin D, 1,25-dihydroxyvitamin D, exhibits an extremely low serum concentration. Thus, its quantification is extremely challenging, even using LC-MS/MS. It requires difficult sample pretreatment procedures, such as solid phase extraction and derivatization. Using an atmospheric pressure chemical ionization (APCI) source to measure the level of vitamins is common, but the research about measuring 1,25(OH)2D3 level using LC-MS/MS with an electro spray ionization (ESI) source is limited. Since vitamin D deficiency may negatively affect graft function in renal transplant recipients, this study was designed to evaluate vitamin D status and graft function before and after transplantation. The level of 1,25-dihydroxyvitamin D3 was also measured using LC-MS/MS with an ESI source and compared with other parameters.

### Materials and Methods

#### Design

Thirty-five living-donor renal transplant recipients were recruited into the study (12 female, 23 male; mean age±SD: 40.30±12.85 years, ≥18 years old). This prospective, longitudinal study was conducted in accordance with the ethical standards of the Akdeniz University Faculty of Medicine ethics committee (approval number: 225) and the Helsinki Declaration, and written informed consent was provided by all of the patients. Five patients who had a transplantation from a cadaveric donor, malignancy, combined (pancreas or liver) transplantation, or graft failure related to surgical causes were excluded from the study. Demographic characteristics, including age, sex, primary disease and dialysis type are presented in Table 1. The kidney graft recipients were managed using the center’s protocol, which includes tacrolimus, mycophenolate mofetil, and basiliximab/everolimus/sirolimus.

#### Samples

The samples were collected immediately before and 6 months after transplantation. The samples were centrifuged at 4000 rpm for 5 minutes. The sera, plasma, and urine were stored until the day of analysis at -80°C. Estimated GFR (eGFR) was calculated using the Chronic Kidney Disease Epidemiology formula and the serum creatinine values of the patients [16].

#### Procedure

Serum creatinine, BUN, calcium, phosphorus, alkaline phosphatase, albumin, and urine creatinine levels were measured using commercial kits and a Roche COBAS-8000 autoanalyzer (Roche Diagnostics, Mannheim, Germany) and colorimetric methods. Urine protein was determined using the turbidimetric method and commercial kits in the COBAS-8000 autoanalyzer. Serum PTH and 25(OH)D levels were measured using commercial kits in a Roche COBAS-e602 autoanalyzer (Roche Diagnostics, Mannheim, Germany) and commercial kits in the COBAS-8000 autoanalyzer. Serum PTH and 25(OH)D levels were measured using commercial kits in a Roche COBAS-e602 autoanalyzer (Roche Diagnostics, Mannheim, Germany) and commercial kits in the COBAS-8000 autoanalyzer.

#### Results

<table>
<thead>
<tr>
<th>Table 1. Demographic features of the patients</th>
<th>All Patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>Donor age (years)</td>
<td>41.43±12.82</td>
</tr>
<tr>
<td>Recipient age (years)</td>
<td>40.30±12.85</td>
</tr>
<tr>
<td>Gender (female/male) (%)</td>
<td>10/20</td>
</tr>
<tr>
<td>Body mass index (kg/m²)</td>
<td>23.74±4.76</td>
</tr>
<tr>
<td>Type of Renal Replacement Therapy</td>
<td>n(%)</td>
</tr>
<tr>
<td>Peritoneal dialysis D</td>
<td>5 (17)</td>
</tr>
<tr>
<td>Hemodyalisis</td>
<td>12 (40)</td>
</tr>
<tr>
<td>Preemptive</td>
<td>13 (43)</td>
</tr>
<tr>
<td>The Etiology of End-Stage Renal Disease</td>
<td>n(%)</td>
</tr>
<tr>
<td>Glomerulonephritis</td>
<td>5 (16.67)</td>
</tr>
<tr>
<td>Diabetes mellitus</td>
<td>4 (13.33)</td>
</tr>
<tr>
<td>Hypertension</td>
<td>3 (10)</td>
</tr>
<tr>
<td>Vesico urethral reflux</td>
<td>3 (10)</td>
</tr>
<tr>
<td>Polycystic kidney disease</td>
<td>3 (10)</td>
</tr>
<tr>
<td>Etiology unknown</td>
<td>9 (30)</td>
</tr>
<tr>
<td>Other</td>
<td>3 (10)</td>
</tr>
<tr>
<td>Total</td>
<td>30 (100)</td>
</tr>
</tbody>
</table>

Results are expressed as numbers and percentages.
Diagnostics, Mannheim, Germany) using electrochemiluminescence immunoassay (ECLIA) methods. The detection limit of ECLIA 25(OH)D was noted as 3.00 ng/mL in the kit insert. ImmunoTube LC-MS/MS Extraction Kit (KM1000; Immundiagnostik AG, Besheim, Germany) was used for the plasma 1,25(OH)2D3 analysis. This method is based on the quantitative measurement of 1,25(OH)2D3 in plasma after extraction. Analysis of 1,25(OH)2D3 was performed using the optimized multiple reaction monitoring method in ESI-sourced LC-MS-8040 triple quadrupole tandem mass spectrometry combined with ultra fast liquid chromatography (LC-20 AD UFLC XR, Shimadzu Corp., Kyoto, Japan). ESI (+) positive mode was used in this analysis.

**Statistical Analysis**

All statistical analyses were performed with IBM SPSS Statistics for Windows, Version 20.0 (IBM Corp., Armonk, NY, USA) and a significance level of 0.05 was adopted. The Kolmogorov-Smirnov test was performed to assess deviation from normal distribution. Quantitative variables were summarized as mean and standard deviation (SD), or as median. Student’s t-test or the Mann–Whitney U test was used for comparison of continuous variables between groups. The analysis of correlation was done with the Spearman or Pearson test.

**Results**

Patient characteristics and demographic features are shown in Table 1. The mean age of patients was 40.30±12.86 years (range: 19-70 years). There were 20 male and 10 female patients. Twelve of the patients received hemodialysis and 5 received peritoneal dialysis treatment before transplantation. A preemptive kidney transplantation was carried out for 13 patients.

Table 2 summarizes the plasma 1,25(OH)2D3, serum creatinine, phosphorus, calcium, PTH, 25(OH)D, BUN, ALP level, and urinary P/C ratio of the patients before and 6 months after transplantation. The calcium-phosphorus product value is also provided. The level of 1,25(OH)2D3 showed a significant increase due to the improvement of kidney function after transplantation (p=0.0001), while the serum 25(OH)D level did not increase significantly (p=0.102) (Table 2). The serum creatinine, phosphorus, PTH, BUN, and ALP levels significantly decreased after transplantation, while the calcium level increased. Also, though the serum PTH level was significantly lower after transplantation, it was still above the reference ranges. The urine P/C ratio was found to have decreased significantly after transplantation (p=0.007).

Prior to transplantation, 87% of the recipients had a vitamin D deficiency (<15 ng/mL), while the proportion was 73% 6 months later. Additionally, vitamin D insufficiency (15-30 ng/mL) was detected in 13% before transplantation and 27% 6 months after receiving the transplant. Significantly positive correlations between calcium and 25(OH)D (r=0.447; p=0.013), as well as PTH and ALP (r=0.843; p=0.0001) levels were observed before transplantation. In addition, significantly negative correlations between PTH, ALP, creatinine and eGFR (r=-0.395, p<0.05; r=-0.421, p<0.05; r=-0.892, p=0.0001, respectively) were found. Significantly negative correlations between PTH and ALP and calcium (r=-0.467, p<0.01; r=-0.431, p<0.05, respectively) were also found. No significant correlation was observed between 1,25(OH)2D3 level and other parameters before transplantation. After the transplantation, a negative correlation was seen between serum 25(OH)D and PTH level (r=-0.365; p=0.047). Significant correlations between proteinuria expressed as P/C ratio and 25(OH)D or 1,25(OH)2D3 were not observed.
Discussion

Metabolic complications began to be more frequently encountered due to prolongation of graft survival after transplantation. Bone and mineral metabolism disorders are very important for renal transplant recipients. Therefore, we evaluated some parameters related to bone metabolism, vitamin D status, and graft function before and after transplantation. However, we were not able to measure bone mineral density in this study. Several studies have reported that 25(OH)D deficiency is very common after renal transplantation. The most important reason is thought to be protection of patients from sunlight due to the increased risk of non-melanoma skin cancer associated with immunosuppressive therapy. In a recent study, a low 25(OH)D level 3 months after transplantation was demonstrated to be associated with renal fibrosis, renal tubular atrophy, and a low GFR after 1 year [17].

A circulating 25(OH)D level of less than 5 ng/mL is defined as severe deficiency, 15-29 ng/mL is insufficiency, above 30 ng/mL is normal, and more than 150 ng/mL is vitamin D intoxication, according to the National Kidney Foundation Kidney Disease Outcomes Quality Initiative (KDOQI) guideline. This guideline recommends beginning replacement therapy with vitamin D2 (ergocalciferol) when the 25(OH)D level is less than 30 ng/mL [18]. The Kidney Disease: Improving Global Outcomes guide also suggests measurement of the level of 25(OH)D in CKD patients in stages 1-5 and treatment of vitamin D deficiency like the general population [19]. In our study, the 25(OH)D level was 7.74±6.59 ng/mL before transplantation, and this value increased to 10.46±5.79 ng/mL after transplantation. However, this increase was not statistically significant. No patient had a vitamin D level greater than 30 ng/mL in our study. In the study patients, 13% had vitamin D insufficiency and 87% had vitamin D deficiency before transplantation. Six months after transplantation, 27% had vitamin D insufficiency and 73% had vitamin D deficiency. The assessment of vitamin D deficiency remains difficult due to seasonal variations in serum 25(OH)D. Analysis of seasonal changes in 25(OH)D has revealed that the serum level demonstrated a sinusoidal fluctuation throughout the year and was significantly higher in the summer. The response of 25(OH)D to seasonal variations was found to be 50% in the Turkish population [20]. Our data did not reveal a strong seasonal influence on vitamin D status in renal transplant recipients, likely related to avoiding exposure to sunlight because of the increased risk of non-melanoma skin cancer associated with immunosuppressive therapy. Therefore, we believe that vitamin D deficiency and insufficiency observed in renal transplant patients is independent of seasonal effects.

A high prevalence of vitamin D insufficiency in patients with chronic renal disease has also been documented in literature. Our study focused on the association of a low vitamin D level with proteinuria and graft function. A low 25(OH)D level has been associated with an increased prevalence of albuminuria among the general adult population [21]. An inverse relationship has been reported between vitamin D level and proteinuria [12, 22]. It is not well explained whether only urinary loss of vitamin D binding protein or 25(OH)D spontaneously contributed to vitamin D insufficiency. Vitamin D deficiency may also cause proteinuria by suppressing activation of the renin-angiotensin system and it contributes to the reduction of proteinuria through hemodynamic mechanisms. Additionally, vitamin D deficiency leads to podocyte loss and glomerulosclerosis through direct cellular effects [23]. As seen in the literature, the results of this study indicated that proteinuria decreased with transplantation (Table 2). However, no significant correlation was observed between 25(OH)D and proteinuria. The reason may be the small sample size and limited number of transplanted patients with severe proteinuria.

Vitamin D may provide improvement in graft function and protect graft function. Keyzer et al. [24] reported that a low 25(OH)D level was associated with all causes of mortality and a marked annual reduction in renal function in their large scale study. We also evaluated the relationship between 25(OH)D level and graft function as assessed by eGFR. No significant correlation was shown between these parameters either before or after transplantation. Although the 25(OH)D level was measured at two time points, we did not observe change over time. Longer monitoring may be needed to fully evaluate any correlation between 25(OH)D level and graft function.

We observed a moderate level of 1,25(OH)2D3 before transplantation although there was the absence of adequate kidney function. This might be due to non-renal synthesis of 1,25(OH)2D3. The 1,25(OH)2D3 level showed a significant increase after transplantation due to the improvement of kidney function (p=0.0001) (Table 2). The plasma 1,25(OH)2D3 level was similar to the post-transplantation findings of Keyzer et al. [24]; however, they reported that the association of low 1,25(OH)2D3 with mortality and graft failure depends on renal function.

Another point of our study was to examine the association between 25(OH)D, 1,25(OH)2D3 and PTH. In our study, the mean PTH level was higher than expected before transplantation. Although a significant and marked reduction in the PTH level was observed with transplantation, the level was still above the reference ranges. The low 25(OH)D level seen, even though renal function returned to normal, was related to the existence of hyperparathyroidism in our study. These findings may be explained according to the KDOQI. In the first year following renal transplantation, hyperparathyroidism persists in nearly 50% of recipients, despite the decrease in PTH level [8, 9].

Imunoassays are widely used for the measurement of serum/plasma vitamin D metabolites. However, accurate quantification of these metabolites is difficult due to cross-reactivity. LC-MS/MS is the gold standard method of analyzing these metabolites due to its high sensitivity and selectivity. The level of 1,25-dihydroxyvitamin D3 was measured using LC-MS/MS with an ESI source and compared with other parameters in our study. As a result of financial limitations, we could not measure vitamin D using LC-MS/MS in this study.
In conclusion, vitamin D insufficiency was still common after renal transplantation. Although it was associated with proteinuria among kidney transplant recipients, we could not significantly demonstrate this result in the present study due to some limitations, such as a small sample size and the need for longer monitoring. Additional, larger studies are necessary to evaluate our results and to determine whether vitamin D may play an important role in the improvement of long-term graft function among kidney transplant recipients.

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Conflict of interest: None.

Ethical approval: All procedures performed in studies involving human participants were in accordance with the ethical standards of the Ethics Committee of Akdeniz University Faculty of Medicine (approval number: 225) at which the studies were conducted and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards.

Peer-review: Externally peer-reviewed.


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