ORIGINAL ARTICLE

Pediatrics Sciences Journal (*PedScij*) 2020, Volume 1, Number 2 : 47-53
P-ISSN: 2722-0427, E-ISSN: 2722-1474

Pediatric Sciences Journal

Published by DiscoverSys

Effect Of Vitamin D3 On Transforming Growth Factor - B1 In Pediatric Patients With Stage 1 To 3 Chronic Kidney Disease: An Experimental Study And Randomized Controlled Trials

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ABSTRACT

Introduction: In Indonesia, Chronic Kidney Disease (CKD) is a severe health problem both in terms of treatment and health costs. One way to reduce mortality is by blocking the progression of the disease. Transforming Growth Factor-β1 (TGF-β1) is a marker of progression from CKD through its fibrosis pathway. One mechanism for interfering with the TGF-β1 signaling pathway is by giving vitamin D3(cholecalciferol). This study aims to find out that supplementation of vitamin D3 can increase plasma 25(OH)D levels and reduce TGF-β1 levels in children with CKD.

Methods: The study was approved by the hospital's ethical committee, designed as a randomized clinical trial, double-blind, pre and post-test control group, which involved 30 subjects diagnosed as CKD stage 1-3. Subjects were divided into two groups; the vitamin D3-treated group and the placebo group. TGF-β1 and vitamin D plasma level was measured by ELISA method.

Results: Vitamin D levels increased by 9.35 compared to the placebo group. The magnitude of the increase from the Wilcoxon statistical test results obtained p=0.001 (p<0.05), so there was a significant increase in vitamin D levels after supplementing vitamin D3. In the group given vitamin D3 supplementation, it was found that TGF-β1 levels had decreased by 108.64. The reduction from the Wilcoxon statistical test results obtained p=0.001 (p<0.05), so there was a significant decrease after vitamin D3 supplementation. Based on the Spearman correlation test, there is a meaningful relationship between increased vitamin D levels with a reduction of TGF-β1 levels. The correlation coefficient is -0.753, which is harmful. The correlation coefficient is in a substantial range.

Conclusion: We concluded that vitamin D3 could increase 25(OH) D plasma level and decrease TGF-β1 levels significantly in children with stage 1 until 3 of CKD.

Keywords: chronic kidney disease, TGF-β1, vitamin D3

Cite this Article: Subandyah. K., Aditya, R. 2020. Effect Of Vitamin D3 On Transforming Growth Factor - B1 In Pediatric Patients With Stage 1 To 3 Chronic Kidney Disease: An Experimental Study And Randomized Controlled Trials. *Pediatrics Sciences Journal* 1(2) : 47-53.

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Received: 2020-09-22
Accepted: 2020-11-29
Published: 2020-12-01

INTRODUCTION

Chronic kidney disease (CKD) is a severe health problem with increasing morbidity and mortality, affecting children, causing significant socioeconomic issues. Early detection and intervention are crucial to slow down the disease's progression while maintaining the quality of life.[1] The incidence of CKD is different in each country, and it is estimated that the incidence of CKD in real life is higher than existing data because many cases are not detected. The Italkid-project study reported that the prevalence of CKD in children reached 12.1 patients/ year/ 1 million children with the age range of 8.8- 13.9 years. The prevalence of stage I and II CKD is reported to reach 18.5-58.3 cases/ 1 million children. Multicenter studies conducted in Turkey said that the incidence of CKD got 10.9 cases/ 1 million children, with the majority of stage V (32.5%), stage IV (29.8%), and stage III (25.8%). About 68% of children with CKD develop terminal kidney failure (GGT) by 20 years. Children with GGT have a survival rate of approximately 3% at the age of 20 years. In Indonesia, there are no national data on the incidence of CKD. In 2006 and 2007, the Department of Pediatrics, RSCM Jakarta, reported 382 CKD patients.[2,3]

Holistic intervention is essential for CKD therapy. One proposed method to reduce mortality is by blocking the progression of the disease. Some mechanisms that can cause CKD's advance are hypertension, cytokine, or growth factor mechanisms, the renin-angiotensin-aldosterone system, loss of podocyte cells, dyslipidemia, and proteinuria. Based on some of these mechanisms, the result of progressive pathophysiology in CKD is the formation of fibrotic tissue in the kidney structure, which causes permanent damage. Specific cytokines or growth factors markers of renal progression are transforming growth factor-beta (TGF-β), peroxisome proliferator-activated receptor-γ (PPAR-γ), and plasminogen activator inhibitor-1 (PAI-1). Hence controlling these specific cytokines to prevent the progression of CKD becomes crucial. Based on references,

elevated levels of TGF-β1 would increase the risk of fibrosis tissue formation. In contrast, the control of TGF-β1 cytokines at low levels will inhibit fibrosis progression.[4-8]

One mechanism to interfere TGF-β1 signaling pathway is by administering vitamin D3 (cholecalciferol). In a study by Aschenbrenner et al. in 2011, it was mentioned that supplementation of vitamin D3 in mice had a significant effect on reducing the bioactive effect of TGF-β1. Based on the literature, there are two mechanisms of vitamin D in order to inhibit fibrosis. The first mechanism is the inhibition of TGF-β signaling by vitamin D receptors (VDR) by binding smalls mothers against decapentaplegic homologs (SMAD 3), which will reduce the regulation of pro-fibrotic, pro-tumorigenic phenotypes in cancer-associated fibroblasts (CAF). The second mechanism, stull involving VDR, is by blunting TGF-β signals through genome competition that removes SMAD 3 from SMAD binding element (SBE).[9] To date, research on the role of vitamin D on TGF-β1 regulation in patients with CKD has not been elaborated. Investigation on the effect of vitamin D supplementation on TGF-β1 and IL-6 levels had been done in adult patients with stage V chronic kidney disease who undergo hemodialysis, with the result showed decreasing levels of TGF-β1 and IL-6. This result is essential as the control of CKD progression will reduce mortality and morbidity, especially in children with CKD.[10]

METHODS

This research is an experimental study with a double-blind, randomized clinical trial (RCT) study design, pre and post-test control group with the treatment of vitamin D3 administered via oral, given for 12 weeks. The research group consisted of two groups, namely group 1 (G1), the group was given vitamin D3 supplementation, and group 2 (G2), the group was given a placebo. The study was conducted at the Children's Nephrology Poly and Inpatient Rooms of the Saiful Anwar General Hospital Malang from June 2019 to September 2019. This study was approved by the RSSA Research Ethics Committee in Malang. Based on the calculation of the number of samples, the minimum sample needed for each group is 15 individuals. Inclusion criteria were patients with chronic kidney disease classified as stage 1-3 according to the National Kidney Foundation's Kidney Disease and Outcome Quality Initiative (NKF-KDOQI) classification, age range between 2 to 18 years old, parents/ guardians of the patients allowed their children to be included in the study after explained (informed consent). Exclusion criteria were patients with chronic kidney disease who had received vitamin D therapy before, patients with chronic kidney disease beyond stage 3, age-range less than two years old. The criteria for drop out were when the patients refused to take vitamin D3 supplementation recommended by researchers and when parents or children wanted to stop participating in the study.

Measurement of transforming growth factor β1 (TGF-β1) level

TGF-β1 levels were measured by the ELISA technique using the Human TGF-β1 Immunoassay (Cohesion # CEK1332) unit in ng/ ml. According to the American Journal of Kidney Disease, the levels of 25 (OH) D plasma are classified. Classified as severe deficiency status of vitamin D when the levels are less than 5ng/ ml, mild deficiency 5-15ng/ ml, and insufficiency when vitamin levels are 16-30ng/ ml. Plasma 25 (OH) D levels were measured before and after the administration of vitamin D. Serum 25 (OH) D levels were analyzed by ELISA (Alegria). Vitamin D3 was administered via oral according to the KDOQI protocol for 12 weeks (vitamin D was taken once a week).

Blood samples were collected from the subjects that were included in the inclusion criteria. The samples were taken simultaneously as routine blood evaluation of the CKD patient (monthly evaluation for kidney function). Samples from venous blood were collected in centrifuge tubes. The blood sample was left to clot for 2 hours at room temperature or overnight at 4˚C before centrifugation. The samples were then centrifuged for 20 minutes at a speed of 1000x g. The plasma was removed for further measurement of 25 (OH) D levels, or it can be stored in a refrigerator at -20° C or -80°.

Statistical Analysis

The data were analyzed statistically. Initially, the normality sample data was confirmed with the Shapiro Wilk test (n <50). Data classified as normally distributed if sig> 0.05. Normal distribution of data followed by pairwise testing using paired t-test or using Wilcoxon if the data is not normally distributed. We used the Pearson correlation test to test the relationship if the data are normal or Spearman if the data are not normal. Data were analyzed using 95% confidence level (α = 0.05). All statistical analysis was carried out with the help of SPSS for Windows 26.

RESULTS

The research was carried out in the pediatric nephrology clinic and inpatient of the children's room at Dr. Saiful Anwar Hospital Malang. In this study, Respondents were children with the age

range of 2-18 years old, divided into two groups, namely a group of chronic kidney disease patients who were given oral vitamin D3 and a placebo group (control) of 15 children in each group. As supplementary data, researchers also observed the demographic data, such as gender, age, body mass index (BMI), urea, creatinine, and LFG.

Table 1 showed the balance number of the male and female ratio in the two groups, while based on the age, the average age range of each group was between 9-10 years. The normality test results concluded that only the variable of decreased level of TGF-β1 in the placebo group had fulfilled the normality assumption (sig Shapiro Wilk> 0,05). Meanwhile, the others did not meet the normality assumption. Therefore, all tests were analyzed using non-parametric tests. Wilcoxon test analysis was performed to compare 25(OH)D plasma levels before and after the supplementation with vitamin D in pairs, and the detailed result was presented in Table 2.

Table 1. Baseline characteristics

Characteristics	Placebo (n=15)	Vitamin D3 Supplementation (n=15)
Sex		
• Male	7/15	8/15
• Female	8/15	7/15
Age (years)	9,73 ± 3,879	10,01 ± 3,79
BMI	17,03 ± 1,98	20,33 ± 7,30
Urea	35,35 ± 29,48	25,95 ± 6,84
Creatinine	0,53 ± 0,22	0,48 ± 0,26
LFG	150,83 ± 61,82	215,92 ± 163,98

Table 2. Result of Wilcoxon Test of Pre-Post 25(OH)D Plasma Level

Group	Mean of 25(OH)D Level ± SD		p-value
	Pre	Post	
Placebo	12,16 ± 7,01	7,01 ± 3,13	0,005
Supplementation with Vitamin D3	10,40 ± 6,23	19,75 ± 6,76	0,001

*p-value, significant if < 0,05

Table 3. Results of Wilcoxon Test of Pre-Post TGF-β1 Levels

Group	Mean of TGF-β1 Level ± SD		p-value
	Pre	Post	
Placebo	162,96 ± 133,53	228,96 ± 140,97	0,001
Supplementation with Vitamin D3	282,07 ± 156,18	173,43 ± 91,24	0,001

* p-value, significant if < 0,05

Table 4. Correlation Test between Increased 25 (OH) D Plasma Levels with Decreases TGF-β1 levels

Independent Variable	Dependent Variable	p value	R count
Increased Vitamin 25(OH)D Plasma level	Decreased TGF-β1level	0,000	-0,753

* p-value, significant if < 0,05

Based on **Table 3**, the average level of 25 (OH) D in the placebo group decreased between the pre and post averages with a value of 5,15. The Wilcoxon statistical results showed that the decrease was significant with p-value= 0,005. Simultaneously, the group given vitamin D3 supplementation showed increasing value between pre and posted 25 (OH) D levels by 9,35. Based on the Wilcoxon statistical test results was obtained p = 0,001 (p <0,05); thus, it was concluded that there was a significant increase in levels of 25 (OH) D after supplementation with vitamin D3. The analysis using the Wilcoxon test to compare TGF-β1 levels pre-post in pairs were presented in Table 3.

According to Table 3, it is known that the mean TGF-β1 level in the placebo group between the pre and post-measurement increased with the value of 66. Wilcoxon statistical results stated that the increase was significant with a p-value of 0,001. The group treated with vitamin D3 supplementation demonstrated that TGF-β1 levels had decreased by 108,64. The value of the reduction from the Wilcoxon statistical test results was p= 0,001 (p <0,05), so it was concluded that there was a significant decrease in TGF-β1 levels after vitamin D3 supplementation. To determine whether the relationship between the increasing level of plasma 25 (OH) D and the decreasing level of TGF-β1, the Spearman correlation test was performed with the results shown in Table 4.

Based on Table 5 regarding the Spearman correlation test obtained sig= 0,000 (p <0,05). These results concluded that there was a significant relationship between the increasing plasma 25 (OH) D levels with the decreasing plasma TGF-β1 levels. The value of the resulting correlation coefficient is -0,753. The negative sign's statistical significance explained an inverse relationship between the two variables, that when plasma 25 (OH) D level increases, then the TGF-β1 levels tend to decrease. The correlation coefficient with the value of 0,753 was interpreted statistically as a high correlation coefficient.

DISCUSSION

Baseline Characteristic

In this study, 30 subjects included in this research were divided into two groups: a group of pediatric patients with chronic kidney disease treated according to the cause of CKD and pediatric patients with chronic kidney disease treated according to the cause of CKD with vitamin D3. The characteristics of the data obtained with an age range from 3 to 16 years old, with an average age of 9 to 10 years

old. This is consistent with epidemiological data in the Italkid-project study, which reported that CKD prevalence in children reached 12,1 cases/year/1 million children with an age range of 8,8-13,9 years old.[2]

Subject distribution by sex found that males and females have almost the same prevalence. Data from previous studies had not specifically addressed the ratio of CKD incidence by sex. Our data was based on multicenter research in Turkey, which reported CKD incidence of 10.9 cases per 1 million children, with the majority of stage V (32,5%), stage IV (29,8%), and stage III (25,8%) with an even distribution of sexes.[3]

This study's results were obtained from 30 subjects with chronic kidney disease who had vitamin D levels below the normal range. The criteria were under references stating that patients with CKD could not produce an adequate amount of 1,25 (OH) D. These patients might also have low initial nutritional intake associated with a reduction in appetite caused by uremia, as well as food restrictions from specific nutrients, such as phosphorus, causing inadequate substrates for conversion to calcitriol.[11,12]

Level of 25 (OH) D Plasma

In this study, the average level of 25 (OH) D before being given vitamin D3 supplementation was measured. The placebo group had a slightly higher average than the treatment group, with a difference of 1,76. The p-value obtained from the Mann Whitney test was 0,576, which was higher than 0,05. This concluded that there were no difference in 25 (OH) D levels before vitamin D3 supplementation was provided between the two study groups. After administering vitamin D3 supplementation, the average treatment group produced a much higher average of 19,75, with a difference of 12,74 compared to the placebo group. The p-value obtained from the Mann Whitney test results is 0,000, which is smaller than 0,05. This concluded that there were differences in levels of 25 (OH) D after vitamin D3 supplementation. This is following the physiology of the activation of vitamin D, which is explained as follows, that 25-hydroxyvitamin D3 is the main form of vitamin D found in the circulation. In circulation, 25 (OH) D3 is transported by DBP to the kidneys. There, a receptor named magalin plays a role in internalizing 25 (OH) D3 through endocytosis. After 25 (OH) D3 has internalized the proximal renal tubule, 25 (OH) D3 undergoes hydroxylation at the position of carbon 1 in ring A which results in the formation of the active form of vitamin D, 1,25-dihydroxyvitamin D3 (1,25 (OH) ¬2D3) or calcitriol which has a role in the biological process of vitamin D. In patients with CKD the 25 (OH) D3 activation process does not occur due to kidney damage, resulting in decreased levels of vitamin D in plasma; thus administration of vitamin D supplementation in the active form will increase plasma vitamin D levels physiologically.[13]

Level of Transforming Growth Factor Beta 1 (TGF-β1)

In this study, we measured the average TGF-β1 level before vitamin D3 supplementation was given. The placebo group had an average lower than the treatment group, with a mean difference of 119,11. The p-value obtained from the Mann Whitney test results was 0,019, which was smaller than 0,05. This concluded that there were differences in TGF-β1 levels before vitamin D3 supplementation was given. After administering vitamin D3 supplementation, the mean treatment group had decreased compared to the value before, whereas the placebo group showed an increased value. The difference between the two groups was 55,54. Mann Whitney test results showed the sig value obtained 0,272 (p> 0,05). This concluded that there was no difference in TGF-β1 levels after vitamin D3 supplementation. However, these results clinically indicated that supplementation with vitamin D3 proven to reduce TGF-β1 levels compared to the placebo group that showed an increase. It is known that the mean TGF-β1 levels in the placebo group increased with the difference in the pre and post mean values of 66. Wilcoxon's statistical results stated that the increase was significant with a p-value of 0,001. The group administered with vitamin D3 supplementation demonstrated that TGF-β1 levels had decreased by 108,64. The value of the reduction from the Wilcoxon statistical test results obtained p = 0,001 (p <0,05), so it was concluded that there was a significant decrease in TGF-β1 levels after vitamin D3 supplementation.

The role of TGF-β in kidney inflammation is explained as follows. The occurrence of fibrosis in the kidney, although it is the kidney's direct damage, is not caused by autoimmune disease. However, it still consists of a significant component of inflammatory cells, thus causing overexpression of inflammatory genes, releasing pro-inflammatory cytokines, activation of NF-κB, and infiltration of macrophages lymphocytes. Hence, kidney fibrosis can also be considered as the result of a chronic inflammatory reaction. A continuous kidney damage process is associated with increased expression of pro-inflammatory cytokines TNF-α and IL-1 and is associated with NF-κB activation. Prolonged kidney damage also leads to activation of the intrarenal renin-angiotensin system, mediated

by Ang II, which acts as a pro-inflammatory cytokine in the kidney by activating NF-κB. In addition to NF-κB, Ang II signaling, through its AT1 and AT2 receptors, also increases the expression of other pro-inflammatory genes, including those that encode IL-6 and chemoattractant proteins 1. Increased Ang II production further induces renal oxidative stress, which plays a vital role in inducing inflammatory states due to oxidative stress are the primary activators of the NF-κB pathway. Following the secretion of inflammatory cytokines, kidneys and other organs release TGF-β1 after the damage occurs. TGF-β1 is considered a major anti-inflammatory cytokine. The biological significance of the release of TGF-β1 by kidney cells in damaged kidneys might act to moderate anti-inflammatory reactions and heal damaged tissue. TGF-β1 works against pro-inflammatory cytokines IL-1 and TNF-α in glomerular disease and is a deactivator of macrophages during kidney injury. TGF-β1 is induced by Ang II and is also produced by interstitial fibroblasts and macrophage infiltration.[14]

However, although TGF-β1 acts as an anti-inflammatory cytokine to heal damaged kidneys, TGF-β1 also promotes chronic kidney disease development because it plays a significant role as a fibrogenic agent, as explained above. Therefore, the expression of sustained deviations from TGF-β1 results in the pathological accumulation of ECM material in both the glomerulus and interstitial compartments. TGF-β1 also has several pro-inflammatory properties because of its functions as a chemo-attractant of leukocytes and induces cyclooxygenase-2 in mesangial cells. The pro-fibrotic role of TGF-β1 is also based on its main character for the formation of myofibroblasts through the activation of fibroblasts and the induction of epithelial and endothelial transitions to mesenchymal cells. The inflammation process also seems to aid EMT because Ang II induces the synthesis of TGF-β1 and its receptors in tubular epithelial cells. Thus, TGF-β1, a molecule that is secreted to control inflammation in the kidney, also promotes fibrosis development.[15,16]

Relationship between TGF-β1 levels with Vitamin 25 (OH) D Plasma

Based on the Spearman correlation test, obtained sig = 0,000 (p <0,05). These results concluded a significant relationship between an increase in 25 (OH) D plasma levels with a decrease in TGF-β1 levels. The magnitude of the resulting correlation coefficient is -0,753, which showed a negative value. This means that if the level of 25 (OH) D plasma increases, the TGF-β1 level would decrease. The correlation coefficient is classified as strongly correlated.

The interaction of 1,25-dihydroxy vitamin D3 (1,25- (OH) 2D3) plays an essential role in regulating the immune response and its role in bone metabolism. TGF-β cytokines regulate various biological processes, including proliferation, cellular differentiation, immune modulation, and extracellular matrix deposition modulation. 1,25-dihydroxyvitamin D3 interacts in vitro with the SMAD protein as an essential regulator of TGF-β signal transduction. We suggested that the administration of exogenous vitamin D3 will alter protein levels for TGF-β signaling in kidney tissue. In this discussion, we would like to incorporate several studies regarding the effect of vitamin D3 on TGF-β regulation.[16]

In the studies conducted on asthma, the mediators of inflammatory cells such as macrophages, lymphocytes, eosinophils, fibroblasts, and airway epithelial cells synthesize TGF-β. Cytokines derived from T helper 2 (Th2), including interleukine-4 (IL-4), play an important role in airway remodeling. TGF-β induces the expression of tissue inhibitors of matrix metalloproteinase 1 (TIMP-1) through the Th2 pathway. Vitamin D reduces the production of CD4 + T cells from cytokines Th2, IL-4, IL-5, and IL-13 and promotes the release of IL-10. Calcitriol concentrations in vitro are crucial in determining the effects on the differentiation and function of Th2 cells. In the same way, vitamin D inhibits the proliferation and activation of fibroblasts by inhibiting the TGF-β pathway and reducing the expression of extracellular matrix proteins.[17]

In other studies of TGF-β and vitamin D in the event of malignancies, it is generally known that the involvement of vitamin D3 metabolites (referred to as calcitriol or vitamin D) in inhibiting the growth of cancer cells has been extensively investigated. In this case, a cross mechanism occurs between 1,25-dihydroxy vitamin D3 (1,25- (OH) 2D3) and tumor microenvironment (TME), which signals cancer-associated fibroblasts (CAFs) as targets for anticancer by calcitriol. Phenotypic similarities are shown in myofibroblast, which settled in cancerous tissue and the fibrotic locus of non-neoplastic diseases, so a study was carried out on the mechanism of vitamin D action in chronic non-neoplastic diseases and in cancer to assess mechanical similarities and divergences. Some observations showed that vitamin D or synthetic ligands through vitamin D receptors (VDR) inhibits the growth factor-β / SMADs transformation that signals myofibroblasts regardless of the type of trigger factor.[18]

The role of vitamin D3 in the regulation of TGF-β in systemic sclerosis (SSc) was also explained in another study. VDR expression has been analyzed in SSc disease; VDR signaling is modulated by

siRNA and selective paricalcitol agonists. VDR expression is reduced in fibrosis in patients with SSc. Decreased expression of VDR increases fibroblast sensitivity to TGF-β. In contrast, VDR activation by paricalcitol reduces the stimulating effect of TGF-β on fibroblasts, inhibiting collagen release and myofibroblast differentiation. Paricalcitol stimulates the formation of phosphorylated VDR and SMAD 3 to inhibit SMAD transcription. Treatment with paricalcitol exerts a strong antifibrotic effect induced by TBRI. In conclusion, VDR is a negative regulator of TGF-β / SMAD signaling. Impaired VDR signaling with reduced VDR expression and decreased ligand level can contribute to hyperactive TGF-β signaling and fibrosis activation in SSc disease.[19]

Cell or receptor exposure to 1,25 (OH) 2D3 or the active form of vitamin D results in decreased regulation of expression of large numbers of pro-fibrotic genes induced by TGF-β1. A biomolecular study mentioned that vitamin D does not change fibroblasts' basal expression; it shows that vitamin D selectively interferes with TGF-β signaling. When observed in the biomolecular aspect, vitamin D receptor (VDR) and SMAD 3 are in the same location. The researchers had analyzed that the vitamin D receptor-retinoid acid X heterodimer complex receptor complex (VDR / RXR) interfered with TGF-β1 / SMAD 3 transcriptional regulation of the pro-fibrotic gene by competing in binding to SMAD 3 with the SMAD binding element, therefore, blunting the signaling to the target gene. This genomic competition is enabled by the mechanism of TGF-β1 chromatin dependent remodeling, which is affected by VDR activation, which causes changes in chromatin structure. Changes in the structure of chromatin are triggered by histone acetylation, especially by p300 acetyltransferase. VDR competition against SMAD 3 in chromatin disrupts the pro-fibrotic mechanism by TGF-β1. Another biomolecular mechanism that occurs is that Vitamin D receptor element (VDRE) activates the recruitment of protein co-activators, such as histone acetyltransferases and co-repressors, causing epigenetic changes.

Study limitations

This study only involved one research center with limited subjects and the period of research. Different medication routines taken by each research subject could not be uninformed, so it is difficult to know whether there is an interaction between patients' drugs on vitamin D absorption. Based on epidemiological studies, around 40% of Indonesian children experience vitamin D deficiency. Besides, our trust in patient compliance to take supplementation depends on the patients' honesty, even though we had tried to control it by establishing the drug card to monitor the administration of the medication. The etiology of the primary disease of CKD in children varies widely. It is difficult to establish the effect of therapy with vitamin D on each primary disease. Also, there is no specific reference mentioning that the level of plasma biomarker can accurately reflect the kidney's exact level.

ETHICAL CLEARANCE

Already had agreement from Saiful Anwar hospital ethical clearance team

CONFLICT OF INTEREST

No conflict if interest

FUNDING

Private funding

REFFERENCE

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