**Study of Vitamin D deficiency and CD4**+**CD28null Tcells and their relation to atherosclerosis in chronic kidney disease patients**

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**Abstract: Aim*:*** Our study aimed to detect a link between 25(OH) vitamin D deficiency, inflammation and CD4+CD28null T-cell expansion, which might contribute to atherosclerosis in CKD subjects.

**Background:** Cardiovascular diseases (CVD) are the main cause of mortality in CKD patients. Atherosclerosis is highly prevalent in advanced renal failure and progresses faster in patients with renal dysfunction than in the general population.

**Methods*:*** This study was conducted on newly diagnosed 40 chronic kidney disease patients and 30 normal individuals who served as control. Common carotid artery intima media thickness (CCA-IMT) was measured with an ultrasound system. Highly sensitive C-reactive protein (hsCRP) was measured by immunoturbidimetric assay. The frequency of circulating CD4+CD28null T cells was evaluated by flowcytometry.25(OH) vitamin D was measured in serum by enzyme linked immunosorbent assay.

**Results:** CKD subjects exhibited higher CCA-IMT (0.97±0.21 vs 0.56±0.10mm, P <0.001), hs-CRP (48.7±25.8vs 6.8±1.7mg/mL, P <0.001), CD4+CD28null cell frequency (5.7±1.5 vs 1.5±0.49%, P <0.001) and lower 25(OH) vitamin D levels 14.9±9.05vs 47.2±7.9ng/mL, P < 0.001). In CKD subjects, serum 25 (OH) vitamin D level showed a strong inverse correlation with CCA-IMT (r = -0.699, P < 0.001), with CD4+CD28null cell frequency (r = -0.966, P< 0.001) and with hs-CRP (r = -0.742, P < 0.001).

Conclusion: 25 (OH) vitamin D deficiency is associated with inflammatory activation, increased CD4+CD28null T-cell expansion and increased CCA-IMT, a preclinical marker of atherosclerosis in CKD subjects.

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Key Words: atherosclerosis, chronic kidney disease, intima-media thickness, CD4+CD28null T-cell, vitamin D.

**1. Introduction**

Cardiovascular diseases (CVD) are the main cause of mortality in chronic kidney disease (CKD) patients. Premature CVD, including stroke, peripheral vascular disease, sudden death, coronary artery disease, and congestive heart failure, is a notorious problem in patients with CKD. Atherosclerosis is highly prevalent in advanced renal failure and progresses faster in patients with renal dysfunction than in the general population **(Cheung and William, 2011).**

Traditional CVD risk factors do not adequately explain this disproportionate increase in risk and the role of non-traditional risk factors such as inflammation, oxidative stress and altered immune function have been explored **(Cachofeiro et al., 2008).**

Large epidemiological studies have shown a relationship between vitamin D deficiency and CVD risk and mortality in the general population. In patients with CKD, vitamin D deficiency is associated with increased CV mortality **(Yadav and Jha, 2011).**

Deficiencyof vitamin D is associated with inflammatory activation, decreased arterial compliance and increased intima-media thickness. Increased inflammation, impaired endothelial function and central arterial stiffness are common in patientswith CKD **(Ngo et al., 2010).**

It has been suggested that vitamin D directly modulates immune response, both of Th1 and Th2 type and regulatory T cell function. CD4+ T-cells express vitamin D receptor and can be modulated in response to vitamin D therapy. It is also possible that indirect actions might play a role. It has been suggested that the expansion in CD4+CD28null cell population is part of increased inflammation in CKD. Vitamin D deficiency is indeed regarded as a pro-inflammatory state. However, the exact mechanism by which inflammation might influence the loss of CD28 is not well-understood. Previous study has shown some T cell subsets, in particular the CD4+CD28null cells, to be associated with cardiovascular disease **(Yadav and Jha, 2011).**

Loss of CD28 from CD4+ cells confers on them a cytotoxic phenotype,with the ability to release interferon-γ (INF-γ), perforinand granzyme**(Betjes et al., 2010).**

They infiltrate atherosclerotic plaquesand may contribute to their development and instability bykilling endothelial cells and vascular smooth muscle cells **(Litjens et al., 2011).**

Evidence (in vitro & in vivo) suggests that CD4+CD28null T lymphocytes may be involved in the initiation and progression of atherosclerosis, whereas they subsequently contribute to the destabilization of atherosclerotic plaques **(Liuzzoe et al., 2007).**

**2. Subjects and methods**

The study was inducted at nephrology unit of internal medicine department and clinical pathology department, Menoufiya University Hospital in the period from April 2014 to Oct. 2014. This study was conducted on newly diagnosed 40 chronic kidney disease patients and 30 normal individuals who served as control. Patients with known coronary artery disease, heart failure, malignancies, active or chronic infections, smokers, taking immunosuppressive drugs or vitamin D supplements were excluded. CKD stage was defined according to Kidney Disease Outcome Quality Initiative (KDOQI) criteria and estimated glomerular filtration rate (eGFR) was calculated using Modification of Diet in Renal Disease (MDRD) formula.

All studied groups were subjected to

1. **Full history taking and physical examination.**
2. **Routine laboratory investigations**: including CBC (APX pentra XL80), Kidney function, Serum electrolytes, Liver function tests, Lipid profile, Blood sugar using AU 480 (Beckman, USA).
3. **Highly sensitive C-reactive protein (hs CRP):**

Serum was separated then analyzed by immunoturbidimetric assay (Orion DiagnosticaTurbox).

1. **Detection of CD4+CD28null T lymphocytes by flowcytometry:**

 50μL of each whole blood sample was mixed together with 20μL of FITC conjugated anti-CD4 monoclonal antibody and PE conjugated CD28. Another tube without monoclonal antibody was used as auto-control and incubated for 20 min in dark at 4 °C. 2mL of lysis solution was added to each tube and incubated for 10 minute. The tubes then were centrifuged at 1800rpm for 5 minutes and the supernatant was discarded. The tubes then were washed twice with 2mL P.B.S. (phosphate buffered saline) at 1800rpm for 5minute. The cell bullet then was re suspended in 500mL P.B.S. and kept for flowcytometric analysis.

1. **25 OH vitamin D (by ELISA technique):**

The serum was collected and stored at -20 °C for further analysis by ELISA. Vitamin D status was defined as sufficient (> 30ng/ml), insufficient (15-30ng/ml) and deficient (<15ng/ml) **(Mahon et al., 2003)**

1. **Radiological investigations** for pelvic-abdominal ultrasound and estimation of common carotid artery intima – media thickness.

**Statistical Analysis**

The data collected were tabulated and analyzed by SPSS (statistical package for social science) version 22.0 on IBM compatible computer. Two types of statistics were done:Descriptive statistics: e.g. percentage (%), mean and standard deviation (SD).Analytic statistics: Chi-square test (χ2), Student t-test, Mann-Whitney test, ANOVA (f) test, Kruskal-Wallis test and Spearman correlation (r). P value less than 0.05 was considered statistically significant.

**3. Results**

**1-Clinical and demographic data**

The study was conducted on 40 CKD patients, 25 (62.5%) males and 15 (37.5%) females and also 30 controls, 14 (46.7%) males and 16 (53.3%) females with no significant difference (p-value: 0.19).Mean age of the patients was (60.9±7.8 years) and the controls was (58.1±10.8 years). No significant difference in mean age was observed between patients and control (p-value: 0.22).Twenty one patients (52.5%) were diabetic and 19 patients (47.5%) were hypertensive. All of the 30 controls are free from diabetes mellitus and hypertension at the start of this study (100%) with a highly significant difference (p-value <0.001). **Table (1)**

**2-Biochemical parameters: Table (2)**

* Hb level in the patient group (10.4±1.7) was highly significant lower than control group (13.1±0.56) (p-value > 0.001), also serum albumin in the patients group (3.5±0.65) was highly significant lower than controls group (4.5±0.49) (p-value: > 0.001).
* Urea level in the patient group (76.1±42.4) was highly significant higher than control group (25.4±4.9) (p-value: >0.001), also serum creatinine in the patients group (3.2±1.8) was highly significant higher than controls group (0.72±0.12) (p-value: > 0.001).
* We found that serum calcium level in the patient group (7.9±0.69) was highly significant lower than control group (9.4±1.4) with (p-value <0.001), also serum phosphorus was highly significant higher in the patients group (4.3±0.57) than controls group (3.03±0.47) (p-value >0.001).
* There was no significant difference in the patients’ Cholesterol level (154.3±34.2) when compared to control group (141.3±21.4) with (p-value: 0.07), also we didn’t find significant difference in the patients’ serum Triglyceride (136.5±36.1) when compared to control group (120.9±33.8) with (p-value: > 0.06), also we didn’t find significant difference in the patients’ serum HDL (34.3±7.9) and LDL (92.3±28.3) when compared to control group (HDL 32.5±10.1) and ( LDL 84.4±17.3).

**3-Comparison between CKD patients and healthy control groups as regarding hs-CRP, CCA-IMT, CD4+ CD28 null T cells and 25 (OH) vitamin D. Table (3)**

* Serum hs-CRP was highly significant higher in the patient group (48.7±25.8) than control group (6.8±1.7) (p-value <0.001).
* CCA-IMT in the patient group (0.97±0.21) was highly significant higher than control group (0.56±0.10) (p-value: <0.001).
* We found that CD4+ CD28 null T cells in the patient group (5.7±1.5) was highly significant higher than control group (1.5±0.49) (p-value <0.001). **table3 & figure1**
* Serum 25 (OH) vitamin D level in the patient group (14.9±9.05) was highly significant lower than control group (47.2±7.9) (p-value <0.001).Vitamin D level among CKD patients were divided into three categories, sufficient, insufficient and deficient and hs-CRP, CCA-IMT, CD4+ CD28 null T cells were analyzed according to these categories **(figure 2)**

**4-Correlation analysis**

* hs-CRP showed no significant correlation with age (p-value: 0.75**).** However**,** itshowed a significant negative correlation with eGFR (r = -0.523**,** p-value = 0.001**),** highly significant negative correlation with 25 (OH) vitamin D (r = -0.742, p-value <0.001) and highly significant positive correlation with CCA-IMT (r= 0.570, p-value <0.001) and with CD4+ CD28 null T cells (r= 0.759, p-value <0.001). **Table (4)**:
* CD4+ CD28 null T cells showed a significant positive correlation with age (r**=** 0.455**,** p-value: 0.003**),** highly significant negative correlation with 25 (OH) vit D (r**=** -0.966**,** p-value: <0.001**)** and with eGFR (r= -0.780, p-value: <0.001), highly significant positive correlation with CCA-IMT (r**=** 0.866**,** p-value: <0.001) andwith hs-CRP (r**=** 0.759**,** p-value: <0.001). **Table (5)**
* 25 (OH) vitamin D showed a significant negative correlation with age (r= -0.478**,** p-value = 0.002), highly significant positive correlation with eGFR (r= 0.816, p-value <0.001) and highly significant negative correlation with hs-CRP (r= -0.742**,** p-value<0.001), with CD4+ CD28 null T cells (r= -0.966**,** p-value <0.001**)** and with CCA-IMT (r= -0.699,p-value <0.001). **Table (6)**

**Table (1):** Demographic characteristics of groups

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
|  | **CKDpatients****(No=40)** | **Healthy controls****(No=30)** | **t-Test** | **P value** |
| **Age (years)**Mean±SD | 60.9±7.8 | 58.1±10.8 | 1.24 | 0.22 |
| **Gender** Male  Female | **No** | **%** | **No** | **%** | **X2 test** | **P value** |
| 2515 | 62.537.5 | 1416 | 46.753.3 | 1.74 | 0.19 |
| **Chronic diseases**No DM Hypertension | 02119 | 0.052.547.5 | 3000 | 1000.00.0 | 70.00 | <0.001 |

**Table (2):** Comparison between CKD patients and healthy control groups as regarding routine laboratory investigations

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
|  | **CKDpatients****(No=40)** | **Healthy controls****(No=30)** | **t-Test** | **P value** |
| **Mean±SD** | **Mean±SD** |
| Hemoglobin (Hb) (g/dl) | 10.4±1.7 | 13.1±0.56 | 8.23 | <0.001\*\* |
| Albumin (ALB) (g/dl) | 3.5±0.65 | 4.5±0.49 | 7.25 | <0.001\*\* |
| Urea (mg/dl) | 76.1±42.4 | 25.4±4.9 | 6.71# | <0.001\*\* |
| Creatinine (mg/dl) | 3.2±1.8 | 0.72±0.12 | 7.15# | <0.001\*\* |
| Cholesterol (mg/dl) | 154.3±34.2 | 141.3±21.4 | 1.84 | 0.07 |
| Triglyceride(mg/dl) | 136.5±36.1 | 120.9±33.8 | 1.86 | 0.06 |
| HDL (mg/dl) | 34.3±7.9 | 32.5±10.1 | 0.79 | 0.43 |
| LDL (mg/dl) | 92.3±28.3 | 84.4±17.3 | 1.35 | 0.18 |
| Calcium (Ca) (mg/dl) | 7.9±0.69 | 9.4±1.4 | 5.32 | <0.001\*\* |
| Phosphorus (PO4)(mg/dl) | 4.3±0.57 | 3.03±0.47 | 10.37 | <0.001\*\* |

#Mann-Whitney test \*\*Highly significant difference

**Table (3)**: Comparison between CKD patients and healthy control groups as regarding hs-CRP,CCA-IMT, CD4+ CD28null T cells and 25 (OH) vitamin D.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
|  | **CKD****patients****(No=40)** | **Healthy controls****(No=30)** | **Mann Whitney****Test** | **P value** |
| **Mean±SD** | **Mean±SD** |
| **hs-CRP(mg/dl)** | 48.7±25.8 | 6.8±1.7 | 7.14 | <0.001\*\* |
| **CCA-IMT** | 0.97±0.21 | 0.56±0.10 | 9.83 | <0.001\*\* |
| **CD4+ CD28 null cells (%)** | 5.7±1.5 | 1.5±0.49 | 14.89 | <0.001\*\* |
| **25 (OH) vitamin D (ng/ml)** | 14.9±9.05 | 47.2±7.9 | 7.13 | <0.001\*\* |

\*\*Highly significant correlation

|  |  |
| --- | --- |
| 1a | 1b |

**Fig1**: flowcytometric analysis of CD4+CD28 null in control (1a) and CKD patients (1b**)**

**Fig2:** Comparison between three 25 (OH) vitamin D statuses among CKD patients

**Table (4)**: Spearman correlation between hs-CRP and age, eGFR, 25 (OH) vit, D, CCA-IMT and CD4+ CD28 null cells among cases

|  |  |
| --- | --- |
|  | **hs-CRP** |
| **R** | **P value** |
| **Age** | 0.052 | 0.75 |
| **eGFR** | -0.523 | 0.001\* |
| **25 (OH) vit, D** | -0.742 | <0.001\*\* |
| **CCA-IMT** | 0.570 | <0.001\*\* |
| **CD4+ CD28 null cells** | 0.759 | <0.001\*\* |

\*\*Highly significant correlation

**Table (5)**: Spearman correlation between CD4+ CD28 null T cells and age, 25 (OH) vit D, eGFR, CCA-IMT and hs-CRP among CKD patients

|  |  |
| --- | --- |
|  | **CD4+ CD28 null T cells** |
| **R** | **P value** |
| **Age** | 0.455 | 0.003\* |
| **25 (OH) vit, D** | -0.966 | <0.001\*\* |
| **eGFR** | -0.780 | <0.001\*\* |
| **CCA-IMT** | 0.866 | <0.001\*\* |
| **hs-CRP** | 0.759 | <0.001\*\* |

**Table (6):** Spearman correlation between 25 (OH) vit, D and age, eGFR, hs-CRP, CD4+ CD28 null cells and CCA-IMT among cases

|  |  |
| --- | --- |
|  | **25 (OH) vit, D** |
| **R** | **P value** |
| **Age** | -0.478 | 0.002\* |
| **eGFR** | 0.816 | <0.001\*\* |
| **hs-CRP** | -0.742 | <0.001\*\* |
| **CD4+ CD28 null cells** | -0.966 | <0.001\*\* |
| **CCA-IMT** | -0.699 | <0.001\*\* |

\*Significant correlation \*\*Highly significant correlation

**4. Discussion**

Cardiovascular diseases (CVD) are the main cause of mortality in CKD patients**(Cheung and William, 2011).**Traditional CVD risk factors do not adequately explain this disproportionate increase in risk and the role of non-traditional risk factors such as inflammation, oxidative stress and altered immune function have been explored **(Cachofeiro et al., 2008).**

Vitamin D deficiency has been associated with a number of diseases including cancers, diabetes, infections, hypertension and CVD **(Yadav et al., 2012).** Our data further add to the emerging literature about the role of vitamin D in cardiovascular health in CKD patients.

In the present study there are no statistically significant differences between the studied groups as regard age and gender

We found that Hb level in the patient group was highly significant lower than that of the control group because erythropoietin deficiency**(Brugnara and Eckardt 2011).**

Also serum albumin was highly significant lower in the patient group than that of the control group. The decline in nutritional status during the course of progressive kidney disease may be caused by disturbances in protein and energy metabolism **(Astor et al., 2011).**

It was found that urea and creatinine levels in the patient group were highly significant higher than that of the control group as the kidneys are not able to filter waste efficiently, there will likely be a rise in urea and creatinine levels in the blood **(Miller, 2009).**

It was found that serum calcium level in the patient group was highly significant lower than that of the control group.Also, serum phosphorus was highly significant higher in the patient group than that of the control group. The fractional excretion of phosphate drops, leading to an increase in the serum phosphate level. This is accompanied by a reciprocal decrease in serum calcium concentration. Also the 1α-hydroxylation in the kidney of 25-hydroxyvitamin D [25-(OH)D] declines, leading to lower serum levels of 1,25-dihydroxyvitamin D [1,25-(OH2)D]. This lack of 1,25-(OH2)D contributes to the development of hypocalcemia**(National Kidney Foundation, 2003).**

We found that serum hs-CRP was highly significant higher in the patient group than that of the control group. This finding is consistent with **MeenakshiSreeramet al, 2013**who reported that the level of CRP was significantly higher in CKD patients. CRP directly modulates the production of endothelium derived vasoactive factors, including down regulating endothelial NOS-derived NO while augmenting the production of the potent endothelium-derived vasoconstrictor endothelin. Additionally, CRP facilitates endothelial cell apoptosis and attenuates angiogenesis, which is an important compensatory mechanism in ischemia (**Vermaet al, 2003).**

It was found in the present study that the CCA-IMT level in the patient group was higher than that of the control group.**Lubomirova et al.,2014** found that Intima-media was thickened in patients with CKD the mean value was over 0.75mm. The values in the CKD group were significantly higher than those in the healthy controls the mean value was over 0.59mm. CIMT can be considered as an early index of generalized atherosclerosis, the development of atherosclerosis can be useful to evaluate the efficacy of eventual therapeutic strategies by reducing endothelial damage **(LAI et al, 2014).**

In our study, hs-CRP showed highly significant positive correlation with CCA-IMT this was in agreement with **Zoccali et al., 2002**, **Szeto et al., 2003, Covic et al., 2005 and Kim et al., 2013**. This was against study done by **Yadav et al., 2012**, who did not find any association of hs-CRP with CCA-IMT.

In our study, the CD4+CD28null T cells level in the CKD patients group showed higher levels when compared to the control group. Similar finding were reported in the work of **MichielandBetjes et al, 2013** who reported that These CD4+CD28null T cells are a rare population in most healthy individuals and usually do not exceed a few percent of the total CD4+ T cell population .

In the present study, there was significant positive correlation between CD4+CD28null T cells and CRP and this against by reported by **Yadav et al., 2012**. Also, there was significant positive correlation between CD4+CD28null T cells and IMT and this is agree with **Yadav et al., 2012** and **Zhiping et al in 2013** who found positive correlation between CCA-IMT and CD4+CD28null T cell frequency.

Loss of CD28 from CD4+ cells confers on them a cytotoxic phenotype,with the ability to release interferon-γ (INF-γ), perforinandgranzyme**(Betjes et al., 2010)**.

They infiltrate atherosclerotic plaquesand may contribute to their development and instability bykilling endothelial cells and vascular smooth muscle cells **(Litjens et al., 2011)*.***

Evidence (in vitro & in vivo) suggests that CD4+CD28null T lymphocytes may be involved in the initiation and progression of atherosclerosis, whereas they subsequently contribute to the destabilization of atherosclerotic plaques **(Liuzzoe et al., 2007)**.

It has been suggested that vitamin D directly modulates immune response, both of Th1 and Th2 type and regulatory T cell function. CD4+ T-cells express vitamin D receptor and can be modulated in response to vitamin D therapy. ***(*Yadav and Jha, 2011).**So,the present study aimed to detect a link between 25(OH) vitamin D deficiency, inflammation and CD4+CD28null T-cell expansion, which might contribute to cardiovascular disease in CKD subjects.

We found that serum 25 (OH) vitamin D level in the patient group was significantly lower than that of the control group. Similar findings were reported by **Yadav et al., 2012.**

The research groups of **Targher et al.** and **Liu et al**., 2012 demonstrated an inverse correlation between vitamin D levels and cIMT severity.

For instance, studies focusing on carotid intima-media thickness (cIMT), a well-recognized biomarker of subclinical atherosclerosis also associated with a wide range of CV risk factors and CV diseases showed a potential relationship between vitamin D deficiency and atherogenesis**(Bauer et al., 2012).**

We also showed an association of vitamin D deficiency with inflammatory activation and expansion of the CD4+28null T-cell population, which has been implicated in the genesis of atherosclerosis, where 25 (OH) vitamin D level showed highly significant negative correlation with hs-CRP, CD4+CD28null cells and CCA-IMT. Similarly**Yadav et al., 2012** showed a correlation between vitamin D deficiency and the expansion of CD4+CD28null cell population in CKD subjects and a strong association between vitamin D deficiency and increased CCA-IMT, where the vitamin D level was inversely correlated with CCA-IMT, CD4+CD28null T cell frequency and hs-CRP levels.

Vitamin D deficiency is indeed regarded as a pro-inflammatory state. However, the exact mechanism by which inflammation might influence the loss of CD28 is not well-understood. Many studies have shown some T cell subsets, in particular the CD4+CD28null cells, to be associated with cardiovascular disease ***(*Yadav and Jha, 2011).**Accordingto our knowledge, this is the first study to be done on Egyptian population but it is limited by the small sample size**.** So, further studies with larger size and investigation of vitamin D supplementation on CD4+CD28null T-cell are recommended.

**In conclusion:** Based on these findings, we suggest that 25 (OH) vitamin D deficiency is associated with inflammatory activation, increased CD4+CD28null T-cell expansion and increased CCA-IMT, a preclinical marker of atherosclerosis in CKD subjects.

**Conflicts of Interest**

The authors state no conflicts of interest.

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