BRIEF REPORT

Clinical and hematological data to group different chronic kidney disease patients: A practical approach to establish different groups of patients

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Background: Chronic kidney disease (CKD) is the convergent point of several pathological processes, and its evolution is insidious and characterized by a progressive and irreversible loss of kidney function. This impaired function induces the accumulation of uremic toxins and individuals with terminal CKD often have altered physiological responses, including a persistent state of immuno-suppression and development of diseases. A better characterization and stratification of these patients with CKD in different immuno-compromised groups would contribute to more effective and personalized treatments. The focus of this study was to use two parameters to stratify patients with CKD into four separate groups that are representative of different immunological status.

Methods: Patients with CKD were chosen randomly and stratified into four separate groups according to the period of time receiving dialysis treatment and leukocyte blood counts. The amount of apoptotic CD4 T cells were measured in each group of patients, and clinical/hematological parameters were correlated by multivariate analysis with each group.

Results: Observations reveal that one of the four groups of patients with CKD (group 3) had more apoptotic CD4 T cells than the other group; this group also had an increased malnutrition inflammation score (MIS), an elevated Kt/V, and a higher incidence of smoking.

Conclusion: A simple two-parameter-based stratification strategy could be used to design effective immunological therapies that differentiate the degrees of immuno-suppression across groups of patients with CKD.

KEYWORDS

apoptosis, CD4 T cells, hemodialysis, immuno-suppression, multivariate analysis, smoking

1 | INTRODUCTION

Chronic kidney disease (CKD) is the convergent end-point of several pathological processes,¹ being the most prevalent disease for patients with hypertension and diabetes mellitus.² Its evolution is insidious and characterized by progressive and irreversible loss of kidney function.^{1,3}

As such, there is a linear correlation between loss of nephrons and progressive increase in uremic toxin levels in the blood.^{1,3} Therefore, patients with CKD are maintained in a state of residual uremia.⁴⁻⁶

Individuals with terminal CKD often have anemia, cachexia, and advanced atherosclerotic disease, associated with a persistent state of immuno-suppression that leads to the development of infections,

tumors, and lower vaccine response rates.^{7,8} This observed deterioration may be due to a state of prolonged and dysfunctional activation of the innate immune system and the reduction in lymphocyte functional activity, because of chronic exposure to uremic toxins.⁸⁻¹⁰

During hemodialysis, contact between antigen presenting cells (APCs) and the dialyzer membrane occur, causing mechanical stress and APCs activation.^{5,11,12} There is also exposure to other factors, such as oxidized lipoproteins (oxidized LDL), bacterial degradation products (eg, LPS), or nonenzymatic glycosylation of proteins, which may contribute to immune system activation.¹¹

Chronic activation of APCs induces strong CD4 T-cell activation and differentiation^{7,13}; however, over a long period, this chronic activation could lead to a reduction in the peripheral CD4 T lymphocyte counts. This reduction could be related to a reduced production of naïve T cells in the thymus, a reduction in lymphocyte proliferative capacity, a decreased production of effector-memory CD4 T cells (TEM), and an increased apoptosis rate.^{7,13} However, it is still to be determined which of these mechanisms contribute to the immunodeficiency observed in some patients with CKD, or if hematological/clinical parameters could be useful to categorize CKD patients in groups with different immune responses.

Therefore, the main objective of our study was to assess whether easily obtainable clinical factors, such as the period undergoing dialysis treatment and the leukocyte/CD4 T cells counts, could be used to stratify patients with CKD into different immuno-compromised groups (groups with different amounts of apoptotic CD4 T cells). For this purpose, a multivariate analysis was used to correlate CD4 T-cell apoptosis levels of the different groups with the clinical characteristics of patients with CKD within each group. Based upon a positive correlation, the clinical data could be used to stratify patients with CKD and design personalized treatment options and follow-up care.

2 | MATERIALS AND METHODS

2.1 | Patients

The amounts of CKD and control patients, as well as the criteria used for their selection, are described in the Appendix S1. All procedures followed the Helsinki Declaration of 1975, as revised in 2008, and were in accordance with the local laws and regulations (protocol number 65896316.8.0000.5064).

The participants were divided into four groups (Table 1), according to leukocyte count and the amount of time receiving dialytic therapy.

2.2 | FACS staining

Preparation of blood samples before staining is described in the Appendix S1. After RBC removal, cells were re-suspended in FACS buffer (Thermo Fisher Scientific Inc., Waltham, MA, USA) and counted. About 5×10^6 cells were labeled with an anti-CD4-APC antibody (BD Biosciences, San Jose, CA, USA) for 30 minutes at 4°C, and then centrifuged (390 g) and re-suspended in Annexin V binding buffer (Thermo Fisher Scientific Inc.). Each sample received Annexin-V-PE

TABLE 1 Division of patients by dialysis time and leukocyte count

Groups ^a	Dialysis ^b	Leukocyte count ^c	N. patients
1 (<4 y/<3000)	666 ± 605	2466 ± 730	n = 4
2 (<4 y/>3000)	647 ± 380	4400 ± 773	n = 5
3 (>4 y/<3000)	2589 ± 485	1957 ± 262	n = 6
4 (>4 y/>3000)	1806 ± 179	4573 ± 585	n = 3

All data are presented as averages ± standard deviations (SD).

^aThese groups were established according to the dialysis time in years (under or over 4 y), and counts of peripheral blood leukocytes (higher than or lower than 3000 leukocytes/mm³ of blood).

^bDialysis time was measured in days. Each period of 365 d was considered equivalent to 1 y; therefore, 1460 d would correspond to 4 y of dialysis.

^cLeukocyte count was performed in a Neubauer chamber after red blood cell lysis.

(BD Biosciences) and was incubated for 10 minutes at 4°C. After that, samples were read in a flow cytometer (FACS Calibur, BD Biosciences) where the percentages of total CD4⁺ T cells (CD4⁺) and CD4⁺ T cells in apoptosis (CD4⁺/Annexin-V⁺) were calculated. The total counts of CD4⁺ T and apoptotic CD4 T cells were determined by applying each percentage of these cell types to their respective leukocyte counts.

2.3 | Clinical data from medical records

The variables used from each patient are described in the Results section. Smoking and erythropoietin use was quantified by assigning indices, for which the values 0 ("non-smoker" or "non-user") or 1 ("smoker" or user) were assigned to each patient. The means and standard errors of these indices were calculated for each group; the closer the index was to the value 1, the higher were the proportions of smokers or erythropoietin users found in each group.

2.4 | Multivariate analysis

As mentioned above, there were a large number of variables obtained from each patient. To simultaneously compare and identify the list of factors that are most relevant for each group, we performed a multivariate analysis with relevance vectors. In this representation, the importance of a characteristic is determined by the magnitude and direction of its corresponding vector; more important vectors will point to the associated group. By ordering all such vectors, a table consisting of the order of relevance for variables within a group was constructed. This grouped vector model was carried out with the FITOPAC software (Unicamp, Campinas, Brazil).

2.5 | Statistical analysis

FACS data were compared by ANOVA, followed by Tukey's correction. Some of the patients' clinical data were tested against reference values within a normal population. The values were the following: the index of the adequacy of dialysis treatment (Kt/V index) was 1.2, the phosphorus concentration (4.5 mg/dL), the malnutrition information

FIGURE 1 Hemodialysis effect on CD4 T cells from patients with CKD. Patients with CKD were divided into 4 groups according to time duration in hemodialysis (above or below 4 y) and leukocyte numbers/mL of blood (higher or lower than 3.0×10^6 cells/mL). A, numbers of leukocytes/mL; B, percentages of CD4 T cells; C, numbers of CD4 T cells/mL; D, percentages of apoptotic CD4 T cells ($\text{CD4}^+/\text{Annexin V}^+$ T cells); E, numbers of apoptotic CD4 T cells. These results are representative of 2 independent experiments of repeated measurements, $*P < .01$

score (MIS) is 10.0, the Albumin concentration was 3.7 g/dL, the anti-HbsAg concentration (10 mIU/mL), total/corrected calcium concentration (10.2 mg/dL), and body mass index (BMI; 21.8). For parathyroid hormone (PTH), a reference value of 385 pg/mL for patients with CKD was used. The P values $< .05$ were considered statistically significant.

3 | RESULTS

3.1 | Variations in leukocyte and CD4 T-cell numbers, and amounts of apoptotic CD4 T cells in CKD patient groups

We observed 11% of patients with CKD have an apoptotic CD4 T-cell count of approximately 200 cells/ mm^3 , which is typical for patients having an acquired immunodeficiency syndrome.^{14,15} Therefore, this parameter may be an indicator for groups of patients with CKD having an impaired immune response.

The patients were divided into 4 groups according to Dialysis Time and Leukocyte Count, as described in Table 1. The group receiving dialysis for a longer time (>4 years) and a lower number of leukocytes ($<3.0 \times 10^6$ leukocytes/ mm^3) would have higher immune response impairment than the other 3 groups due to elevated CD4 T-cell apoptosis (Figure 1). Groups 1 and 3 had fewer leukocytes when compared to groups 2 and 4 (Figure 1A). The percentages and total numbers of

FIGURE 2 Multivariate analysis for the correlation of clinical/hematological data among groups of patients with CKD. The clinical and/or hematological data collected during screening and dialysis therapy were correlated with 4 groups of defined patients. The proximity and size of each vector were used to measure the influence of each parameter on a particular group. Data from all patients were compiled for this analysis ($n = 18$)

TABLE 2 Correlation between clinical/hematological data for each group of patients with CKD

Group 1	Autovector ^a
Residual diuresis	-0.3942
Hemoglobin	-0.3455
Albumin	-0.1565
BMI	-0.0560
Age	-0.0052
Group 2	Autovector
Smoking	-0.4022
Albumin	-0.4017
Anti Hbs	-0.3055
Kt/V	-0.2715
Phosphorous	-0.0190
Group 3	Autovector
MIS	0.3947
Phosphorous	0.3943
Erythropoietin	0.3504
Kt/V	0.3423
PTH	0.2668
Creatinine	0.1814
Smoking	0.1571
Total calcium	0.1305
Anti Hbs	0.0238
Group 4	Autovector
Total calcium	0.4119
BMI	0.3066
PTH	0.285
Hemoglobin	0.2673
Erythropoietin	0.209
Age	0.1385
Creatinine	0.1164
Residual diuresis	0.0988
MIS	0.0837

^aAutovector: intensity and proximity vector calculated by multivariate analysis; for positive autovectors (groups 3 and 4), the larger an autovector value is, the stronger is its correlation with that group; for negative autovectors (groups 1 and 2), the smaller an autovector value is, the stronger is its correlation with that group.

CD4 T cells (Figure 1B,C), as well as numbers of apoptotic CD4 T cell (Figure 1E), were always higher in group 3; this difference was not observed with respect to percentages of apoptotic CD4 T cells (Figure 1D).

3.2 | Clinical/hematological data are correlated to patients in different groups of patients with CKD

After finding differences in numbers of apoptotic CD4 T cells among patients with CKD, we evaluated how clinical/hematological data

FIGURE 3 Changes in Kt/V, PTH, and smoking index in patients with CKD. Levels in Kt/V, PTH, and smoking were measured in patients with CKD and compared with standard values. These patients were divided into 4 groups according to period of hemodialysis (higher or lower than 4 y) and number of leukocytes/mL of blood (higher or lower than 3.0×10^6 cells/mL). A, average values of Kt/V; B, average concentration (pg/mL) of parathyroid hormone (PTH) dosage; C, smoking index. The results are representative of 2 independent experiments of repeated measurements, * $P < .01$

could be used to distinguish the group 3 patients from patients in other groups.

A multivariate correlation analysis of the clinical/hematological data from patients with CKD (Figure 2, Table 2) was carried out to discover salient factors from each group. In group 1, residual diuresis, hemoglobin, and serum albumin were correlated. The distinguishing factors for group 2 were a history of smoking, serum albumin, anti-HbsAg, and Kt/V. The factors found in group 3 were MIS, serum phosphorus, the use of erythropoietin, Kt/V, and intact PTH. Finally, the salient factors found in group 4 were calcium, BMI, intact PTH, hemoglobin levels, and the use of erythropoietin.

3.3 | Changes in Kt/V and PTH levels and influence of smoking inside groups of patients with CKD

Kt/V, PTH, and smoker index were the only altered parameters observed in some patients in this study when compared with reference values. Patients in groups 2 and 3 presented Kt/V higher indices than

the reference levels of 1.2, indicative of a good dialysis (Figure 3A). PTH levels were lower in patients from group 2 only when compared with the average reference level (385 pg/dL) for patients with CKD (Figure 3B). An interesting measurement was the smoker index (Figure 3C). Group 3 had the highest number of smokers, and there were no smokers in group 4.

4 | DISCUSSION

Patients with CKD have widely different degrees of immuno-suppression,^{7,8} which in some cases cannot be correlated to the amount of CD4 T cells in their blood¹⁶ but it may be correlated to the quality of these cells. Therefore, their stratification into groups with distinct clinical/hematological characteristics (Figures 2 and 3; Table 2) could help to elaborate more effective and personalized treatment plans. We found that patients could be separated according to relatively simple parameters that are easily obtained in lab screening, namely their leukocyte counts, and their time period receiving dialysis therapy (Table 1). The group receiving dialysis for a longer time (>4 years) and displaying a lower number of leukocytes ($<3.0 \times 10^6$ leukocytes/mm³) would have a higher immune response impairment than the other 3 groups due to elevated CD4 T-cell apoptosis (Figure 1). Thus, patients stratified by these two criteria could receive differentiated treatments according to their "immunological status."

Future studies could perform prospective evaluations of each patient group, taking into consideration the correlation between immuno-suppression and occurrence of infections, neoplasm, and renal transplantation outcomes. Nonetheless, we believe our results are useful to establish a criterion for further studies with CKD patients.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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