

Evaluation of klotho expression on peripheral blood lymphocytes among hemodialysis patients and its possible contribution to their immuno-compromised status

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Abstract

Infection is the second most common cause of mortality among end-stage kidney disease (ESKD) patients. Uremic toxins are the main cause of impaired immune response among ESKD patients. Klotho gene, the anti-aging gene, encodes the transmembrane alpha klotho (α KL) protein which acts as an obligate coreceptor for fibroblast growth factor 23 (FGF23). Klotho protein may play a role in immune cell functions, particularly in anti-inflammatory response; however, its role is still incompletely understood. In the present study, we aimed to measure α KL protein expression on peripheral blood lymphocytes (PBLs) among hemodialysis (HD) patients, and we assumed that decreased α KL expression on PBLs may contribute to the impaired immunity among HD patients. This case-control study included 20 ESKD patients on regular hemodialysis for more than 3 months. Their ages ranged from 24 to 69 years. Patients with primary immunodeficiencies, those on systemic immunosuppressive drugs, those with ongoing infections or who had recently recovered from infections, and those with malignancies on active treatment were excluded. A control group of 20 normal subjects of comparable age and gender were also included. We compared α KL protein expression on PBLs by flow cytometry between both groups. Significant reductions in percentages of α KL protein expression on B lymphocytes (CD19), T lymphocytes (CD3), and natural killer cells (CD56) were observed among HD patients compared to controls. We also noticed a significant reduction in the percentages of natural killer cells among HD patients. The present study suggests that decreased α KL expression on PBLs may contribute to the immunocompromised status among HD patients, highlighting the importance of understanding the exact function of α KL protein on immune cells. This

may offer a future diagnostic and therapeutic tool to improve the immune response among HD patients.

Keywords: Alpha klotho protein, ESKD, FGF23, Hemodialysis, Infection, PBLs

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Introduction

Chronic kidney disease (CKD) is a progressive disease that affects more than 10% of the population all over the world. It is a leading cause of morbidity and mortality worldwide. Due to its economic burden, high prevalence and the significant adverse outcome, enhanced efforts are performed to achieve better prevention and management of CKD.¹ Cardiovascular disease and infections are the leading causes of death among CKD patients, together, they account for more than 60% of all-cause mortality among end-stage kidney disease (ESKD) patients.² The morbidity and mortality profiles among CKD population resemble that of geriatric population, especially in the pathophysiological changes in the vascular and immune systems.³ This is most evident among ESKD group of patients.

CKD is associated with immune stimulation, systemic inflammation, and immune deficiency. The altered immune response is due to affection of both innate and acquired immunity.⁴ Many factors contribute to the impaired immune function among CKD population especially ESKD group of patients e.g.: uremia, malnutrition, systemic inflammation, vitamin D deficiency and dialysis-related factors. Intestinal dysbiosis, iron deficiency anemia and subsequent iron overload upon treatment may also contribute.⁵ Systemic inflammation leads to atherosclerosis, cardiovascular disease, cachexia, and anemia, while immune deficiency causes impaired response to vaccination, and increased incidence and severity of infections.⁵

The Klotho (KL) gene was first described in 1997 by Kuro and colleagues in a transgenic mouse. Klotho knockout mice showed phenotypes simulating premature aging in humans, like arteriosclerosis, osteoporosis, senile skin changes and ectopic calcifications, together with a short life span and infertility. To

date, the KL gene is the first discovered gene to cause numerous premature ageing- associated diseases by a single gene mutation.⁶ Klotho gene encodes a single pass transmembrane protein, it is mainly expressed in the distal tubules of the kidneys.⁷ There are two types of described klotho proteins, transmembrane klotho (alpha, beta, and gamma subtypes) and the secreted klotho. The membrane klotho (mKlotho) acts as an obligatory coreceptor for fibroblast growth factors (FGFs) to perform their functions, while the secreted klotho acts as a circulating hormone with FGF dependent and independent manner.⁸

In 2000, Yamashita et al., isolated and described a novel fibroblast growth factor 23 (FGF23), which remains the last member of FGF family till now.⁹ FGF23 is an osteocytes-derived hormone. Under normal physiological conditions, it stimulates phosphate excretion by the kidneys through decreasing the apical expression of type II sodium phosphate cotransporters in proximal tubules. In addition, FGF23 suppresses active vitamin D synthesis.¹⁰

Alpha klotho (α KL) is an obligatory coreceptor for FGF23 and increases its affinity to FGF receptor 1 (FGFr1). Upon attachment of α KL to FGFr1, a specific attachment site for FGF23 is formed and so FGF23 can perform its function properly.¹¹ There are two forms of α KL described, single pass transmembrane protein named membrane-bound klotho and soluble klotho which is formed after cleavage of the extracellular part of the membrane bound klotho by transaminases disintegrin A and metalloprotease 10 (ADAM10), ADAM 17 and β -secretase 1.¹²

Studying Klotho protein in CKD patients showed significant decrease of its values with the progression of the disease with lowest levels among ESKD on hemodialysis.¹³ Alpha-Klotho/ FGF-23 axis dysregulation increases morbidity and mortality among ESKD patients

through causing chronic kidney disease- bone mineral disorders (CKD-BMD), atherosclerosis, left ventricular hypertrophy and systemic inflammation.¹⁴

Several studies, involving both animal and human subjects, have indicated that klotho might play a potential function within the immune system. In 2000, Okada et al., found that klotho deficient mice suffered from thymic atrophy, reduced number of splenocytes and severe B lymphopenia with preserved hematopoietic stem cell function suggesting that abnormal hematopoietic microenvironment of the bone marrow may be responsible for the described findings.¹⁵ A study was carried out to investigate the immunological response of klotho deficient mice to sepsis. The finding revealed lymphopenia, increased cell apoptosis, impaired bacterial clearance, decreased recruitment of innate immune cells, in addition to a decreased survival rate among klotho deficient group compared to the control group.¹⁶

In 2016, Nakashima and colleagues studied the expression of klotho/ FGF23 on splenic cells of mice and concluded that klotho/FGF23 was expressed on B cells and plasmacytoid dendritic cells (pDCs) suggesting that klotho/FGF23 may participate in immune system processes.¹⁷ In 2007, a study illustrated the klotho expression in rheumatoid arthritis (RA) group of patients, and discovered that Klotho expression at the mRNA level significantly decreases in resting human CD4+ T lymphocytes with aging as well as among RA patients regardless of the patients' age.¹⁸ Karami and colleagues investigated klotho gene expression in peripheral blood mononuclear cells (PBMCs) among relapsing-remitting multiple sclerosis (RRMS) patients, they found decrease in klotho gene expression by 2.5 folds among RRMS patients compared to healthy individuals. They suggested a possible role of klotho in PBMCs among MS patients.¹⁹ Investigating renal interstitial fibrosis, in diabetic nephropathy CKD patients, showed that soluble Klotho binds to type II transforming growth factor β (TGF β) receptors preventing TGF- β 1 attachment to its cell-surface receptors, thus klotho may have an anti-inflammatory function by inhibiting the profibrotic process.²⁰

A recent study suggested an abnormal α -klotho/ FGF23 axis in immune cells as a potential cause of immunodeficiency among CKD patients.²¹

The aim of this study was to test the hypothesis that decreased α KL expression on peripheral blood lymphocytes (PBLs) may contribute to impaired immunity among hemodialysis (HD) patients. Therefore, we measured the expression of α KL protein on PBLs among HD patients compared to normal control subjects.

Subjects and Methods

The present case-control study included 40 subjects, who were classified into two main groups. Group I: comprised 20 ESKD patients on maintenance hemodialysis and group II: comprised 20 apparently healthy individuals of comparable age and gender who represented the control group. All patients were recruited from those on regular hemodialysis at Ain Shams University Hospitals Dialysis Centre during December 2021 till April 2022.

The criteria for eligible study patients included patients on regular hemodialysis for $\geq$ 3 months who were committed to 3 sessions/week for at least 4 hours/session. Their ages ranged from 24 to 69 years. However, patients with primary immunodeficiency diseases, those who were receiving systemic immunosuppressive therapy, those with ongoing infections or recently recovered, patients with malignancies on active treatment, pregnant females, and those who refused or who were unable to sign an informed written consent, were all excluded from the study.

Sample collection

From each participant, a venous blood sample (2 ml) was collected by venipuncture under an aseptic technique in EDTA tube for complete blood count analysis. And for flowcytometric analysis, a venous blood sample (4 ml) was collected in a heparinized tube. The samples were transferred to the laboratory within one hour of collection, and immuno-stained.

Complete blood count

The hematological parameters were measured using an automated blood analyzer (XP-300 Sysmex analyzer, Biochem Diagnostics, USA), according to the manufacturer's instructions.

Direct immunofluorescence staining of blood cells with α -Klotho

The expression of α -Klotho protein was measured on three populations of lymphocytes: B-cell (CD19+), T-cell (CD3+), and natural killer (NK) cells (CD56+), using multi-color flow cytometry analysis. The flowcytometric analysis was performed on Beckman Coulter Navios EX SM: BE14548 software version (Beckman Coulter Diagnostics, California, USA).

The reagents used included phosphate buffered saline containing 1% bovine serum albumin (PBS/BSA) (Invitrogen, ThermoScientific, USA). PBS, erythrolyse a red blood cell lysing buffer (Invitrogen, ThermoScientific, USA) and heparin as an anticoagulant.

Cell preparation and data analysis

For cell preparation, 100 μ L of heparinized blood was suspended in 100 μ L of cold (4°C) phosphate buffer saline/ bovine serum albumin PBS/BSA buffer.

Immunostaining: from each blood sample aliquots of suspended cells were stained with four monoclonal conjugated antibodies including: anti-CD19-APC (Beckman coulter, USA), anti-CD3-PC5.5 (Beckman coulter, USA), anti-CD56-PE (Beckman coulter, USA), anti- α -Klotho antibody-FITC (Thermo Scientific, USA). For each staining, 5 μ L of each antibody were added to 200 μ L of the cell suspension, mixed well, and incubated at 4°C for at least 30 minutes, avoiding direct light.

At the end of incubation period, the cells were washed with 2 ml cold (4°C) PBS/BSA, then centrifugated at 300-400g for 5 minutes at 4°C, and the supernatant discarded. To each blood suspension, 2 ml of freshly prepared erythrolyse red cell lysing buffer was added, mixed, and incubated for 10 minutes at room temperature.

Then the blood suspension was centrifugated at 300-400g for 5 minutes at room temperature and supernatant was discarded. The cell suspension was washed in 2 mL of PBS/BSA, centrifugated at 300-400g for 5 minutes and the supernatant was discarded. Finally, the cells were resuspended in 200 μ L cold (4°C) PBS and analyzed within 2 hours by flowcytometry.

For flow cytometry cells were gated based on their monoclonal antibody staining. Appropriate isotype controls were kept for each set. Forward and side scatter patterns were gated to exclude debris. A total of 50,000 events were collected and analyzed and Navios software BE14548 (Beckman Coulter Diagnostics, California, USA) was used to analyze flow cytometry data. The expression of α -Klotho was determined on each population of cells and the results were presented as a percentage of gated cells.

Statistical Analysis

The collected data were revised, coded, tabulated, and introduced to a personal computer using Statistical package for Social Science (SPSS version 22 for windows; SPSS Inc. Chicago). Data were expressed as mean $\pm$ standard deviation (SD) and range for parametric data, and as median and interquartile range (IQR) for non-parametric data, respectively. For comparison of the two groups, Student's *t*-test was used for parametric data, and Mann-Whitney *U* test was used for non-parametric data. The Chi-square test was used to analyze categorical data. Area under the curve (AUC) calculations of non-parametric receiver operating characteristic (ROC) curves were used to assess the sensitivity and specificity of α -klotho protein expression on immune cells in hemodialysis patients. A *p*-value of less than 0.05 was considered significant.

Results

Demographic data of the studied groups

As shown in Table 1, the age, gender, and smoking status were comparable in both groups.

Table 1. Demographic characteristics of the studied groups.

Variable		Hemodialysis group (n=20)	Control group (n=20)	<i>p</i> value
Age (years)	mean± SD	44.35±12.85	47.7±15.69	NS
	Range	24.0 – 69.0	19.0 – 69.0	
Gender				
Male	n (%)	10 (50.0)	11(55)	NS
Female		10 (50.0)	9 (45)	
Smoking				
Non-smoker	n (%)	12 (60.0)	15(75.0)	NS
Smoker		8 (40.0)	5 (25.0)	

n: number of subjects in each group, SD: standard deviation, $p > 0.05$ is not significant (NS).

The etiology of chronic kidney disease (CKD) and distribution of co-morbidities among the hemodialysis group

As shown in Table 2, the underlying cause of CKD was identified in most cases, however, in 30% of cases, the cause was unknown. In the hemodialysis group, 25 % of cases had

hypertensive nephrosclerosis. Regarding the associated comorbidities, most HD patients were hypertensive (65 %), whereas patients with diabetes mellitus (DM), cardiovascular diseases, cerebrovascular diseases constituted the minor group among patients.

Table 2. The etiology of chronic kidney disease (CKD) and distribution of the co-morbidities among the hemodialysis group.

Variable	Hemodialysis group (n=20)
Etiology of CKD	n (%)
Congenital bladder disease	1 (5)
ADPKD	1 (5)
HTN nephrosclerosis and DKD	1 (5)
HTN nephrosclerosis	4 (20)
Obstructive uropathy	1 (5)
ADPKD with renal stone disease	1 (5)
Reflux nephropathy	2 (10)
Pyelonephritis	1 (5)
Preeclampsia	1 (5)
Proteinuria of unknown etiology	1 (5)
Unknown	6 (30)
Associated co-morbidities	
HTN	13 (65)
Diabetes Mellitus	1 (5)
Cardiovascular disease	4 (20)
PVD	1 (5)
Cerebrovascular disease	1 (5)

All values are presented as number (%), n: number of patients, HTN: hypertension, PVD: peripheral vascular disease, CKD: chronic kidney disease, ADPKD: adult polycystic kidney disease, DKD: diabetic kidney disease.

Comparative analysis of the hematological parameters between the hemodialysis (HD) and control groups

Mild anemia, leukopenia, and mild thrombocytopenia were significantly associated with hemodialysis patients. (Table 3)

Table 3. Comparative analysis of the hematological parameters between the hemodialysis (HD) and control groups.

Variable		Hemodialysis group (n=20)	Control group (n=20)	p value
Hemoglobin (gm/dL)	mean± SD	11.4±0.83	12.89±1.14	0.0001
	Range	10.5– 13.2	11.2 – 15.2	
WBC (×10 ³)/μL	mean± SD	6.76±1.52	8.18±1.63	0.010
	Range	3.2– 9.9	5.8 – 11.2	
Platelets (×10 ³)/μL	mean± SD	209.2±67.67	256.9±57.0	0.006
	Range	92– 401	189 – 381	

n: number of subjects in each group. SD: standard deviation. Analysis was performed by Student's t test. $p < 0.05$ is significant.

Comparative analysis of the number of gated lymphocytes and the percentage of different phenotypes of gated lymphocytes between the hemodialysis (HD) and control groups

The total gated lymphocytes were divided into three main subtypes, based on the immunophenotype, including B-cell: CD19+, T cell: CD3+, and natural killer: CD56+ cells. The percentage of each cell subpopulation was calculated by flow cytometry as percentage

from the total gated lymphocytes. As shown in Table 4, there was a significant reduction in the number of total gated lymphocytes (cells/μL) as well as a significant reduction in the percentage of NK cells among hemodialysis patients compared to the control group ($p=0.001$) (Figure 1). However, the percentages of B and T cells were not different between the hemodialysis patients and the control group ($p > 0.05$).

Table 4. Comparative analysis of the number of gated lymphocytes and the percentage of different phenotypes of gated lymphocytes between the hemodialysis (HD) and control groups.

Variable	Hemodialysis (n=20)	Control (n=20)	p value
Number of gated lymphocytes (cells/μL)	374.5 (192, 930.5)	1571 (1391, 1902)	<0.001
B lymphocytes (CD19+) %	4.5 (3.2, 6.4)	6.45 (4.7, 7.8)	NS
T lymphocytes (CD3+) %	62.25 (58, 70)	66 (59, 72)	NS
NK lymphocytes (CD56+) %	4.1 (1.6, 5.6)	13.7 (12.0, 16.0)	0.001

All values are presented as median (interquartile range). Analysis was performed by Mann-Whitney U test. n: number of subjects in each group. $p > 0.05$ is not significant (NS).

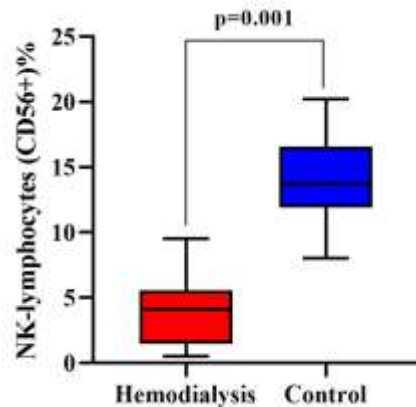


Figure 1. Boxplot graph illustrating a significant reduction of the percentage of CD56 positive cells expression in hemodialysis patients compared to the control group ($p=0.001$).

Comparative analysis of the percentage of α -Klotho protein-positive immune cells between the hemodialysis (HD) and control groups

We measured the α -Klotho protein expression on three populations of immune cells: B-cell, CD19; T-cell, CD3; and NK cells: CD56 by flow cytometry using human monoclonal antibodies.

The values were compared between the two studied groups. As shown in Table 5, significant reductions in the percentage of the α -Klotho protein expression on CD19, CD3, and CD56 cells, were observed among hemodialysis patients as compared to controls ($p<0.001$).

Table 5. Comparative analysis of the percentage of α -Klotho protein-positive immune cells between the hemodialysis (HD) and control groups.

Variable	Hemodialysis group (n=20)	Control group (n=20)	p-value
CD19/ α -Klotho+ (%)	0.39 (0.2, 0.5)	1.07 (0.9, 1.8)	0.001
CD3/ α -Klotho+ (%)	14.36 (1.2, 20)	58.4 (49, 68)	0.001
CD56/ α -Klotho+ (%)	3.5 (1.5, 6.7)	11.50 (9.4, 13)	0.001

All values are represented as median (interquartile range). analysis was performed by Mann-Whitney U test. n: number of subjects in each group. $p<0.05$ is significant.

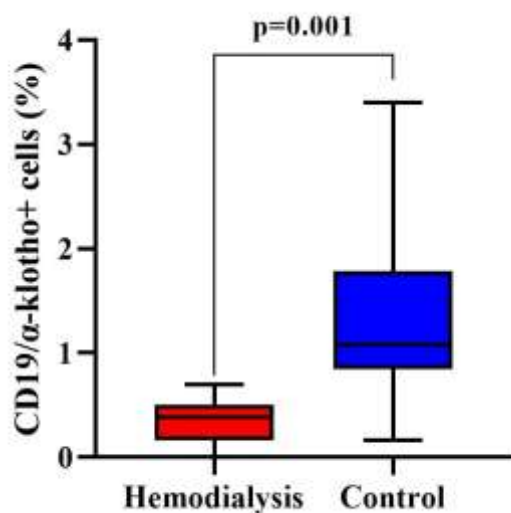


Figure 2. Boxplot graph illustrating a significant reduction of the percentage of CD19/ α -Klotho+ positive cells expression in hemodialysis patients compared to the control group ($p=0.001$).

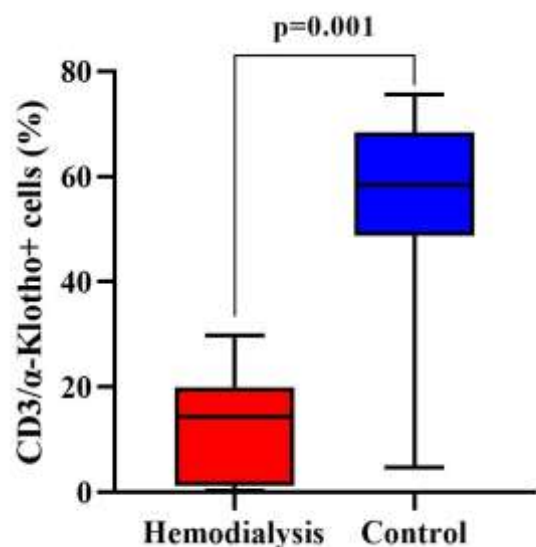


Figure 3. Boxplot graph illustrating a significant reduction of the percentage of CD3/ α-Klotho+ positive cells expression in hemodialysis patients compared to the control group ($p=0.001$).

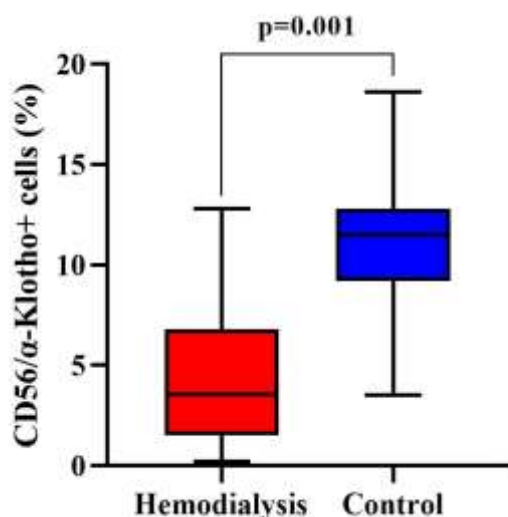


Figure 4. Boxplot graph illustrating a significant reduction of the percentage of CD56/ α-Klotho+ positive cells expression in hemodialysis patients compared to the control group ($p=0.001$).

Diagnostic potential of α-Klotho expression on immune cells as a non-invasive biomarker for immunocompromised state in hemodialysis patients

To determine the diagnostic value of α-Klotho protein expression on lymphocytes in hemodialysis patients, we plotted a receiver operating characteristics curve. When the expression level of α-Klotho on immune cells of hemodialysis patients was compared with the controls; we found that the percentage of

CD19/ α-Klotho+, CD3/ α-Klotho+, and CD56/ α-Klotho+ cells were good diagnostic biomarkers for the immunological status in hemodialysis patients. At the optimum cut-off values of 0.65, 39.0, and 8.2; the percentage of CD19/ α-Klotho+, CD3/ α-Klotho+, and CD56/ α-Klotho+ cells; respectively had a sensitivity ranging from 80% to 85% and a specificity ranging from 90 to 95% to discriminate hemodialysis patients from controls ($p<0.01$). The data are presented in Table 6.

Table 6. Receiver operating characteristic (ROC) curve analysis to determine the diagnostic potential of percentage of α -Klotho positive immune cells in hemodialysis patients.

α -Klotho %	AUC (95% CI)	Cut-off value	<i>p</i> value	Sensitivity (%)	Specificity (%)
CD19/ α -Klotho+	0.93 (0.82– 1.00)	0.65	0.01	85	95
CD3/ α -Klotho+	0.92 (0.83 – 1.00)	39.0	0.01	80	95
CD56/ α -Klotho+	0.90 (0.801 – 0.99)	8.2	0.01	85	90

CI: confidence interval, AUC: area under the curve.

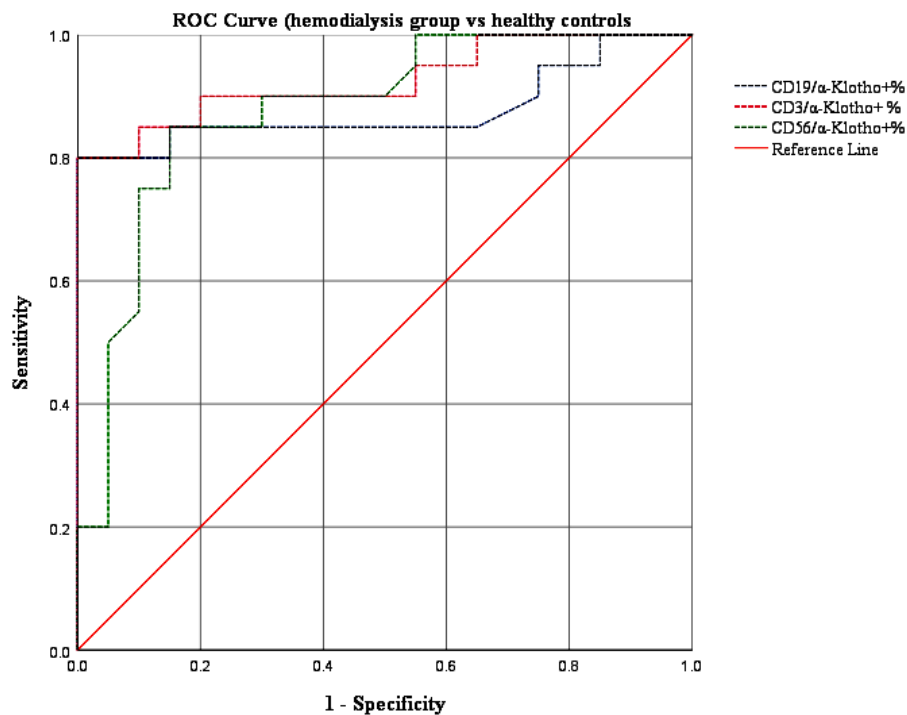


Figure 5. Receiver operating characteristic (ROC) curve analysis showing a significant diagnostic potential of percentage of CD19/ α -Klotho+, CD3/ α -Klotho+, CD56/ α -Klotho+ positive cells in discriminating hemodialysis patients from controls ($p=0.01$).

Discussion

In our study, to determine FGF23-klotho abnormalities that may contribute to the immunocompromised status among HD patients, we evaluated the total number of peripheral blood lymphocytes and the expression of α -Klotho protein on three populations of lymphocytes: B-cell (CD19+), T-cell (CD3+), and NK cells (CD56+), using multi-color flowcytometry analysis among hemodialysis patients compared to control subjects. The study included 40 subjects, who were classified into two main groups; group I:

comprised 20 ESKD patients on regular hemodialysis and group II: comprised 20 apparently normal individuals as the control group. Group I (HD group) comprised 10 males (50%) and 10 females (50%), while group II (control group) comprised 11 males (55 %) and 9 females (45 %).

In our study, hemoglobin concentration, white blood cell count, and platelet count were significantly lower among the hemodialysis group compared to the control group. This coincides with that reported by Habib et al., 2017, who studied the hematological changes in CKD patients and the effect of hemodialysis on

it. They found a significant reduction of hemoglobin levels and platelet counts in CKD patients which further decreased after hemodialysis.²² Our findings also agreed with those of Modi et al., 2021, who conducted a prospective cross-sectional study that showed a significant decrease in hemoglobin levels and platelet counts among hemodialysis patients compared to the control group.²³ Another cross-sectional study conducted by Baloglu et al., 2021, concluded that hemoglobin level is significantly lower among hemodialysis patients compared to CKD patients who underwent renal transplant.²⁴

In our study, the number of gated lymphocytes were significantly lower among the hemodialysis group compared to the control group. The gated lymphocytes were divided into three main subtypes based on immunophenotype; B cell: CD19+, T cell: CD3+ and natural killer (NK) cell: CD56+. B and T cells showed lower percentages among the hemodialysis group compared to the control group; however, this difference did not reach statistical significance. The percentages of NK cells were significantly lower among the hemodialysis group compared to the control group. This finding partially coincides with that of the study conducted by Ayna et al., 2017, who evaluated T and B lymphocyte percentages in 103 patients- donor couples: 42 CKD patients for preemptive kidney transplant, 45 patients on hemodialysis, and 16 subjects on peritoneal dialysis. Both T and B cells were numerically higher among healthy donors compared to CKD groups, with statistical significance in T cell subtype.²⁵

All-cause mortality among 104 hemodialysis patients was evaluated by Molina et al., 2018, they stated that a low total lymphocyte count (below the normal laboratory range) was found in 45.2% of the studied hemodialysis patients. The CD19+ B cell was the most frequent lymphopenias (57.7%) followed by CD3+ T cell (40.4%) and CD56+CD16+CD3- natural killer (8.7%). This matches our study finding of low percent of B, T and NK lymphocytes among hemodialysis group with statistically significant lower NK cells percentage.²⁶ A total of 410 patients, stage 3 to 5 non-dialysis CKD, were

enrolled by Xiong et al., 2021, in a study to evaluate the T lymphocyte subsets alteration, infection, and renal outcome in advanced CKD. They found a significant decrease in the level of CD3+, CD4+, CD8+ T cells, and the CD4+/CD8+ T cell ratio among CKD patients compared to the healthy group. Despite being conducted on CKD non dialysis patients, this study presents evidence that not only ESKD on hemodialysis patients are more susceptible to infections but also CKD patients in stages 3-5.²⁷

In our study, a comparative analysis of the percentage of α -klotho protein positive immune cells between hemodialysis and control group showed a significant reduction ($p=0.001$) of the percentage of α -klotho protein expression on all studied lymphocyte subtypes; CD19, CD3 and CD56 cells. To our knowledge, the only study which discussed klotho- FGF-23 axis on immune cells among hemodialysis was conducted by Yashiro et al., 2020. They evaluated the lymphocyte profile of 16 hemodialysis patients and 5 healthy subjects. They found a low level of lymphocytes among hemodialysis patients with a reduced number of T, B and NK cells compared to healthy subjects. They also found that the number of α KL-positive B cells was reduced among hemodialysis patients compared to healthy subjects.²¹

Based on our study data we may conclude that several hemodialysis patients have lymphopenia which may contribute to their immunocompromised status compared to healthy individuals. We also noticed significant reductions in the percentage of the α -Klotho protein expression on the examined three populations of lymphocytes: B-cell (CD19+), T-cell (CD3+), and NK cells (CD56+) among hemodialysis patients compared to control group.

Author Contributions

RE, was responsible for conception of the study, patient recruitment, data collection, and manuscript writing. MNF, designed the study protocol, shared in manuscript writing, and critically revised the manuscript. NE, was responsible for conducting flow cytometry and analysis and interpretation of research data. MSA, critically revised the manuscript. HME, critically revised the manuscript.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Ethical approval

The study protocol was reviewed and approved by the Research Ethical Committee of the Faculty of Medicine, Ain Shams University, Cairo, Egypt (number: FMASU MD 21/2021).

Informed consent

A signed informed written consent was obtained from all participants prior to their participation in the study.

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