Determination of the level of CD4CD69 T cells in hemodialysis patients

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Determination of the level of CD4CD69 T cells in hemodialysis patients

Azza A.I. Elmenyawi, Ahmed A.A. Megeed, Nehad R. Ibrahim, Mohamed Salah

Introduction
The CD4 helper T cell is responsible for humoral, cellular immunity, and inflammation. CD69 may be a sensitive marker indicating that the CD4 T cell is activated. The aim of this study is to determine CD4CD69 T cells for estimating the activation of CD4 T cells and to monitor hemodialysis (HD) outcome.

Participants and methods
Sixty-two HD patients and 50 controls were enrolled in our study. CD3, CD4, CD3CD69, and CD4CD69 T cell percentages were assessed by flow cytometry. Transferrin was determined using ELISA.

Results
The lymphocyte count, the CD3 T cell percentage and CD4 T cell percentage were highly significantly lower, while the CD3CD69 T cell percentage and the CD4CD69 T cell percentage were highly significantly elevated. Mean fluorescence intensity of CD4CD69, 13.89 ± 2.38 (±SD), in the HD group was significantly increased than that of the controls, 13.12 ± 4.93 (±SD) (P < 0.001). On univariate regression analysis, the CD4CD69 T cell was negatively related to albumin (odds ratio (OR), 95% confidence interval (CI): 0.159, 0.037–0.677; P = 0.013) and HD duration (OR, 95% CI: 1.187, 1.062–1.327; P = 0.003 and OR, 95% CI: 2.364, 1.004–5.564; P = 0.049, respectively).

Conclusion
CD4CD69 T cell and CD3CD69 T cell percentages were increased in HD patients despite lymphopenia. Reducing the concentration of transferrin and good nutrition may decrease CD4 T cell activation and, consequently, complications in HD patients.

Keywords: CD3 T cells, CD4CD69 T cells, helper T cells, hemodialysis

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activation during the process. At the same time, ESRD leads to a decrease and dysfunction of plasmacytoid dendritic cells that are further exacerbated by the HD procedure [4].

HD patients have an increased risk of cardiovascular diseases and cerebrovascular diseases and increased susceptibility to infectious diseases because of the immunodeficiency caused by uremia, especially T-cell defects, leading to considerable clinical effects on morbidity and mortality [5].

There are changes due to premature immunological aging [6] such as thymic function depletion and expansion of memory T cells, leading to susceptibility to infection, decreased vaccination rate and increased risk of cancer and atherosclerotic vascular disease [7]. Lymphopenia is a classic finding in ESRD patients [8] and its level is highly correlated with the stage of CKD. The gradual involution of the thymus with increasing age is characteristic of an aging adaptive immune system. The reduction in thymic output is directly proportional to age [9] and results in reduced production of naive T cells, observed by the count of circulating CD31-positive naive T cells, which consequently results in a decrease in the extent of the T cell receptor reserve and decreases the reaction to microorganisms [10].

Two theories have been suggested for the thymic output loss due to aging: the 'soil' theory, which postulates reduction in stromal niches for the growth of progenitor T cells in the bone marrow and the thymus occurring with age [11], and the 'seed' theory, which suggests a shift to myeloid precursor cells rather than lymphoid precursor cells, leading to intact innate immunity as granulocytes and monocytes [12].

The association of the proinflammatory state and the premature immunological aging leads to inflammation, and may play a role in morbidity and mortality in old patients [13].

There is an increased expression of IL-2 receptors (CD25) on naive T cells of patients with renal failure and a pro-apoptotic state exists, resulting in activation-promoted cell destruction. This process of peripheral deletion can play a role in the reduction of total naive T cells as it decreases the effectiveness of the homeostatic reproduction reaction, which balances thymus involution [14]. The memory T cell reserve is unstable in patients with renal failure. The decrease in naive T cells and central-memory T cells results in decreased total count of CD4 T cells [15]. Aging exerts minimal action on CD8 T cells because the increase in well-differentiated memory T cells compensates for the decrease in naive CD8 [16].

There is an aging-associated reduction of telomere length in CD4 and CD8 cells due to the proliferative history of the cells. The T cell telomere length decreases and is positively correlated with age and is less in patients with renal failure than in normal individuals [14].

The CD4 helper T cell is responsible for humoral, cellular immunity, and inflammation. However, CD8 cytotoxic T cells destroy infected cells [17]. The CD4 T-cell portion is a risk factor for cardiovascular disease [18] and the activated T cells are linked to atherosclerosis [5]. Therefore, modulating the activation of CD4 T cells may be helpful in decreasing the effects of some complications in HD cases.

CD69 is the first marker that arises on the activated T cell surface compared with CD25 CD71 [19]. The CD69 signaling in CD4+ cells leads to CD4+ cell migration, cytokine output and rapid reproduction. Also, it is permanently present on T cell-infiltrated inflammation sites in different chronic inflammatory disorders [20]. CD69 expression is limited to positively selected thymocytes, some types of memory cells [21] and white blood cells of chronic inflammatory infiltrates. It is well known that CD4 T cells play a role in the immune disturbance in patients on HD. CD69 expression is minor (marginal) on typical (normal) CD4+ cells, but can be increased in these cells rapidly when subjected to stimulation [22]. A study [23] using CD69-lacking models revealed that the lack of CD69 leads to increased susceptibility to various inflammatory or autoimmune diseases. CD69 can be a good effective marker for peripheral blood monocyte cells in HIV-infected patients, and the decrease in CD69 generally indicates the usefulness of antiretroviral therapy [24]. However, CD69 can also be a useful marker to recognize drug-reactive T cells in case of drug allergy [25]. Therefore, CD69 may be a sensitive marker indicating that the CD4 T cell is activated.

The aim of this study is to determine CD4CD69 T cells for estimating the activation of the CD4 T cells. Also, some factors are investigated to monitor CD4CD69 T cell activation to decrease HD complications.

**Participants and methods**

The study group included 62 patients [26] (41.5%) males and 36 (58.5%) females, mean age 46.52 ± 9.9 (±SD); 50 age-matched and sex-matched normal individuals [20] (40%) males and 30 (60%) females) were also recruited, mean age 43.95 ± 11.22 (±SD). The patients had been on HD for more than 6 months at the National Institute of Urology and Nephrology, Cairo, Egypt, 4 h, thrice weekly. Exclusion criteria were a history of tumors, hematopoietic disease, acute illness and chronic infectious diseases such as hepatitis B. None of them had received immunosuppressive drugs or undergone transplantation before. All the patients were informed of the objectives of the study and signed an informed consent form. The study was approved by the institute ethics committee.

We obtained the clinical data of enrolled patients from the start of the regular HD till December 2019. We achieved dialysis duration and HD-related heart failure and arrhythmia or other related cardiovascular from the medical history system. Primary renal disease included diabetic nephropathy (18), chronic glomerular nephritis (14), hypertensive nephropathy (13), polycystic kidney (seven), and unknown (10).

Whole blood was collected after cannulation of the vascular access before the start of dialysis. Complete blood count was
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analyzed using a celltac analyzer (Nihon Kohden, Tokyo, Japan). Total protein, albumen, globulin, and magnesium were measured on a Vitros 350 auto analyzer (Ortho Clinical Diagnostics, Mumbai, India). Transferrin was determined using a sandwich ELISA technique using the Human Transferrin ELISA Kit (AssayMax) provided by Assay Pro (Saint Charles, Missouri, USA) according to the manufacturer’s instructions. For assessment of CDs, blood was incubated for 30 min in the dark at room temperature with fluorescein-conjugated monoclonal antibodies: human anti-CD3-APC (Allophycocyanin), anti-CD4-FITC (Fluorescein isothiocyanate) and anti-CD69-PE (phycoerythrin) (Immunotech SAS, Beckman Coulter, Marseille, France). Subsequently, the blood was lysed with RBC lysis buffer (9 g NH4Cl, 1 g KOH, 200 µl EDTA dissolved in 1 l distilled water; Beckman Coulter) incubated for 30 min in the dark at room temperature. Then, the cells were analyzed on a Navios Ex cell analyzer (Beckman Coulter Life Sciences, Indianapolis, Indiana, USA). The data analysis was carried out using Navios Ex software.

Statistical analysis
Analysis was carried out using the statistical package software IBM SPSS, version 24 (IBM Corp., Armonk, New York, USA) using means ± SD, t test, χ2 test or Fisher’s exact test. The median [interquartile range (IQR)] was determined and analysis was also carried out using the Mann–Whitney U test, Spearman correlation analysis, univariate and multivariate logistic regression. A P value less than 0.05 was considered to be significantly different.

Results

After adjusting for sex and age between the HD and control groups, we found that total protein, albumen, magnesium and transferrin are significantly different in HD patients compared with the control group (P < 0.001). We also found that the lymphocyte count was highly significantly lower [median 1.6, IQR (0.9–1.9) vs. median 2.3, IQR (1.7–2.9), P < 0.001]; the CD3 T cell and the CD4 T cell percentages were also highly significantly lower [median 60.25 and 49.2, IQR (22.4–66.7) and (34.4–55.6), respectively, P < 0.001, Figs 1 and 2]. However, the CD3CD69 T cell and CD4CD69 T cell percentages were highly significantly elevated [median 14.5 and 9.55, IQR (5.8–29.8) and (3.0–15.7) vs. median 3.5 and 2.65, IQR (1.8–5.2) and (1.1–3.1), respectively, P < 0.001, Figs 3 and 4, Table 1]. On comparing the values of the mean fluorescence intensity (MFI) of CD4CD69, 13.89 ± 2.38 (±SD), and CD3CD69, 4.99 ± 1.34 (±SD), in the HD group with the controls, 13.12 ± 4.93 (±SD) and 1.99 ± 1.5 (±SD), we found a significant increase in the MFI of CD4CD69 T cells (P < 0.001) and no significant difference in the MFI of CD3CD69 T cells (P = 0.35) (Fig 5).

We found that CD4CD69 T cells were significantly increased in males (r = 0.285, P = 0.025) and CD4 T cells were marginally associated with old age (r = 0.24, P = 0.06).

Multivariate logistic regression analysis showed that duration of HD and transferrin are independent predictors of the CD4CD69 T cell (OR, 95% CI: 1.187, 1.062–1.327; P = 0.003 and OR, 95% CI: 2.364, 1.004–5.564; P = 0.049, respectively) (Table 4).

Discussion

This study showed decreased lymphocyte count in HD patients, in agreement with the results of Chen et al. [26]. In ESRD, T cell lymphopenia may be due to reduction of circulating naïve CD4+ and CD8+ T cells [27] and central memory CD4+ T cells because of increased percentage of apoptosis stimulated by activation, which was increased by the dialysis process itself [28]. We found a significant decrease of CD3 percentage in HD patients than in the controls, in accordance with some studies [27,28]. The naïve and central memory cell counts are significantly associated with serum creatinine and urea concentrations, leading to more T cell lymphopenia as a consequence of renal dysfunction aggravation. In HD patients, thymic output is impaired compared with controls after adjustment for age [14].

Stress-induced premature senescence may occur due to depletion of the lymphocyte activation ability in prolonged HD [29]. The premature aging of CD4+ and CD8+ T cells worsens renal dysfunction [27]. In old age, it is known that HD is correlated with an increase in memory T cell senescence [29]. At about 20 years, T cell premature aging was found in HD patients compared with healthy individuals [14]. Children with renal disease have a reduction in the proportion of naïve T cells, indicative of T cell exhaustion and senescence [30]. The Crépin et al. [31] reported that CKD was correlated with premature T cell aging. This leads to an increased risk of age-related diseases such as infections. Immunological T cell age can be evaluated by naïve T cell CD31 expression and T-cell receptor excision circle content to assess thymic
output. T cell telomere length and the state of differentiation of T cells can be assessed. In addition, immunological T cell age evaluation can be used to identify kidney transplant recipients who are at risk for transplant kidney rejection or to obviate excess immunosuppression. Patients with renal failure are highly susceptible to infections due to premature aging of the T-cell attributed to uremia-associated proinflammatory status, and are at increased risk for virus-associated cancers, atherosclerotic diseases and weak response to vaccination [32].

After renal transplant, the T cell count is still abnormal despite normal kidney function and decreased oxidative stress [33]; naive T cell counts and thymic output do not increase [34,35]. This may be due to the use of immunosuppressive drugs [34], irreversible thymic function alteration, steady T cell changes and modifications in bone marrow precursor cells [35]. Antithymoglobulin may affect thymic output up to one year after transplant [36]. Luque et al. [37] found that nearly 5% of French patients showed a long-term decrease of CD4+ T cell count less than 300 × 10³/µl and decreased thymic output for 10 years after kidney transplant.

In our study, the duration of HD was an independent predictor of the CD4CD69 T cell percentage increase. Luque et al. [37]

<table>
<thead>
<tr>
<th>Table 1: Demographic and clinical characteristics of the hemodialysis patients and control groups after matching for age and sex</th>
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<tbody>
<tr>
<td><strong>Hemodialysis group</strong></td>
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<td>------------------------</td>
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<tr>
<td>Age (years)</td>
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<tr>
<td>Sex: male [n (%)]</td>
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<tr>
<td>Hb (g/dl)</td>
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<tr>
<td>WBC (×10⁹/µl)</td>
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<tr>
<td>Neutrophil (×10⁹/µl)</td>
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<tr>
<td>Lymphocyte (×10⁹/µl)</td>
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<tr>
<td>PLT (×10⁹/µl)</td>
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<tr>
<td>TP (g/dl)</td>
</tr>
<tr>
<td>Albumin (g/dl)</td>
</tr>
<tr>
<td>Globulin (g/dl)</td>
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<tr>
<td>Mg (mg/dl)</td>
</tr>
<tr>
<td>TRF (mg/ml)</td>
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<tr>
<td>CD3 T cell percentage</td>
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<td>CD4 T cell percentage</td>
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<tr>
<td>CD3CD69 T cell percentage</td>
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<td>CD4CD69 T cell percentage</td>
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CVD, cardiovascular disease; Hb, hemoglobin; HS, highly significant; Insig, insignificant; Mg, magnesium; PLT, platelets; S, significant; TP, total protein; TRF, transferrin; WBC, white blood cells.

<table>
<thead>
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<th>Table 2: Correlation of CD values with other variables</th>
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<tr>
<td><strong>CD4 T cell percentage</strong></td>
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<tr>
<td></td>
</tr>
<tr>
<td>Sex</td>
</tr>
<tr>
<td>Age (years)</td>
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<tr>
<td>Hb (g/dl)</td>
</tr>
<tr>
<td>WBC (×10⁹/µl)</td>
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<tr>
<td>Neutrophil (×10⁹/µl)</td>
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<td>PLT (×10⁹/µl)</td>
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<td>TP (g/dl)</td>
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<tr>
<td>Albumin (g/dl)</td>
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<tr>
<td>Globulin (g/dl)</td>
</tr>
<tr>
<td>Mg (mg/dl)</td>
</tr>
<tr>
<td>TRF (mg/ml)</td>
</tr>
<tr>
<td>Hemodialysis duration (months)</td>
</tr>
<tr>
<td>CVD [n (%)]</td>
</tr>
<tr>
<td>Arrhythmia</td>
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<tr>
<td>Heart failure</td>
</tr>
</tbody>
</table>

CVD, cardiovascular disease; Hb, hemoglobin; Mg, magnesium; PLT, platelets; TP, total protein; TRF, transferrin; WBC, white blood cells.
Table 3: Univariate analysis of CD4CD69 T cells

<table>
<thead>
<tr>
<th></th>
<th>OR</th>
<th>95% CI</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex</td>
<td>2.24</td>
<td>0.799-6.282</td>
<td>0.125</td>
</tr>
<tr>
<td>Age (years)</td>
<td>0.983</td>
<td>0.934-1.034</td>
<td>0.503</td>
</tr>
<tr>
<td>Hb (g/dl)</td>
<td>0.984</td>
<td>0.736-1.315</td>
<td>0.912</td>
</tr>
<tr>
<td>WBC (×10^3/µl)</td>
<td>1.005</td>
<td>0.787-1.283</td>
<td>0.97</td>
</tr>
<tr>
<td>Neutrophil (×10^3/µl)</td>
<td>0.97</td>
<td>0.732-1.287</td>
<td>0.835</td>
</tr>
<tr>
<td>Lymphocyte (×10^3/µl)</td>
<td>0.516</td>
<td>0.129-2.07</td>
<td>0.351</td>
</tr>
<tr>
<td>PLT (×10^3/µl)</td>
<td>0.999</td>
<td>0.991-1.007</td>
<td>0.741</td>
</tr>
<tr>
<td>TP (g/dl)</td>
<td>0.486</td>
<td>0.2-1.182</td>
<td>0.112</td>
</tr>
<tr>
<td>Albumin (g/dl)</td>
<td>0.159</td>
<td>0.037-0.677</td>
<td>0.013</td>
</tr>
<tr>
<td>Globulin (g/dl)</td>
<td>1.074</td>
<td>0.459-2.51</td>
<td>0.869</td>
</tr>
<tr>
<td>Mg (mg/dl)</td>
<td>3.743</td>
<td>0.827-16.941</td>
<td>0.087</td>
</tr>
<tr>
<td>TRF (mg/ml)</td>
<td>3.015</td>
<td>1.779-5.108</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Hemodialysis duration (months)</td>
<td>1.189</td>
<td>1.098-1.288</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>CVD [n (%)]</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Arhythmia</td>
<td>0.225</td>
<td>0.024-2.139</td>
<td>0.194</td>
</tr>
<tr>
<td>Heart failure</td>
<td>0.236</td>
<td>0.045-1.246</td>
<td>0.89</td>
</tr>
</tbody>
</table>

CVD, cardiovascular disease; Hb, hemoglobin; Mg, magnesium; PLT, platelets; TP, total protein; TRF, transferrin; WBC, white blood cells.

We estimated the serum albumin as it is beneficial in evaluating a patient’s nutrition status. Malnutrition reduces immunity and increases the susceptibility to infectious disease. Good nutrition leads to better outcome in HD patients [26]. Our results confirm the increase in CD4CD69 T cells in ESRD patients. CD69 is an element of the C-type lectin-like receptor family [47]. Its expression on T cells is observed in many viral and bacterial infection models [48]. It causes inflow of calcium ions and acts as a signal transducer that stimulates activation of the mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK) pathway [49], causing migration and proliferation of T cells [20]. CD69 activation induces IL-2 and interferon-γ production, which stimulates the cytotoxic function of CD8+ T cells [50].

We estimated the serum albumin as it is beneficial in evaluating a patient’s nutrition status. Malnutrition reduces immunity and increases the susceptibility to infectious disease. Good nutrition leads to better outcome in HD patients [26]. Our results confirm the increase in CD4CD69 T cells in ESRD patients. CD69 is an element of the C-type lectin-like receptor family [47]. Its expression on T cells is observed in many viral and bacterial infection models [48]. It causes inflow of calcium ions and acts as a signal transducer that stimulates activation of the mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK) pathway [49], causing migration and proliferation of T cells [20]. CD69 activation induces IL-2 and interferon-γ production, which stimulates the cytotoxic function of CD8+ T cells [50].

P-glycoprotein expression on CD4CD69 cells in proliferative lupus nephritis patients plays a role in active efflux of intracellular drugs, leading to drug resistance. These cells infiltrate the renal tissue, causing damage. In refractory proliferative lupus nephritis, these cells can be targeted to control disease activity [51].

The MFI is proportional to the cell density of the molecules analyzed per cell. We compared the values of MFI of CD4CD69 and CD3CD69 in the HD group. We found a significant increase in the MFI of CD4CD69 T cells, with no significant difference in the MFI of CD3CD69 T cells. This may be related to the small sample size. In contrast, Almeida et al. [44] found no significant differences in the MFI of CD4CD69 T cells after HD; however, their results confirm ours for CD3CD69.

We found that CD4CD69 T cells were associated with heart failure, but not independently. This confirms the results of Chen et al. [26].

We estimated the serum albumin as it is beneficial in evaluating a patient’s nutrition status. Malnutrition reduces immunity and increases the susceptibility to infectious disease. Good nutrition leads to better outcome in HD patients [26]. Our results revealed that CD4CD69 T cells were negatively related to albumin by univariate regression analysis.

Magnesium plays an important role in energy metabolism and protein synthesis in humans. It is known that magnesium...
modulates the cytokine production of CD4 helper T cells, thus altering the inflammatory state [52]. In addition, the magnesium transporter protein 1 plays a role in T-cell receptor signaling. Its mutation was linked to T-cell deficiency. Hypomagnesemia is present in hereditary diseases as mutations in transient receptor potential melastatin type 6, cyclin M2 and claudin 16. Also, hypomagnesemia may be a result of intake of certain medicines such as diuretics, epidermal growth factor.
receptor inhibitors and calcineurin inhibitors [53]. Our results showed a marginally significant positive correlation between CD4CD69 T cells and magnesium.

Transferrin glycoprotein is responsible for iron transport in blood [54]. It is a growth factor. It activates T cells and induces CD69 expression and proliferation [55]. Thus, higher transferrin would activate more T cells and impair the immune system. However, our study revealed that CD4CD69 T cells were independently associated with transferrin. Therefore, we can decrease CD4 T cell activation by decreasing the transferrin level, but validation of this requires more research.

**Conclusions**

In this study, it was found that HD influences CD69 expression on T cells.

The activated CD4 T cell, CD4CD69 T cell and CD3CD69 T cell percentages were increased in HD patients despite lymphopenia. Also, CD4CD69 T cells were independently associated positively with serum transferrin and HD duration. Serum albumin was statistically significant in univariate analysis with CD4CD69 T cell percentage. Reducing the concentration of transferrin and good nutrition may decrease CD4 T cell activation and, consequently, complications in HD patients.

**Recommendation**

The main limitation of this study is the limited sample size. Thus, to confirm the consistency of these results, it is necessary to include a larger number of patients and further research is required.

**Conflicts of interest**

None

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