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Haemodialysis in Diabetic Patients Modulates Inflammatory Cytokine Profile and T Cell Activation Status

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Abstract

Diabetic nephropathy (DN) is a common complication in patients with diabetes, and most of them need renal replacement therapy such as haemodialysis (HD). These patients have a high tendency to develop infections and exhibit anomalies in the immune system. The objective of this study was to assess the expression of activation-related markers on T cells, as well as to quantify inflammatory cytokines, before and after a single HD session in DN patients. The study involved DN patients under HD treatment who signed an informed consent form. Blood samples before and after one HD session were collected, to analyse the expression of CD25, CD69 and CD71 in T cells. We also quantified IL-12p70, IL-8, IL-10, IL-1β, TNF-α and IL-6 in serum samples using the cytometric bead array technique. After the HD session, there was an increase in the CD4/CD8 ratio due to significant alterations in both subsets. The relative percentage of CD25+ cells and CD8+ CD25+ increased significantly after the HD session, while the relative percentage of CD69 T cells decreased. There was a significant decrease in the CD25 mean fluorescence intensity values for CD4+ T, as well as in the case of CD71 in T cells after the HD session. Regarding cytokine synthesis, we found a significant increase in IL-10 and IL-6 and a decrease in IL-8 after HD session. This study showed that a HD session in DN patients affects the T cell activation status in the two major subpopulations and differentially modulates the production of inflammatory cytokines.

Introduction

Worldwide prevalence studies indicate that there are at least 347 million individuals with diabetes. Published data indicate that 20–50% of patients with type 2 diabetes develop diabetic nephropathy (DN) during their lives [1].

DN is characterized by kidney damage, which occurs as the result of both direct and indirect actions of glucose. These include oxidative stress, advanced glycation and activation of some cytokines [2, 3]. DN is thus a major cause of chronic kidney disease that progresses to end-stage renal disease (ESRD) [4].

One of the features of renal function loss is the elevated concentration of blood urea, which leads to the presence of hypercytokinemia, likely due to the accumulation of proinflammatory cytokines as a consequence of decreased renal elimination [5–7]. Th1, Th2 and Th17 cytokines also take part in the pathophysiology of DN [8].

Both innate and adaptative immunity are dysregulated in patients with ESRD [9, 10]. Regarding cellular adaptative immune response, it was shown that ESRD is characterized by anomalies in different features, such as in the CD4/CD8 ratio, CD4 T cell function, T cells response to stimuli, their activation profile and impaired signals from accessory molecules [11–13]. The pro-inflammatory cytokines IL-1, IL-6 and TNF-α were studied in patients under haemodialysis [14, 15]. Moreover, it was demonstrated that among patients with ESRD, T effector memory CD4 and CD8 T cells, as well as total CD8 T cells, were higher in diabetic than in non-diabetic patients, strengthening the idea that different disease aetiologies can show different haemodialysis effects on T cells [16].

Although a number of studies were published on the role of T cell in type 2 DN haemodialysis [17], publications reporting activation-related parameters on T cells and cytokine secretion using samples from patients with ESRD undergoing dialysis and healthy individuals generated different results. Moreover, very few studies have focused on the effect of a single HD treatment on T cell physiology and cytokine production [16, 18–20]. Therefore, the main

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objective of this study was to compare the following parameters in an homogeneous group of DN patients regarding aetiology and treatment before and after a dialysis session: (1) the proportions of T cells and their major subpopulations CD4 and CD8, (2) the expression of activation markers (CD25, CD69 and CD71) in total T cells and CD4 and CD8 T cell subpopulation and (3) the serum levels of the cytokines IL-8, IL-1β, IL-6, IL-10, TNF-α and IL-12p70. To our knowledge, this study is the first to analyse these parameters in a homogeneous group of DN patients.

Materials and methods

Patients. The study group included 17 patients (7 men/10 women) with diabetic nephropathy (DN) undergoing haemodialysis at the Dialysis Center Fresenius Medical Care in Covilhã (a Portuguese inner city) for more than one year. All patients were non-smokers and had diabetes mellitus type 2. All patients had the same vascular access (intravenous fistula), and none of them had had a transplant. Individuals who received immunosuppressive drugs were excluded. The mean age ($\pm$ SD) was 72.4 $\pm$ 10.2. All the patients were informed of the objectives of the study and signed an informed consent form. The project was submitted and approved by the Fresenius Medical Care ethics committee in accordance with both the Declaration of Helsinki and the Declaration of Istanbul 2008.

T cell activation markers analysis by flow cytometry. Peripheral venous blood samples were collected before and after HD, into EDTA tubes. Analysis of activation markers analysis was carried out by four-colour flow cytometry using phycoerythrin (PE)-conjugated anti-CD25, anti-CD69 and anti-CD71 (Becton-Dickinson Pharmingen, San Jose, CA, USA), peridinin chlorophyll protein (PerCP)-conjugated anti-CD8 (Miltenyi Biotec, Bergisch Gladbach, Germany), allophycocyanin (APC)-conjugated anti-CD3 (Becton-Dickinson Pharmingen) and fluorescein isothiocyanate (FITC)-conjugated anti-CD4 (Becton-Dickinson Pharmingen). The total blood was incubated with each of the above antibodies on ice for 15 min in the dark, following the manufacturer's instructions. The cells were then incubated in 1 ml of BD FACS (BD Biosciences, San Jose, CA, USA) lysing solution for 15 min in the dark. After the lysis of the red blood cells, the cell suspension was washed twice with PBS to remove red blood cell remnants and the leucocytes were resuspended in 400 μL of PBS and placed on ice in the dark until acquisition. Analysis of activation markers was carried out by four-colour analysis using a FACSCalibur flow cytometer and CELLQUEST software (BD Biosciences). For each sample, data from 15000 cells were collected and analysed.

Cytokine analysis. The blood samples were collected before and after HD session into tubes without anticoagulant. Serum was separated after centrifugation at 20 °C, 3000 *g* for 10 min. These samples were frozen in cryopreservation tubes until assessed by flow cytometry. Serum IL-8, IL-1β, IL-6, IL-10, TNF-α and IL-12p70 levels were determined using the Cytometric Bead Array Human Inflammatory Cytokines kit (Becton-Dickinson Pharmingen, 551811), following the manufacturer's instructions.

Statistical analysis. To compare the relative percentage of CD3, CD4 and CD8 cells; activation markers CD25, CD69 and CD71 in T subsets; and cytokines IL-8, IL-1β, IL-6, IL-10, TNF-α and IL-12p70, before and after HD, we used the Wilcoxon signed-rank test. Pearson's and Spearman's rank correlation test was performed to determine the relationships between continuous variables. P values <0.05 were considered statistically significant. SPSS version 22 was used for data analysis.

Results

Baseline laboratory characteristics of the study population

Data regarding routine laboratory blood tests and dialysis efficacy performed at the time of the blood collection for the present work are presented in the Table 1. Specific haematological parameters are presented in Table 2.

Analysis of activation markers on T cells

We analysed the effect of HD on T cells and their major subpopulations, CD4 and CD8. There was an increase in the percentage of CD3CD4 cells after the HD session, 62.3 (43.8–81.2) versus 68.1 (53–89) P = 0,042. Inversely, the relative percentage of CD3CD8 decreased (33.5 (15.5–49.7) versus 28.7 (9.2–40.8), P = 0,000056) after the HD session. As a consequence, there was a significant increase (26%) in the CD4/CD8 ratio, after the HD session (1.9 (0.9–5.2) versus 2.4 (1.3–9.6), 0,042. The increase in the relative percentage of CD3-positive cells observed after the HD was not statistically significant (Fig. 1).

Regarding the expression of activation markers on T cells and their major subpopulations, we found statistically significant differences in the T cell CD25 population [33.7 (4.3–58.2) versus 34.3 (11.5–64.1), P = 0,013], T CD8 CD25 cells [6.7 (1–15.4) versus 6.9 (1.1–25.4), P = 0,002] and T CD69 cells [9 (0.4–30.2) versus 6.4 (1.1–14.7), P = 0,022], where there was a 29% decrease in the relative percentage after HD session (Fig. 2).

We also analysed the mean fluorescence intensity (MFI) of the expression of activation markers in total T cells, and CD4 and CD8 subpopulations. We found that after the HD session, there was a significant decrease in CD4 T cells expressing CD25 [62.5 (23.8–88.1) versus 55.3 (23–71.6), P < 0,037]. Moreover, CD71 MFI also decreased in total T cells [25.5 (10.9–84) versus 18.6 (6.6–49), 0,022], as well as in CD4 T cells [25.4 (10.9–59.3) versus 18.2 (6.5–54.9), P = 0,012] and in CD8 T cells [27.8 (0–120.8)

Table 1 Laboratory characteristics and haemodialysis-related parameters of the population studied.

Patient ID	Creatinine (mg/dl)	C reactive protein (mg/l)	PTH (pg/ml)	Transferrin (mg/dl)	HbA1c (%)	Glycaemia (mg/dl)	Erythropoietin (μg/month)	eKt/V
1	8.96	6	55.39	156	7.6	155.8	40	1.67
2	8.45	4.2	133.3	216	7.4	95.8	80	1.79
3	5.17	4.6	88.44	163	7.6	129.5	80	1.80
4	7.41	3.8	175.9	167	9.1	164.4	40	1.84
5	5.71	3.4	40.11	312	8.5	263.9	No data	2.14
6	7.42	11.2	329	178	7	67.8	40	1.71
7	7.52	1	176.9	136	5.8	47.9	80	1.68
8	8.15	8.1	336.1	139	5.7	183.7	120	1.69
9	7.34	5.5	224.6	154	8.9	109.1	80	1.75
10	7.07	4.8	424.1	153	7.4	202.5	80	2.17
11	8.03	17.3	414.5	182	9	220.6	40	2.46
12	5.14	2.6	228	224	9	163.8	40	1.57
13	4.14	6.4	220.6	118	7	265.2	160	1.10
14	7.01	3.6	264.7	123	6.5	192.1	40	2.20
15	5.55	2.9	42.29	180	6.5	171.9	80	1.94
16	7.61	10.4	276	165	8	182.1	No data	1.33
17	5.56	5.8	265.6	159	8.4	255.2	80	1.83
Med [min–max]	7.3 [4.1–9.0]	4.8 [1.0–17.3]	224.6 [40.1–424.1]	163.0 [118.0–312.0]	7.6 [5.7–9.1]	171.9 [47.9–265.2]	80 [40–120]	1.8 [1.1–2.5]

PTH, parathyroid hormone; Med, median.

Table 2 Haematological parameters of the population studied.

Patient ID	Haemoglobin (g/dl)	WBC (n/μL)	Neutrophils (%)	Basophils (%)	Monocytes (%)	Lymphocytes (%)	Eosinophils (%)	NLR
1	11.1	10000	71.1	0.1	4.1	21.4	3.3	3.3
2	11.2	5300	45.1	0.3	7.7	44.2	2.7	1.0
3	11.6	6700	66.5	0.4	6.1	19.8	7.2	3.4
4	13.8	6700	67.2	0.2	9.1	20.0	3.5	3.4
5	10.8	12000	61.3	0.2	5.8	30.0	2.7	2.0
6	10.9	6500	62.6	0.2	6.1	27.7	3.4	2.3
7	10.7	4900	63.4	0.1	5.4	26.1	5.0	2.4
8	12.2	6700	73.6	0.2	7.6	15.1	3.5	4.9
9	11.4	9900	59.8	0.1	7.5	30.4	2.2	2.0
10	11.1	5400	65.3	0.0	7.0	25.1	2.6	2.6
11	12.9	6000	63.5	0.2	6.9	26.0	3.4	2.4
12	12.9	6200	59.0	0.3	5.1	24.8	10.8	2.4
13	10.8	4900	61.3	0.0	2.9	34.2	1.6	1.8
14	10.5	8200	67.7	0.2	5.8	23.6	2.7	2.9
15	11.9	11400	82.7	0.1	5.8	8.9	2.5	9.3
16	12.7	10600	67.2	0.2	5.2	25.3	2.1	2.7
17	13.4	6500	71.5	0.1	9.7	16.8	1.9	4.3
Med [min–max]	11.4 [10.5–13.8]	6700 [4900–12000]	65.3 [45.1–82.7]	0.2 [0–0.4]	6.1 [2.9–9.7]	25.1 [8.9–44.2]	2.7 [1.6–10.8]	2.6 [1.0–9.3]

NLR, neutrophil to lymphocyte ratio; WBC, white blood cells.

versus 21.4 (5.8–59.8), $P = 0.03$]. Concerning the other cell populations expressing CD25, CD69 and CD71, no significant differences were observed (Fig. 3).

Cytokine analysis

Figure 4 (A, B and C) represents serum levels of IL-10, IL-6 and IL-8 in samples taken before and after HD which were analysed by cytometric bead array. IL-10 (2.1 (1.4–3.8) pg/ml versus 2.4 (1.6–4.2) pg/ml, $P < 0.05$) and IL-6 (6.7 (2.4–24.9) pg/ml versus 7.9 (3.3–31.4) pg/ml, $P < 0.05$) concentrations increased after the HD session. Data on IL-8 concentration showed that after the HD session, the levels decreased significantly [18 (9.1–26.9) pg/ml versus 10.3 (5.8–20.2) pg/ml, $P < 0.05$]. Significant differences were not observed for IL-12p70, TNF-α and IL-1β serum concentration between pre- and post-dialysis samples (data not shown). We analysed the possible

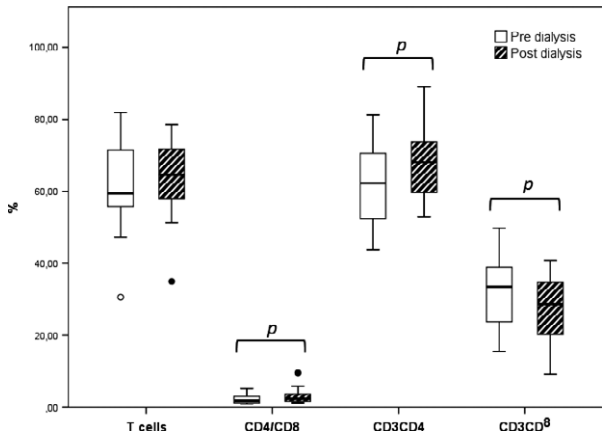

Figure 1 Analysis of the effect of haemodialysis on the percentage of peripheral blood CD4 and CD8 T cells by flow cytometry. Median values of the relative percentages of total T cells, CD4 and CD8 subpopulations and CD4/CD8 ratio before and after HD are shown (the letter '*p*' means that predialysis and post-dialysis data are statistically different ($P < 0.05$) $n = 17$).

correlation of cytokine levels with the haematological parameters, and no significant correlations were obtained.

Discussion

A significant proportion of patients with diabetes ultimately develop diabetic nephropathy [21]. Dialysis causes some relevant changes in the immune system, namely in the activation of complement [22], in the function of monocyte-derived dendritic cells [23] and in the release of various pro-inflammatory cytokines [24]. In this study, we set forth to further investigate some aspects associated with the activation of T lymphocytes, and with the levels of serum inflammatory cytokines. Many studies have focused on the comparison between healthy volunteers and HD patients on these parameters, but very few have focused on changes that occur after a single HD session in the same individual.

Regarding the relative percentage of CD8 T cells, we found a significant reduction after the HD session, consistent with the previous studies [16]. However, the results also revealed an increase in the T CD4 subset, probably caused by the duration of the HD procedure (about 4 h), which promoted a continuous state of activation of T cells, affecting CD4 and CD8 subsets differently. Also, it must be considered the probable consequences of the blood contact with the membrane of the dialyser and whether this contact can lead to T cell apoptosis not equally affecting both compartments [20]. Overall, our results were consistent with other authors regarding the CD4/CD8 ratio, showing an increase in this value after HD session.

Due to the crucial importance of T cells in the immune response, the study of the expression of their surface activation markers is very important to establish immunocompetence [25]; this was performed before and after the HD session. It is important to bear in mind that antigen- and mitogen-driven T cell activation is known to result in the expression of activation markers in an orderly sequence: CD69, CD25, CD71 and HLA-DR [26]. Our results showed that HD caused an increase in the activation of total T cells, and particularly in CD8 compartment as measured by the CD25 expression. CD8 T cell subset could be more susceptible to the stimulus caused by the dialyser membrane when compared to CD4 T cells. As CD69 is the earliest activation marker to be expressed after T cells are stimulated and our samples were analysed at an interval of 2–6 h after blood collection, this may be a possible reason to justify the decrease in percentage when compared to the expression of CD25 on total T cells, which is expressed in minutes to hours after activation. Probably time between the collection of blood samples and flow cytometric analysis was a limitation of the study, and future

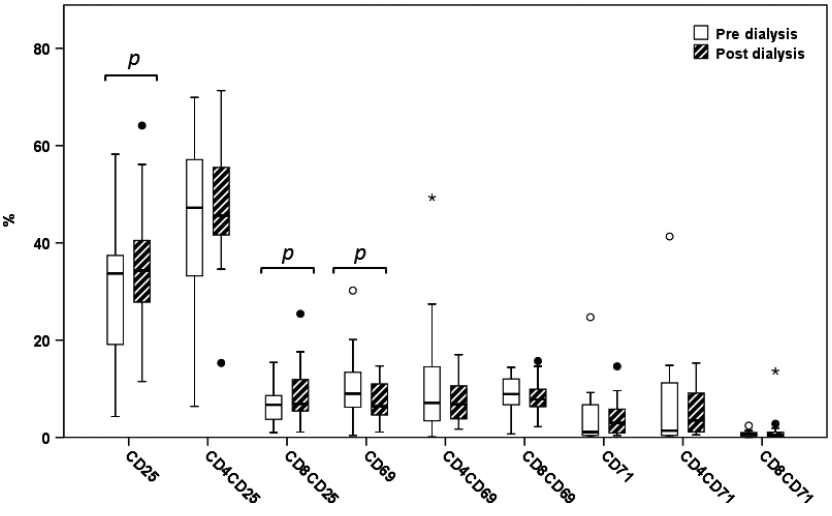

Figure 2 Analysis of the effect of haemodialysis on the percentage of activation markers on T cells and their major subpopulations by flow cytometry. Median values of the relative percentages of expression of CD25, CD69 and CD71 in total T cells and CD4 and CD8 subpopulations, before and after HD, in 17 patients with diabetes are shown (the letter '*p*' means that predialysis and post-dialysis data are statistically different ($P < 0.05$) $n = 17$).

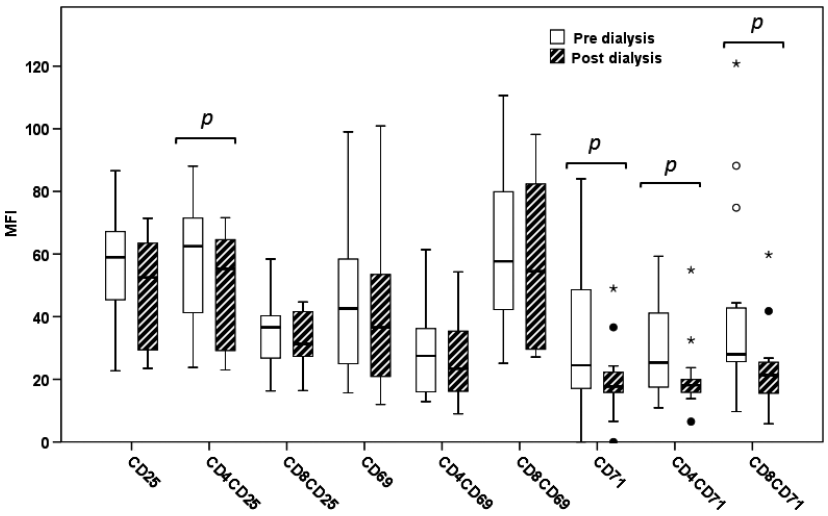

Figure 3 Analysis of the effect of haemodialysis on the level of expression of activation markers in total T cells, CD4 and CD8 subpopulations by flow cytometry. Values of the mean fluorescence intensity (MFI) of T cells and their subpopulations (CD4 and CD8) that express CD25, CD69 and CD71, before and after HD, in 17 patients with diabetes are shown (the letter '*p*' means that pre- dialysis and post-dialysis data are statistically different ($P < 0.05$) $n = 17$).

experiments have to be carried out in order to understand whether this constraint may or may not have an influence on the immunophenotyping results.

The values of mean fluorescence intensity (MFI) were also compared before and after HD for each activation-related molecule. This parameter is proportional to the cell density of the molecules analysed per cell. We observed a reduction in the MFI of T CD4CD25 cells and a decrease in both T cell subsets regarding CD71 expression levels, after the HD session. CD71 binds the complex Fe (APO)-transferrin by endocytosis, delivering iron, an important co-factor of enzymes necessary for cell growth and metabolism. Consistent with this analysis are the results of the last examinations performed before blood collection, where it was verified that approximately 82% of the patients had transferrin values below the reference values (200–360 mg/ml). Although the value of MFI after HD did not change significantly concerning the CD25 marker, a *P* value was yielded near 0.05 ($P = 0.054$), which may be an indicator that there is a tendency for this value to decrease. To confirm this hypothesis, a higher number of volunteers would be required.

It is well known that inflammatory cytokines are involved in the development and progression of diabetic nephropathy [8, 27]. We analysed the synthesis of IL-12p70, IL-8, IL-10, IL-1β, TNF-α and IL-6 in serum samples taken before and after a single dialysis session for each volunteer. We found a significant increase in IL-10 and IL-6 and a decrease in IL-8 after the HD session. It was previously reported that serological concentration of IL-6 does not undergo significant alterations, but IL-8 concentration decreases following haemodialysis [18]. Regarding IL-6, our findings are not concordant with these; however, in this previous study, only 6 patients in a total of 29 had diabetes mellitus, and cytokine detection was determined by chemiluminescence enzyme immunometric assays, contrasting with flow cytometry used in the present report. Rysz's study with 15 patients observed an increase in the concentrations of IL-1β, IL-6, IL-8 and TNF-α after the 4-h HD [19]. Adsorption evaluations of IL-1beta, IL-6 and TNF-alpha by different membrane dialysers showed that the HD effects are not the same [28]. Also, continuous haemofiltration increases IL-6 plasma clearance, but not TNF-alpha clearance [29]. These data were obtained with diverse experimental approaches, making it impossible to clearly understand what is really happening during HD.

An increase in IL-10 levels appears to function as a regulating mechanism to control uraemia and the activation induced by dialysis [30]. Through the inhibition of IL-1β, TNF-α and IL-8, involved in the activation of granulocytes, monocytes–macrophages and NK, T and B cells, IL-10 regulates inflammatory process [31]. Our results are consistent with those findings, where no significant differences occurred in the concentration of IL-1β and TNF-α after HD, and there was a decrease in the concentration of IL-8. Probably the fact that IL-8 has a low molecular weight makes its diffusion through the dialysis membrane easy to occur.

Other than our study, we found no other study reporting IL-12p70 levels pre- and post-dialysis in the literature. We should also consider that the fact that dialysis did not significantly affect the concentrations of IL-1β, TNF-α and IL-12p70 differences in our study may be due to methodological limitations. However, the range of detectable values by the CBA is rather wide, 20–5000 pg/ml, making it important to determine whether values below 20 pg/ml may or may not be physiologically relevant. A limitation of this study is the sample size. Thus, to confirm the consistency of these results, it is necessary to include a larger number of patients. Nevertheless, it is worth mentioning that IL-1β and TNF-α also remained unchanged in the Tarakçioflu report [18].

Finally, we also studied the possible correlation of cytokine levels with haematological parameters presented in Table 2. Regarding the neutrophil to lymphocyte ratio (NLR) that has been gaining interest as a marker of

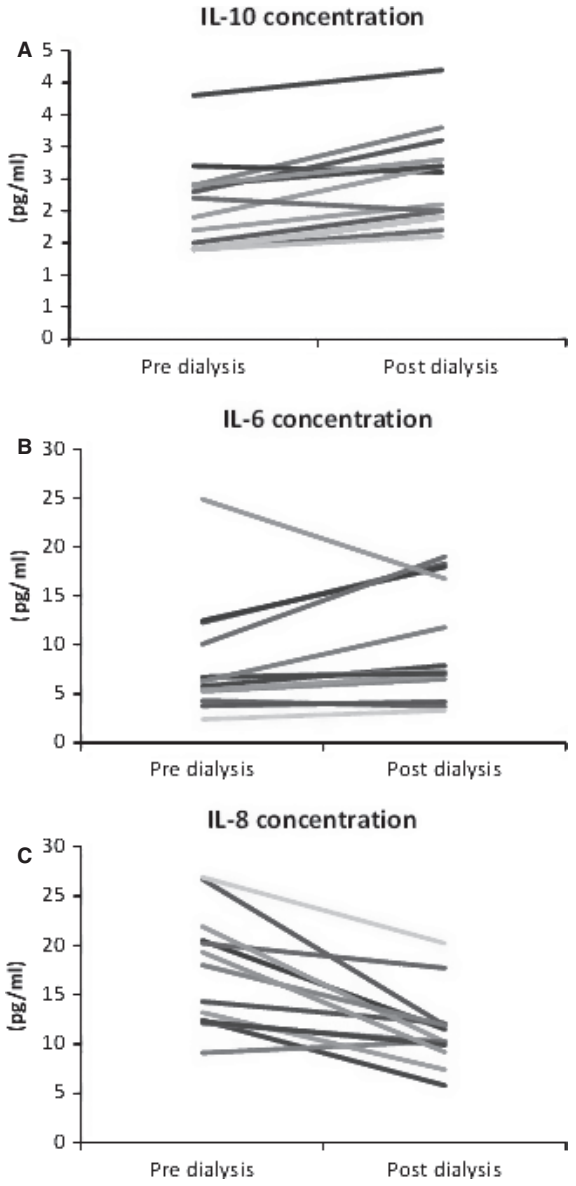

Figure 4 Quantifications of serum levels of IL-10, IL-6 and IL-8 from blood samples taken before and after the haemodialysis session analysed by cytometric bead array. Individual values of cytokines IL-10, IL-6 and IL-8 concentration, before and after HD, expressed in pg/mL are shown.

subclinical inflammation in chronic kidney disease such as dialysis patients [32], we did not find any correlation between NLR and inflammatory cytokines.

To our knowledge, our work is the first with a homogeneous group of DN patients to analyse the expression of activation markers on T cells and their major subpopulations, as well as the serum levels of inflammatory cytokines, in samples taken before and after dialysis in the same individuals. Through this study, it was found that HD does influence the expression of the marker CD25 in T cells and CD8 and CD69 expression on T cells. It was also found that after one HD session, density of CD71 molecules per cell decreased in T cells and CD25 molecules in CD4 T cells. Moreover, it was also verified that the HD session resulted in the increase in the concentration of IL-10 and IL-6 and in a reduction in the concentration of IL-8 after one HD session, possibly suggesting that these amendments are due to contact with the dialyser membrane. Nevertheless, the fact that there is an increase in the concentration of IL-10 during the HD session implies that this treatment has immunomodulatory effect in the patients.

Author statement

All authors have contributed significantly for the paper. Fonseca A.M. and Lourenço O. gave substantial contributions to conception and design, and all three authors contributed to the acquisition, analysis and interpretation of data, drafting the article and revising it critically for important intellectual content.

Conflict of interest statement

All authors have declared no competing interest.

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