Original Article

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High flux haemodialysis with polysulfone dialysers reduces peripheral blood lymphocyte apoptosis

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ABSTRACT

Background: Apoptosis appears to be the major mechanism by which mature lymphocytes maintain homeostasis. This study aims to analyse whether different types of haemodialysis (HD) therapies (low and high flux dialysers) could modify peripheral blood lymphocyte activation status and apoptosis death.

Methods: Forty-six stable chronic HD patients were enrolled in this clinical study (median age 60.6±17.4 years-old). They were randomly split into three groups – Group 1 (n=20) dialyzed with cellulose derivated low-flux dialysers (KUF<10ml/h/mmHg); Group 2 (n=10) dialyzed with polysulfone (PS) low flux dialysers (KUF<10ml/h/mmHg); Group 3 (n=16) dialyzed with PS high-flux dialysers (KUF>20ml/h/mmHg). There was a control group of 30 healthy subjects (61.6±19.8 years-old).

Peripheral blood specimens were collected before and after a dialysis session and the following variables were analysed: membrane IL2–R (CD25) and HLA-DR T-lymphocyte expression; lymphocyte dipeptyldipeptidase (CD26), *Fas* and *Fas*L expression; lymphocyte expression of a cytoplasmic membrane apoptosis marker (flipping of the phosphatidylserine residues on cell surface/Annexin V-FITC assay); IL1β, TNFα, IL2 cytokines plasmic concentrations and plasmic enzymatic ac-

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tivity of caspase-1. Methods used for this experiment were flow cytometry and enzyme-linked immunoassay. Circulating mononuclear cells lymphocyte enriched were also cultured for 36 hours *in vitro* and supernatants analysed to measure IL1β, TNFα, IL2 cytokines concentrations and enzymatic activity of caspase-1.

Results: Lymphocytes of HD patients presented higher HLA-DR expression and Annexin V affinity than the control group (HLA-DR: 11.6±7%, Annexin V: 32.2 ± 20.5% *versus* 8.2±5% and 19.4±9.5%; p<0.001, p<0.01 respectively). After a dialysis session there was a significant reduction of Annexin V and *Fas* labeled lymphocytes in the high flux group (Annexin V: preHD: 34.5±21.3%; postHD: 25.6±17.3%, p=0.02; *Fas*: preHD: 57.3±8.3%; postHD: 52.7±7.1%, p=0.001). This was associated with a significant increase of CD25 and CD26 expression in peripheral blood T lymphocytes and higher IL1β mononuclear supernatant concentrations.

Conclusions: We concluded that HD patients presented increased lymphocyte apoptosis compared to a normal population with the same age. Lymphocyte apoptosis was improved by high flux haemodialysis with PS dialysers. A more significant improvement of apoptotic lymphocyte death after high-flux HD compared to low flux HD coupled with an elevation of pro-inflammatory mediators such as IL1β suggest that this treatment may counteract immune cell death by continuous generation of cell survival factors and/or removal of high molecular weight pro-apoptotic factors ("death factors").

Keywords: Lymphocytes; apoptosis; haemodialysis; high-flux.

INTRODUCTION

Lymphocytes are the main cells of the immune system. They are committed to specific or adaptive immunity but they also play a main role in innate immunity, as proved recently[1]. Key links between these two main systems could be presenting dendritic cells, natural killer cells, γδ T cells. The identification of T cells as a mediator of early antigen–independent tissue injury demonstrates the extensive functional capacity of lymphocytes beyond adaptive immunity. At the periphery, outside thymus and bone-marrow, lymphocytes can suffer essentially three types of cellular death: programmed cellular death (genetically determined and related to the cell lifespan: 5 to 7 weeks for naïve lymphocytes, 20 to 30 years for memory cells); apoptotic cell death ("death-by-instruction" or "death-by-neglect") and finally, necrotic cell death when there are sudden or acute modifications of biological conditions, life non-compatible. In a stable state, the balance between the withdrawal of positive signals, that is, signals needed for continued survival, and the receipt of negative signals would determine apoptotic lymphocyte death[2]. Important positive extrinsic signals for lymphocyte viability are cytokines such as IL2 and IL7 and low-affinity MHC interactions. Clearance of these proinflammatory signals can bring cells to apoptosis death (death-by-neglect"). Negative signals ("death-by-instruction") consist of activation of death activators (such as TNF-α, Fas ligand (FasL)) which bind to respective receptors (TNF receptor, Fas) at the cell surface or simply threaten cell viability by accumulation of dangerous reactive oxygen species[3].

End-stage renal patients (ESRD) suffer from cellular immunodeficiency that can be linked in part to modifications of lymphocyte apoptosis, as some recent studies[4-7] have suggested. Enhanced lymphocyte apoptosis can also cause

immunodeficiency through cell loss and this parameter (lymphopenia) has been described as a determinant of survival in those patients[8,9].

High-flux haemodialysis is characterized by back-filtration of dialysis solution in the distal part of the filter which contributes to what we could call "obligatory interne haemodiafiltration" (median of 1.5 to 1.8 litres per hour during a high flux dialysis session). This is accompanied by an increase in proinflammatory mediators and also cell survival factors such as interleukin 1 (IL1) due to mononuclear activation. This process is naturally dependant on the endotoxin and bacterial contamination of the dialysate and is not totally prevented by the adsorptive dialysis membranes properties[10,11]. In addition, high-flux haemodialysis contributes by the same mechanism (internal filtration) to the removal of bigger molecules such as proapoptotic factors, which are not completely known, such as polyamines, advanced glycosylation end-products (AGEs) and some hypoproliferative citokines such as IL10[12,13]. These two mechanisms, generation of proinflammatory signals associated with haemodialysis and improved clearance of middle molecules by large pore membranes and internal filtration, could have some impact on lymphocyte survival in haemodialysis patients and, consequently, on the immunological state of ESRD patients. This study aimed to evaluate peripheral blood lymphocyte apoptosis parameters during low and high-flux haemodialysis. Our primary objective was to conclude if dialyser permeability has a definite influence on these parameters.

PATIENTS AND METHODS

Assay Principle / Study patients

We aimed at analysing whether different types of haemodialysis (HD) therapies (low and high flux, cellulose-derivated and synthetic membranes), could modify peripheral blood lymphocyte activation status and apoptosis death.

Forty-six stable chronic HD patients were enrolled in this clinical study. They were randomly split into three groups (patients of **Group 1**, n=20, median age 69±11.8 years-old were dialyzed with cellulose derivated low-flux dialysers (KUF<10ml/h/mmHg) / 9 patients dialyzed with cuprophan and 11 patients dialyzed with hemophan membranes; patients of **Group 2**, n=10, median age 48.5±16.3 years-old were dialyzed with polysulfone (PS) low flux dialysers (KUF<10ml/h/mmHg); patients of **Group 3**, n=16, median age 57.8±18.6 years-old were dialyzed with PS high-flux dialysers (KUF>20ml/h/mmHg). There was a **control group** of 30 healthy subjects, median age 61.6±19.8 years-old. Table I shows haemodialysis and dialysers' characteristics.

Peripheral-blood lymphocytes / Supernatants of *in vitro* peripheral mononuclear cells (PMC) cultures

In the study, patients' peripheral blood specimens were drawn before and after a dialysis session (average length 210 minutes) and the following variables were analysed: lymphocyte subpopulations including T$\gamma\delta$ lymphocytes; membrane IL2–R (CD25) and HLA-DR T-lymphocyte expression; lymphocyte dipeptyl-dipeptidase (CD26), Fas (CD95) and FasL (CD95L) expression; lymphocyte expression of a cytoplasmic membrane apoptosis marker (flipping of the phosphatidylserine residues on cell surface / Annexin V-FITC assay); IL1β, TNFα and IL2 citokines plasmic concentrations and enzymatic activity of caspase 1. Methods used for this experiment were flow cytometry and enzyme-linked immunoassay. Circulating mononu-

clear cells lymphocyte enriched were also cultured for 36 hours *in vitro* and supernatants analysed to measure IL1β, TNFα, IL2 and enzymatic activity of caspase 1 concentrations.

Blood specimens of the control group were collected and lymphocytes and mononuclear cells analysed by the same methods.

Our study used the flow cytometric detection of FITC-conjugated Annexin V on the surface of lymphocytes plus supravital exposure to propidium iodide. The phospholipid phosphatidylserine, which is normally hidden within the plasma membrane, is exposed on the surface on apoptotic cells early on this process. Annexin V as a phospholipid–binding protein helped us to identify the phosphatidylserine residues. The combination of Annexin V assay with supravital propidium iodide staining allowed the simultaneous detection of living, apoptotic and necrotic cells (Figs.1, 2).

Mononuclear cells were isolated by centrifugation in *Ficoll-Lymphoprep* (density gradient selective for lymphocytes). Cellular preparations obtained by this method at a concentration of 2 to 5 x10^5/ml were then incubated with fluorescein-conjugated Annexin-V and propidium iodide and analysed by flow cytometry.

Cellular preparations obtained by centrifugation on *Ficoll–Lymphoprep* at concentrations of 10^6/ml were incubated in an oven at 37º, at an atmosphere with 5% CO2 during 36 hours (RPMI as culture medium). After incubation mononuclear cells were picked up and supernatants analysed to measure IL1β, TNFα and IL2 cytokines and enzymatic activity of caspase 1.

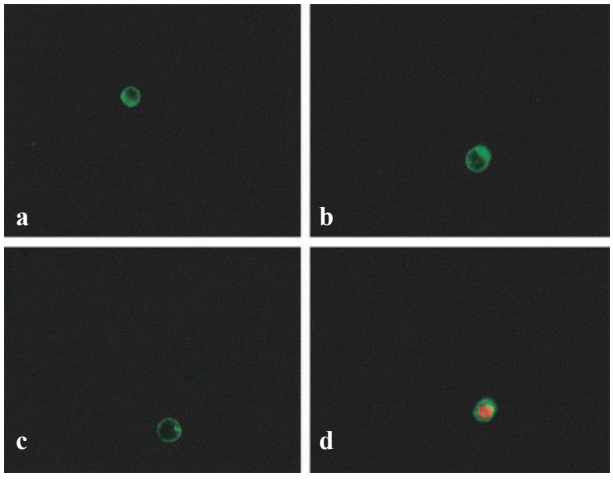

Fig. 1 – Fluorescence microscopy of lymphocytes staining with Annexin-FITC and propidium iodide (PI).
a,b and c: apoptotic lymphocytes with membrane fixed by Annexin V-FITC (green fluorescence light);
d: necrotic lymphocyte with double staining: Annexin-FITC and propidium iodide (PI) (orange fluorescence light).

Statistical analysis

All presented values are mean (m)±standard deviation(SD). Comparisons between mean values from the same group of patients, before and after haemodialysis, were assessed using a paired *t*-test. For comparison of mean values between two different groups we used one-way analysis of variance with a Student's *t*-test (independent samples *t*-test or two-sample *t*-test). Statistical significance levels were fixed at α 5% ($p<0.05$).

RESULTS

Peripheral-blood T lymphocytes (Haemodialysis patients and Control group)

The results of this study show that T lymphocytes of haemodialysis patients presented higher HLA-DR, CD26, CD95γδ, CD95CD95L expression than control group (Table II, IV and V). Increased T lymphocyte expression of HLA-DR and CD26 markers in ESRD patients illustrates immune system alteration as a chronic and non-

During the flow cytometric analysis (*Becton Dickinson FAC-Star cytometer*), green fluorescence of Annexin-FITC was detected after a 530/30 nm filter and the orange emission of propidium iodide after a 585/42 nm filter.
"Dot-plot" graphs of the results of simultaneous 2-color immunofluorescence were analysed by "Paint-a-gate" program (Fig. 1).
Results are presented as the percentage of Annexin V-positive cells.

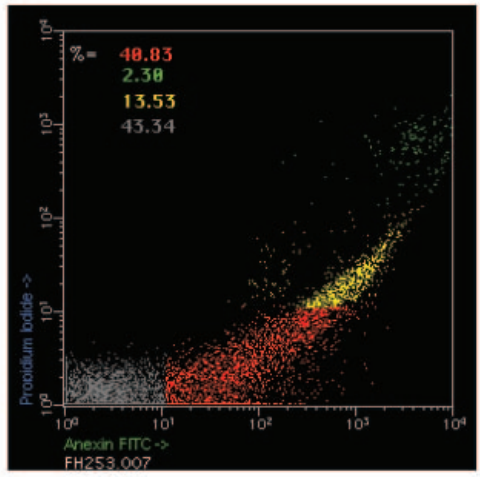

Fig. 2 – Flow cytometry image of lymphocytes incubated with fluorescein conjugated Annexin V and propidium iodide. Percentages of apoptotic (red and yelow colors) and necrotic cells (green color).

specific state of lymphocyte activation. CD25 T lymphocyte expression did not differ between HD patients and the control group (Table III). Compared to the control group, peripheral blood T lymphocytes from haemodialysed patients show a trend towards increased *Fas* (CD95) and *Fas*L (CD95L) receptors expression. This difference did not reach statistical significance except for the Tγδ lymphocyte sub-population (Table V).

Peripheral blood lymphocytes from haemodialysed patients presented increased Annexin V affinity or phosphatidylserine-binding protein levels, which indicates increased *in vivo* apoptotic rates (Fig. 3).

Evaluations pre and post haemodialysis (Overall population, Groups 1, 2 and 3)

Expression of CD25 and CD26 receptors (early expression markers in lymphocyte activation: CD25 is the α chain of IL2 receptor and CD26 suit to an exopeptidase that stimulate Th1 lymphocytes to produce cytokines such as INF-γ and IL2) increased significantly overall immediately after a dialysis session, regardless of the dialysis membrane composition. On the other hand, the lymphocyte membrane HLA-DR receptor expression levels did not change significantly after dialysis in the overall population (Table VI).

T-lymphocyte membrane expression of CD25 and CD26 receptors increased significantly immediately after a dialysis session in the group of patients undergoing high-flux haemodialysis (Group 3) (Table VII). This was precisely the same group of patients that presented significantly decreased *Fas,* FasL and Annexin V peripheral blood lymphocyte expression after a dialysis session (Table VII and Fig. 4).

Overall, the population studied shows a marked decrease in the plasma enzymatic activity of caspase-1 after one dialysis session (preHD: 645.4±319; post HD 529.3±340, p=0.03). Plasma concentrations of IL1β, TNFα and IL2 cytokines were not significantly different after a dialysis session for overall population and for each group (Table VIII).

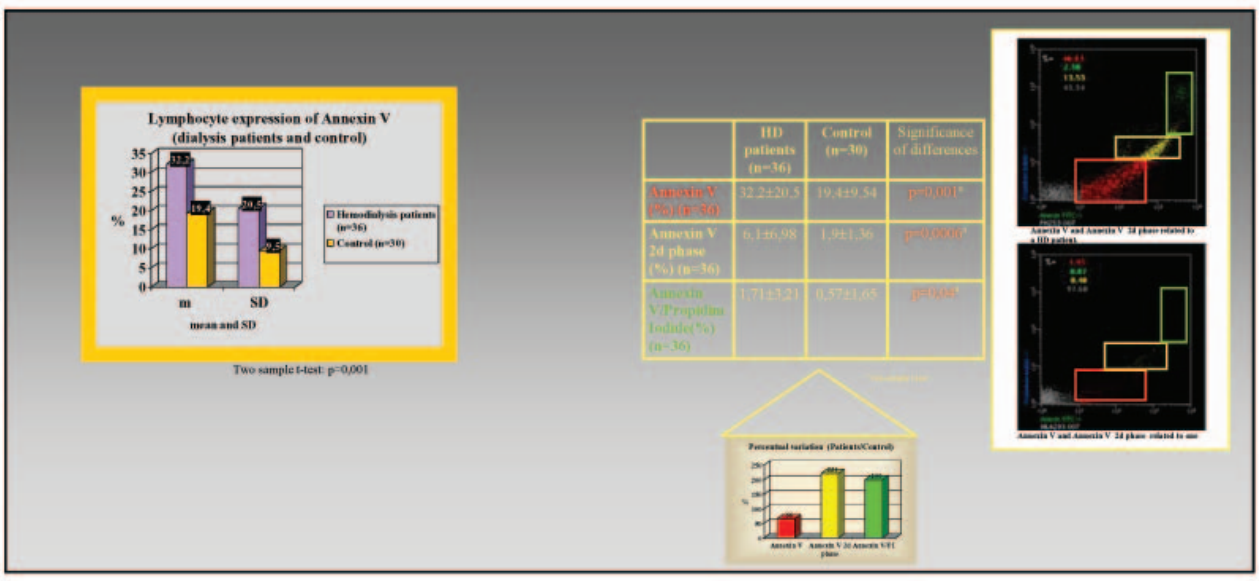

Fig. 3 – Lymphocyte expression of Annexin V (haemodialysis patients and control group)

Measurement of *in vitro* peripheral-blood mononuclear cell IL1β, TNFα and IL2 cytokines levels suggest that dialyzed patients produce and secrete significantly more cytokines than individuals from the control group (**supernatant IL1β**: haemodialysis patients (n=46) preHD 238±191.1 pg/ml, control group (n=30) 105±214.8 pg/ml, p=0.03; **supernatant TNFα**: haemodialysis patients (n=46) preHD 75±72.8 pg/ml, control group (n=30), 25± 39 pg/ml, p=0.003; **supernatant IL2**: haemodialysis patients (n=46) preHD 8±5.7 pg/ml, control group (n=30) 2.8±1.6 pg/ml, p=0.0001). Furthermore, compared to the control group, the enzymatic activity of caspase 1 in peripheral-blood mononuclear cell cultures in dialyzed patients was increased (**supernatant activity of caspase 1**: haemodialysis patients (n=46) preHD 7.6±5 pg/ml, control group (n=30) 2.6±3 pg/ml, p=0.001) (Table IX).

After a single dialysis session, there was a significant decrease of IL1β concentrations in peripheral-blood mononuclear cells supernatants obtained from low-flux membrane haemodialyzed patients (Group 1 and Group 2, n=30 - supernatant IL1β preHD 155.1±151.3 pg/ml, postHD 65.5±52.8 pg/ml, p=0.04). No IL1β supernatant concentration alterations were observed for patients undergoing high-flux membranes dialysis (Group 3) (Table IX).

DISCUSSION

(ESRD) patients present different abnormalities of the immune system that are not totally corrected by haemodialysis treatments[6,14-16]. This particular condition of immunodeficiency of ESRD patients is characterized by functional modifications of elements of innate immunity such as polymorphonuclear[17-20], macrophage[21-24] and NK cells[25-27]. The essential actors of spe-

TABLE I
Haemodialysis and dialyzers' characteristics

Type of dialysis membrane/Flux	**Cuprophan/ low-flux**	**Hemophan/ low-flux**	**Polysulfone/ low-flux**	**Polysulfone/ high-flux**
Dialysis solution (composition/ bacteriologic quality)	Bicarbonate standard 3 mEq/L of Ca2+ / <0.1 UFC/ml and LAL<0.025 U/ml	Bicarbonate standard 3 mEq/L of Ca2+ / <0.1 UFC/ml e LAL<0.025 U/ml	Bicarbonate standard 3 mEq/L de Ca2+ / <0.1 UFC/ml e LAL<0.025 U/ml	Bicarbonate standard 3 mEq/L de Ca2+ / <0.1 UFC/ml e LAL<0.025 U/ml
Qd (Dialysis solution flux, ml/m)	500	500	500	800
Qb (blood flux, ml/m)	250-350	250-350	250-350	400-500
Anticoagulation	Discontinuous: heparine 50U/Kg at beginning and 1000U/hour, (stopping at last hour)	Discontinuous: heparine 50U/Kg at beginning and 1000U/hour, (stopping at last hour)	Discontinuous: heparine 50U/Kg at beginning and 1000U/hour, (stopping at last hour)	Discontinuous: heparine 50U/Kg at beginning and 1000U/hour, (stopping at last hour)
Ultrafiltration (UF) control/rate of UF per hour, ml/h	Volumetric/ 200-1000	Volumetric/ 200-1000	Volumetric/ 200-1000	Volumetric/ 200-1000
Dialyzer model/dialysis membrane polymer	*Alwall GFE-12*, *Gambro*/ Cuprophan	*Lundia Aria 700, Gambro*/ Hemophan	*F6 HPS, Fresenius*/ Polysulfone	*Hemoflow HF 60L, Fresenius*/ Polysulfone
KUF (ml/h/mmHg)	6.0	8.5	8.5	33
Area (m2)	1.3	1.3	1.3	1.3
KoA urea (ml/m)[a]/ Ko urea/m2(ml/m)[a]/ Kurea Qb 300ml/m, UF=0[a]	545 / 419 / 221	730 / 561 /244	581/ 447 / 237	615 / 473 / 253
Kcreat. (ml/m) (Qb 200ml/m, Qd 500 ml/m)[a]	151	170	167	172
Priming volume	65	120	82	82
Residence time (Priming volume/Qb 350ml/m)	0.18	0.34	0.24	0.23
(Membrane thickness (μm) /Fiber diameter (μm)	8 / 200	6.5 / Plate dialyzer	40 / 200	40 / 200
Sterilization	Ethylene oxide	Ethylene oxide	Steam	Ethylene oxide

cific immunity, lymphocytes, are also functionally vulnerable in uraemia as demonstrated by the modifications in humoral and cellular immunity that have been exhaustively studied over the last 15 years. A short overview of some important studies into immunological abnormalities of classical adaptative response associated with uraemia is presented in Tables X and XI.

In recent years, apoptosis has been reported as a major pathway by which mature lymphocytes maintain homeostasis[3,28]. Lymphocyte cellular death regulation is a form of immune system modulation and our understanding of this mechanism could help to explain certain immu-

TABLE II
HLA-DR lymphocyte expression in hemodialysis patients and control group

	CD3/DR (%)	**Significance of differences**
Haemodialysis patients (n=46)	11.6±7.2	**p=0.009[a]**
Control group (n=30)	**8.2±5**	

[a] two-sample *t*-test

TABLE III
CD25 lymphocyte expression in haemodialysis patients and control group

	CD25/CD3 (%)	**Significance of differences**
Haemodialysis patients (n=46)	18±6.1	**n.s.[a]**
Control group (n=30)	**20.8±6.4**	

[a] two-sample *t*-test.

TABLE IV
CD26 (dipeptyldipeptidase) lymphocyte expression in HD patients and controls

CD26 (dipeptyldipeptidase)			
	HD patients (n=46)	**Control group (n=30)**	**Significance of differences**
CD26 (%)	45.9±10,6	39.8±9.46	**p=0.006[a]**
CD26CD3 (%)	40.9±10,5	3.,3±9,3	**n.s. [a]**
CD26CD4 (%)	32.1±9.2	3.,3±9,3	**n.s. [a]**
CD26CD8 (%)	9.51±4.21	7.52±3.81	**p=0.02[a]**
CD26CD3CD25 (%)	**15.6±6.03**	**16.5±5.79**	**n.s. [a]**

[a] two-sample *t*-test

nological abnormalities such as immunodeficiencies and autoimmune diseases.

There are experiments that suggest that lymphocyte apoptosis is disrupted in ESRD patients and this fact could contribute to the cellular immunodeficiency of these patients[4-6,8,29-32]. Our study aimed at concluding if there are differences in lymphocyte apoptosis death parameters after haemodialysis with dialysers of different water permeability (low and high flux, derivatized cellulose and synthetic membranes). As mentioned before, lymphocyte death could present apoptotic characteristics due to activation of the normal genetic programme of cellular survival or to a process of activation-induced cell death (AICD), called "death-by-instruction" or "death-by-neglect"[3]. On the other hand, there is the process of necrotic lymphocyte death due to acute aggressions, not life compatible. During haemodialysis treatments, the aggression or pre-lethal stimulus that lymphocytes could suffer would induce apoptosis death and not necrosis, as these cells resist extreme conditions of cellular hypoxia (ATP synthesis in these cells is based on glycolysis). In addition, removal of uremic apoptotic molecules or instead generation of cell survival factors could contribute to a decrease of lymphocytes apoptosis markers after a haemodialysis session[10-13]. The question could be raised whether the final balance between pro and anti apoptotic stimulus contributes or not, to the cellular immunodeficient state observed in end stage renal patients treated by haemodialysis. We also might be sure if the differences between high and low flux modalities impact on lymphocyte death.

The results of our study showed that haemodialysis patients presented a slight lymphopenia but no significant differences in lymphocyte subpopulations compared with a normal population of the same age (results not showed). There was no difference in the IL2 receptor

TABLE V
CD95 (Fas) and CD95L (FasL) lymphocyte expression in HD patients and controls

CD95 (Fas)			
	HD patients (n=41)	**Control group (n=30)**	Significance
CD95 (%) (n=41)	56.2±9.81	51.7±13.1	**n.s.**[a]
CD95CD3 (%) (n=41)	46.5 ±9.4	45.6±11.75	**n.s.**[a]
CD95 γδ (%) (n=32)	2.9±1.8	2.1±1.57	**p=0.04**[a]
CD95 L (FasL)			
CD95L (%) (n=12)	2±1.9	1.4±1.86	**n.s.**[a]
CD95LCD3 (%) (n=12)	1.46 ±1.92	0.57±0.59	**n.s.**[a]
CD95LCD95(%) (n=12)	**1.81±2.52**	**0.49±0.58**	**p=0.049**[a]

[a] two-sample *t*-test

TABLE VI
% of peripheral blood lymphocytes CD25, HLA-DR, CD26, CD95 and CD95L positive (pre and post haemodialysis session).

Overall Group (n=46)		
	Before HD	**After HD**
CD25 (%) (n=46)	21.4±7	24.2±8 p<0.001[a]
HLADR (%) (n=46)	22.3±10	21.9±10 n.s.[a]
CD26 (%) (n=46)	45.9±11	49.7±11 p<0.001[a]
CD95 (%) (n=41)	56.2±10	53.7±9 p=0.01[a]
CD95L (%) (n=12)	2±1.9	2.9±2.3 p=0.02[a]

[a] paired *t* test

(CD25) peripheral blood lymphocyte expression in haemodialysis patients compared with the control group but there was a sustained increase in HLA-DR expression in peripheral T-lymphocytes (CD3 receptor positive). Compared to the control group, CD26 lymphocyte expression was increased in haemodialysis patients, particularly in cytotoxic lymphocytes (CD8 positive). CD26, a membrane associated peptidase (dipeptidyl-peptidase IV), is characterized by costimulatory properties of activation of lymphocytes. The importance of signalling via CD26 has been proved by researchers into specific inhibitors that suppress T cell proliferation *in vitro*, particularly Th1 lymphocytes[33]. Our observations confirm previous findings that describe a state of lymphocyte preactivation in uraemia that persists even after the beginning of regular haemodialysis[14,30]. The knowledge of molecular mechanisms of lymphocyte activation had allowed to conclude that T cell activation can occur totally independently of antigen[1]. Chemokines such as RANTES and oxygen free radicals are some products that could be playing an important role in this process of non-specific T cell activation[1,34]. In ESRD these products accumulate and could contribute to the up-regulation of pro-inflammatory genes on lymphocytes and to this state of cell pre-activation that compromises adequate immune response[12,13]. Impaired lymphocyte calcium metabolism in end-stage renal disease could also contribute to the presence of some activation signals in those patients. Enhanced calcium intracellular influx and sustained elevation of intracellular calcium acts as an important trigger for nucleus transcriptional activation of cytokine genes such as the IL2 gene[35,36].

TABLE VII
% of peripheral blood T lymphocytes CD25, CD26, CD95 and CD95L positive
(pre and post haemodialysis session).

	Group 1 Cellulose based membranes, low-flux (n=20)		Group 2 Polyssulfone low flux (n=10)		Group 3 Polyssulfone high-flux (n=16)	
	Before HD	After HD	Before HD	After HD	Before HD	After HD
CD25CD3(%) (n=46)	17,0±6	18,9±7 n.s.[a]	18,7±7	22,1±9 n.s.[a]	18,8±6	21,8±8 p=0,01[a]
CD26CD3(%) (n=46)	40±11	43,7±11 n.s.[a]	43,6±12	48±13 n.s.[a]	40±10	44,3±11 p=0.01[a]
CD25CD26CD3 (%) (n=46)	14,7±6	16,2±6 n.s.[a]	15±6,4	18±8 n.s.[a]	17±6,4	19±8 p=0,01[a]
CD95CD3 (%) (n=39)	45,9±9	44,9±9 (n=15) n.s.[a]	46±10	47±8 (n=8) n.s.[a]	47,4±10	43,7±9 p=0.009[a]
CD95LCD3 (%) (n=15)	0,3±0,4	0,4±0,2 (n=6) n.s.[a]	1,5	2,1 (n=3) --	1,9±2,3	1,4±1,7 (n=6) p=0.04[a]

[a]paired *t*-test.

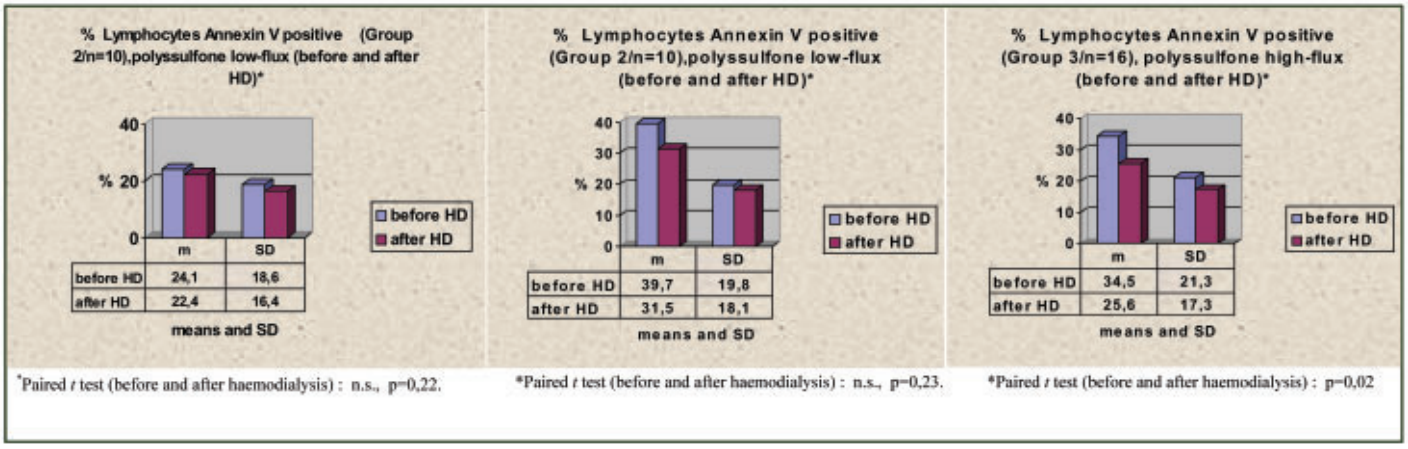

Fig. 4 – Peripheral blood lymphocytes Annexin V positive (pre and post haemodialysis session)

TABLE VIII
Plasmic concentrations of IL1β, TNFα and IL2 cytokines and enzymatic activity of caspase 1.

	Plasmic IL1β (pg/ml)		Plasmic TNFα (pg/ml)		Plasmic IL2 (pg/ml)		Plasmic activity of caspase 1 (pg/ml)	
Haemodialysis patients (n=46)	Before HD 17.6±65.4	After HD 3.5±4.3	Before HD 9.7±7.1	After HD 11.8±4.8	Before HD 6.2±5.2	After HD 6.1±5.6	Before HD 645.4±319	After HD 529.3±340
	[a]n.s., p=0.11		[a]n.s., p=0.14		[a]n.s., p=0.49		[a]p=0.03.	
Group 1 (n=20)	Before HD 5±9	After HD 1.3±1.5	Before HD 7±4.6	After HD 9.2±4	Before HD 10.4±6.2	After HD 11±6	Before HD 535±326.7	After HD 495±306.4
	[a]n.s., p=0.16		[a]n.s., p=0.16		[a]n.s., p=0.42		[a]n.s., p=0.1	
Group 2 (n=10)	Before HD 2.7±3.1	After HD 4.3±3.2	Before HD 10±12.6	After HD 11.3±6	Before HD 3±1.1	After HD 2.8±1.5	Before HD 523±322	After HD 322±237
	[a]n.s., p=0.21		[a]n.s., p=0.43		[a]n.s., p=0.39		[a]n.s., p=0.27	
Group 3 (n=16)	Before HD 7.8±12.8	After HD 3.5±5.4	Before HD 9±4.5	After HD 10.5±5	Before HD 8.3±6.1	After HD 8±6.5	Before HD 691±318	After HD 543.3±365
	[a]n.s., p=0.11.		**[a]n.s., p=.031**		**[a]n.s., p=0.48**		**[a]n.s., p=0.15**	

[a]paired *t*-test.

TABLE IX
Supernatant concentrations of IL1β, TNFα and IL2 cytokines and enzymatic activity of caspase 1.

	Supernatant IL1β (pg/ml)		**Supernatant TNFα (pg/ml)**		**Supernatant IL2 (pg/ml)**		**Supernatant activity of caspase 1 (pg/ml)**	
Control group (n=30)	105±214.8		25±39		2.8±1.6		2.6±3	
Haemodialysis patients (n=46)	Before HD 238±191.1	After HD 180.5±213	Before HD 75±72.8	After HD 85.4±118	Before HD 8±5.7	After HD 9±8	Before HD 7.6±5	After HD 8.6±6.4
	[a]n.s., p=0.07		[a]n.s., p=0.22		p=0.13		[a]n.s., p=0.2	
Group 1 (n=20)	Before HD 179±177.4	After HD 75.7±56	Before HD 66.3±33.6	After HD 93.4±52.7	Before HD 16.2±5.5	After HD 15.1±4.2	Before HD 9.1±8	After HD 3.4±8
	[a]n.s., p=0.10		[a]n.s., p=0.16		[a]n.s., p=0.35		[a]n.s., p=0.23	
Group 2 (n=10)	Before HD 172.5±147	After HD 23.1±26.5	Before HD 72±35.4	After HD 54.5±51.4	Before HD 10.3±7.3	After HD 12.1±6.7	Before HD 4±3.1	After HD 3.4±4.2
	[a]n.s., p=0.04		[a]n.s., p=0.27		[a]n.s., p=0.34		[a]n.s., p=0.2	
Group 3 (n=16)	Before HD 269±198	After HD 259±232	Before HD 76.6±93.4	After HD 99±153.1	Before HD 5.5±4.3	After HD 6.9±5.2	Before HD 7.1±4.7	After HD 8.8±6.2
	[a]n.s., p=0.44		[a]n.s., p=0.17		[a]n.s., p=0.2		[a]n.s., p=0.22	

In our study we observed that haemodialysis treatments, independent of the dialyser membrane composition, cellulose-based or synthetic such as polysulfone, maintained and increased the immune cell activation state. We observed increased expression of CD25 and CD26 lymphocyte activation markers after a dialysis session (**CD25** - 21.4±7% before HD; 24.2±8% after HD, p<0.001; **CD26** - 45.9±11% before HD; 49.7±11% after HD, p<0.001) (Table VI). The

TABLE X
Abnormalities of specific immunity in pre-terminal uraemia.

Modification	Reference
* **Lymphocytopenia**	**Hoy WE *et al*, 1978** **Bansal KO *et al*, 1979** **Wakabayaashi Y *et al*, 1989** **Gogo KO *et al*, 1998** **Fernandez-Fresnedo G *et al*, 2000**
* **Cellular immunodeficiency (LT)** **- Increase of membranous and soluble receptor IL2R**	**Beaurain G *et al*, 1989** **Schachowski J *et al*, 1991** **Caruana RJ *et al*, 1992** **Descamps-Latscha B *et al*, 1995**
- Decrease of *in vitro* lymphocyte proliferative response	**Schachowski J *et al*, 1991** **Puppo F et al, 1991, Lauricella AM *et al*, 1991,** **Donati D et al, 1992, Kaul H *et al*, 2000**
- Decrease of lymphocitotoxicity	**Lang I *et al*, 1982**
* **Deficit of TRC/CD3 Lymphocyte T CD4 expression**	**Schachowski J *et al*, 1991**
* **Increase of CD4 and CD8 soluble receptors concentrations**	**Matsumoto Y *et al*, 1998**
* **Rise of *in vitro* IL1b mononuclear production**	**Pereira BJ *et al*, 1992** **Higuchi T *et al*, 1997**
* **Increase of plasmic concentration of IL1R and IL1R antagonist**	**Pereira BJ *et al*, 1992 e 1994** **Descamps-Latscha B *et al*, 1995** **Higuchi T *et al*, 1997**
* **Increase of *in vitro* mononuclear apoptosis**	**Matsumoto Y *et al*, 1995 e 1997** **Heidenreich S *et al*, 1996** **Martin Malo A *et al*, 2000** **Fernandez-Fresnedo G *et al*, 2000**
* **Decrease of lymphocyte Bcl2 concentration; increase of plasmic Bcl2 concentration**	**Buemi M *et al*, 1998** **Fernandez-Fresnedo G *et al*, 2000**

lymphocyte membrane HLA-DR receptor lymphocyte expression levels do not change significantly after dialysis in the overall population. This difference might be explained by the different categories of genes, CD25 and CD26 being early genes, expressed within 1-2 hours after stimulation and HLA-DR gene being a late gene, expressed more than 2 days after cellular activation[37]. The number of studies that demonstrated an increase of lymphocyte expression of IL2 receptor (α chain or CD25 protein) in ESRD patients after a haemodialysis session and on the steady state[19,38-41] is smaller than those that showed an increase in the same soluble receptor (soluble CD25)[42-52]. Only a few studies report no difference in IL2R membrane expression on lymphocytes after haemodialysis [53,54]. Probably this is an effect of the transitory cellular expres-

TABLE XI
Abnormalities of specific immunity in ESRD patients.

Modification	Reference
* **Lymphocytopenia (LB, LT CD8)**	**Hoy WE WT *et al*, 1878 and 1981; Ramirez G *et al*, 1988 Tchorzewski H *et al*, 1989; Hory B *et al*, 1991 Baj Z *et al*, 1992; Ueki Y *et al*, 1993 Rabb H *et al*, 1994; Deenitchina SS *et al*, 1995; Gogo KO *et al*, 1998**
* **Increase of CD4 and CD8 soluble receptors concentrations**	**Matsumoto Y *et al*, 1998**
* **Increase of soluble CD23 receptor**	**Descamps-Latscha B *et al*, 1993**
* **Cellular immunodeficiency (LT)**	
- **Decrease of T lymphocyte IL2 production**	**Chatenoud L *et al*, 1986; Raskova J *et al*, 1986 Beaurain G *et al*, 1989; Revillard JP *et al*, 1990 Gerez L *et al*, 1991**
- **Increase of membrane and soluble IL2R**	**Chatenoud L *et al*, 1986, Beaurain G *et al*, 1989 Walz G *et al*, 1990, Zaoui P *et al*, 1991 Donati D *et al*, 1991, Vaziri ND *et al*, 1991 Stefoni S et al, 1991, Caruano RJ *et al*, 1992 Patarca R *et al*, 1992; Matsumoto Y *et al*, 1993 Koziol-Montewka M *et al*, 1997; Shu KH *et al*, 1998Daichou Y *et al*, 1999; Meier P *et al*, 2000**
- **Decrease of *in vitro* lymphocyte proliferative response**	**Gast GC *et al*, 1977; Morita Y *et al*, 1997 Alevy YG *et al*, 1984; Raskova *et al*, 1986; Zaoui P *et al*, 1991**
* **Increase of CD3/HLA-DR T lymphocyte %**	**Cheng QL *et al*, 1991; Stefoni S *et al*, 1991 Matsumoto Y et al, 1993; Ueki Y *et al*, 1993 Deenitchina SS *et al*, 1995**
* **Decrease of TCRαβ receptor on T lymphocytes**	**Stefoni S *et al*, 1991; Deenitchina SS *et al*, 1995**
* **Deficit of antigen presenting cells (APC)**	**Mirapeix E *et al*, 1985; Girndt M *et al*, 1993 e 2001**
* **Th2 polarization of T cells**	**Daichou Y *et al*, 1999; Sester U *et al*, 2000; Girndt M *et al*, 2001**
* **Decrease of lymphocyte intracellular pH (deficit of Na+/K+ pump)**	**Rombola G *et al*, 1995**
* **Increase of calcium lymphocyte intracellular concentration**	**Alexiewicz JM *et al*, 1990 and 1996**
* **Increase of IL1, IL6 and TNFα production**	**Luger A *et al*, 1987 ; Paydas S *et al*, 1991;Donati D *et al*, 1991; Memoli B *et al*, 1991; Mege JL *et al*, 1991; Dinarello CA, 1992; Patarca R *et al*, 1992; Haubitz M *et al*, 1992Dinarello CA *et al*, 1995; Lonnemann G *et al*, 1995; Sá H *et al*, 1996; Descamps-Latscha B *et al*, 1996; Donati D *et al*, 1997; Higuchi T *et al*, 1997; Morita Y *et al*, 1997; Kaizu Y *et al*, 1998; Kimmel PL *et al*, 1998; Roccatello D *et al* 1998; Rostaing L *et al* 2000.**

sion of CD25 that is rapidly secreted to blood circulation. The data presented in this study on the CD26 receptor, an exopeptidase that stimulates Th1 lymphocytes to produce cytokines such as INF-γ and IL2 and participates in transendothelial lymphocyte migration, led us to conclude that the increase of its expression after haemodialysis, could contribute to the perpetuation of the inflammatory state associated to uraemia and to dysregulation of the normal distribution of blood and tissue lymphocytes[33]. Increased production of IL1β by ESRD mononuclear cultures enriched on lymphocytes in comparison with the control group strengthens the idea of pre-activation of these kinds of leukocytes in uraemia even when patients are undergoing haemodialysis.

High flux haemodialysis treatment resulted in higher peripheral mononuclear activation than low flux haemodialysis in our study. After a dialysis session, peripheral blood mononuclear cells of patients dialyzed with low-lux membranes (cellulose based and polysulfone dialysers) presented a significant decrease in IL1β production and secretion onto *in vitro* cultures. The patients dialyzed with high flux membranes presented no difference in IL1β supernatant concentrations after the dialysis session (Table IX). We concluded that patients dialyzed with high flux dialysers maintained an elevated rhythm of production and secretion of the pro-inflammatory IL1β cytokine. The stimulus to this increased production and extra-cellular secretion of IL1β might be the diffusive and convective transport of bacterial products across the membrane. These processes are more important when the ultrafiltration per hour is lower than two litres per hour and backdiffusion and backfiltration are strengthed[55]. The difference in supernatants PBMC cultures IL1β concentrations data between low and high flux haemodialysis patients can also be explained by different distribution of this cytokine between the intra and the extracellular compartiments. As demonstrated by Lonnemann G *et al,* when patients currently dialyzed with cuprophan membranes change to high flux synthetic membranes, the total *in vitro* IL1β mononuclear production is identical but the percentage of secretion of this cytokine to the extracellular medium increases[56].

Heidenreich S *et al* and Jaber BL *et al* proved that, paradoxically, the increase of pro-inflammatory cytokines such as TNFα or IL1 could temporally inhibit the apoptotic cascat[6,57]. In agreement with these results we also found a significantly decrease of Fas, FasL and Annexin V peripheral blood lymphocyte expression in the same group that presented significantly higher supernatant PBMC cultures IL1β concentrations after a dialysis session: the high flux polysulfone group. This important observation suggests that, after a haemodialysis session with polysulfone high flux dialysers, peripheral lymphocytes presented a significant improvement of susceptibility to apoptosis death (reduction of Fas lymphocyte expression: preHD: 47.4±10%, postHD: 43.7±9%, p=0.009) and of effective apoptosis rate (decrease of annexin V expression: preHD: 34.5±21.3%, postHD: 25.6±17.3%, p=0.02). It is important to mention that the dialyser membrane didn't seem to be as important as water permeability as the Group 2 patients, who were dialyzed with polysulfone low-flux dialysers, didn't show significant variation of lymphocyte Fas expression or Annexin V expression.

Our data confirm that haemodialysis apparently didn't seem to have influence on plasmic cytokine concentrations such as IL1β, TNFα or IL2, independent of the water permeability of the dialyser used. The same results were described by Rostaing L *et al* in a similar study with different methods[24].

Overall, our results demonstrated that apoptosis lymphocytes markers decrease after

a dialysis session in the global population studied and this was more evident and significant in the group undergoing high flux haemodialysis. Heidenreich S *et al* described, as we do, a decrease in the percentage of peripheral mononuclear cells presenting apoptotic morphology after a dialysis session as compared to the beginning but did not observe differences between groups of patients dialyzed with cuprophan or polysulfone or high and low flux haemodialysis[57]. Other researchers presented data suggesting that quality of dialysis membrane could have some influence on apoptotic leukocyte markers. Carracedo et al, Martin Malo *et al* and Rosenkranz *et al* reported an increase of in vitro apoptosis rate if leukocytes were incubated with cuprophan membranes as compared to polysulfone or polyacrilonitril synthetic membranes[58-60]. Jaber *et al* observed a decrease of *in vitro* polymorphonuclear apoptosis after incubation of these cells with plasma of patients dialyzed with cuprophan. It was not verified if instead of cuprophan, patients were dialyzed with cellulose acetate or polysulfone membranes[61]. Recently, Soriano S *et al* conducted a clinical study similar to ours, and concluded that lymphocyte apoptosis in uraemia is influenced by the permeability of the dialysis membrane. They described a decrease in lymphocyte apoptosis in patients dialyzed with high-flux polysulfone membranes as compared to low flux hemophan or low-flux polysulfone[62].

The observation in our study of a decrease in lymphocyte Fas expression after a dialysis session in the overall population and the fact that this reduction was more accentuated in the group of patients undergoing high-flux polysulfone haemodialysis running in tandem with a decrease in Annexin V expression suggest that the dialytic treatment in general, and the high flux modality in particular, contributes to the elimination of cells such as lymphocytes with apoptotic characteristics. The behaviour of plasmic enzymatic activity of caspase 1 proves this. In the overall population studied, plasma enzymatic activity of caspase 1 decreased significantly after dialysis (preHD: 645.4±319; post HD 529.3±340, p=0.03). Experimental studies based on gene deletion demonstrated a major role played by caspase 1 ("interleukin-1β-converting enzyme or ICE) in apoptosis induced by receptor Fas. Most of the caspases are intracellular but active caspase 1 is localized on the cellular membrane[63]. The decrease of the percentage of lymphocytes with Fas expression after high flux haemodialysis associated to the decrease of Annexin V staining could be related to the elimination of cells from peripheral circulation by the mechanism of activation induced cell death (due to the presence of signals promoting cell survival such as pro-inflammatory cytokines as IL1) or to removal of proapoptotic factors of large molecular weight. In addition, high-flux haemodialysis contributes to the removal of bigger molecules such as proapoptotic factors not completely known such as polyamines, advanced glycosylation end-products (AGEs) and some hypoproliferative citokines such as IL10[12,13]. It is not plausible that a cell which exhibits Annexin V staining could return to the primitive stage, as at that point there is no reversibility of apoptotic cascade and death is definitive.

Compared to a control group, peripheral blood mononuclear cells of dialysis patients cultured *in vitro* without stimulus synthetized and secreted significantly more IL1β, TNFα and IL2 cytokines. Peripheral blood mononuclear supernatants of dialysis patients also presented higher enzymatic activity of caspase 1 when compared to a control group. These observations ratify the *in vivo* results: mononuclear cells of end stage renal patients undergo accelerated apoptosis occurring in tandem with a state of cellular activation.

According to our present data, and compared to a normal population, we can affirm that haemodialysis patients show a immune dysregulation that is characterized by a chronic and non-specific state of lymphocyte and monocyte activation (HLA-DR and CD26 exopeptidase increased cellular expression, increased spontaneous secretion of pro-inflammatory cytokines such as IL1β, TNFα and of IL2 lymphokine) and an increased expression of apoptosis lymphocytes markers (Fas/Fas ligand system, membrane annexin-V binding properties). Both cellulose and polysulfone based haemodialysis maintained and stimulated the immune cell activation status (increased expression of CD25 and CD26 lymphocyte activation markers after a single dialysis session independent of the dialysis membrane). Peripheral mononuclear cell activation rate was higher in high-flux haemodialysis than in low-flux haemodialysis.

In conclusion, a significant improvement of lymphocyte apoptosis rate was observed after a single dialysis session in patients undergoing high-flux haemodialysis with polysulfone dialysers as compared to low-flux haemodialysis with cellulose derivated and polysulfone dialysers. This observation was associated to a significant elevation of pro-inflammatory mediators such as IL1β in high-flux group, indicating that this treatment may counteract immune cell death by continuous generation of cell survival factors or by better removal of pro-apoptotic factors ("death factors").

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