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Original Article

Serum and peripheral blood mononuclear cells infectious burden: Correlation to inflammation and atherosclerosis in haemodialysis patients

GEORGE TSIRPANLIS,[1] STYLIANOS CHATZIPANAGIOTOU,[2] ANASTASIOS IOANNIDIS,[2] FOTINI BOUFIDOU,[2] SPYROS MOUTAFIS[3] and CHRYSOULA NICOLAOU[2]

[1]*Department of Nephrology, General Hospital of Athens,* [2]*Department of Medical Biopathology, Eginition Hospital, Medical School, University of Athens and* [3]*Renal Unit, Erikos Dynan Hospital, Athens, Greece*

SUMMARY:

Background: Infectious agents may be implicated in the inflammatory atherosclerotic process. Not only specific microorganisms but also the infectious burden, defined as the number of pathogens to which a patient is exposed, has been associated with atherosclerosis. In the present study, the infectious burden, determined directly (by identification of viable pathogens in peripheral blood mononuclear cells (PBMC)) and indirectly (by serum antibodies detection) is correlated to the inflammatory and atherosclerotic status in haemodialysis (HD) patients, a population at high risk for cardiovascular disease.

Methods: The viable forms of four microorganisms (*Chlamydia pneumoniae*, herpes virus 1 and 2 and cytomegalovirus) were identified in patients PBMC by cell cultures and subsequent polymerase chain reaction. Serum IgG against the above pathogens and *Helicobacter pylori* were also determined. Inflammation was assessed by measurement of C-reactive protein (CRP), serum amyloid A (SAA), three pro- and one anti-inflammatory cytokines and four adhesion molecules. Atherosclerosis was defined by a scoring system using medical history data.

Results: The number of viable pathogens identified in PBMC in the 122 HD patients included in the study were zero in 22.1% of them, one in 33.6%, two in 43.4% and three in one patient. The number of IgG antibodies determined was one in 6.6% of patients, two in 32%, three in 48.4% and four in 13.1%. Seropositivity was not significantly different between patients with or without the respective viable pathogen identified in PBMC. Atherosclerosis was present in 40.2% of patients, and CRP, SAA and interleukin-6 were all increased in these patients. Neither inflammatory indexes nor atherosclerosis were significantly different in patients with a higher number of viable pathogens detected in PBMC or in those with a higher antibodies number.

Conclusions: The direct infectious burden determination (the number of viable pathogens in PBMC) does not coincide with the serum (by IgG detection) infectious burden. Although inflammation correlates to atherosclerosis, neither PBMC nor the serum infectious burden is associated with these two entities in the inflamed and atherosclerotic HD patients.

KEY WORDS: **cell culture, C-reactive protein, IgG antibodies, interleukin-6, vascular cell adhesion molecule-1.**

INTRODUCTION

Atherosclerosis has recently been recognized as an inflammatory process.[1] Infectious agents might be a cause that initiates or promotes this process.[2] Specific pathogens establishing chronic persistent infections, such as *Chlamydia pneumoniae* (*C. pneumoniae*), herpes virus 1 or 2 (HSV-1, HSV-2), cytomegalovirus (CMV) or *Helicobacter pylori* (*H. pylori*) are probable candidates as atherosclerosis-provoking agents.[3] More recently, it has been proposed that the cumulative pathogen load – the pathogen burden – rather than a single pathogen is cor-

Correspondence: Dr George Tsirpanlis, Kriezi 61, Polydroso, Marousi, 15125, Athens, Greece. Email: tsipg@hellasnet.gr

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related to atherosclerotic cardiovascular disease (CVD).[4] Clinical studies in this field have conflicting results.[5,6]

Atherosclerotic CVD morbidity and mortality are particularly significant in end-stage renal failure (ESRF) patients on haemodialysis (HD).[7] Inflammation is also increased in these patients.[8] The infectious burden and its correlation to atherosclerosis and inflammation have not yet been investigated in this population.

The infectious burden, in studies associating this finding to atherosclerosis, is determined indirectly by the number of serum antibodies against the microorganisms examined. Identification of viable pathogens in peripheral blood mononuclear cells (PBMC) is a direct method for this assessment. To our knowledge, it has not been investigated up until now as to whether this later 'PBMC infectious burden' (i.e. the number of viable pathogens in PBMC) coincides with the 'serum infectious burden' (i.e. the number of serum antibodies against these microorganisms), and also whether it is associated with atherosclerosis and inflammation. In the present study, (i) the infectious burden is determined directly, identifying the viable forms of four microorganisms – *C. pneumoniae*, CMV, HSV-1 and HSV-2 – in PBMC by cell cultures and subsequent polymerase chain reaction (PCR), as well as indirectly by measuring serum IgG antibodies against these four pathogens and also, against *H. Pylori*; (ii) this infectious burden is correlated to serum levels of a number of molecules activated and secreted during the inflammatory process; and (iii) the aggregate pathogen load is associated with atherosclerosis in ESRF patients on HD.

By applying the above described approach, the purpose of the present study is to investigate if an association exists between the number of pathogens detected and inflammation or accelerated atherosclerosis observed in HD patients.

METHODS

Patients' characteristics

One hundred and forty-two stable HD patients were initially examined, after informed consent was given. Twelve patients were excluded because of active infection and/or antibiotic treatment or surgical operation in the last month before the onset of the study. In eight patients from the remaining 130 patients, the sample quantity was insufficient for all of the tests performed. Of the 122 HD patients, which were included in the present study, 71 were males, with (mean ± SD) age 61.35 ± 13.86 (minimum 18, maximum 87) years, 13.9% were diabetics, 47.5% had hypertension (in antihypertensive treatment or blood pressure >140/80 mmHg in three measurements in the last month), 23.8% were smokers, 33.4% were on angiotensin converting enzyme inhibitors, 36% on acetylsalycylic acid (80 mg/day) and 1.2% were on statins. The body mass index was 25.68 ± 4.57.

The cause of ESRF was chronic glomerulonephritis in 48 patients, diabetic nephropathy in 12, hypertensive nephrosclerosis in 21, polycystic disease in 19, other causes in 10 and undetermined causes in 12 patients. The average time on HD was 71.56 ± 64.17 months (range, 1–280). Four patients were on haemodiafiltration and the remaining 118 were on conventional HD with bicarbonate. Seventy-eight patients (63.9%) were dialysed with a modified cellulose membrane (Polysynthane, Baxter, McGaw Park, IL, USA) and 44 (36.1%) with a synthetic membrane (Althane, Baxter). Dialysis filters had an ultrafiltration coefficient of <10 mL/h mmHg in 63.9% of patients. The water used in the dialysate was processed from the same system (central reverse osmosis) for the whole group of patients.

Atherosclerotic CVD profiles in each patient were evaluated using the CVD portion of the Index of Co-Existing Diseases for this entity (Table 1), a standardized four-level scale tested in multiple studies.[9]

Table 1 Estimation of atherosclerotic cardiovascular disease (scoring system)

Score	Definition
Coronary heart disease	
0	Absence of disease in past or present
1	Diagnosis of coronary heart disease in the past or present
	Ischaemia on electrocardiogram or other diagnostic tests
	Stable or exertional angina or angina during haemodialysis
2	History of MI
	Evidence of MI on electrocardiogram
	History of coronary revascularization procedures
3	Angina at rest
	Acute MI within the past 3 months
Cerebrovascular disease	
0	Absence of disease in past or present
1	Diagnosis of cerebrovascular disease in the past
	Asymptomatic carotid stenosis or history of TIA
2	History of carotid endarterectomy
	Multiple TIA in the past
	Current usage of anticoagulants for cerebrovascular disease
3	History of stroke with no or mild residual neurological deficits
	History of stroke with major residual neurological deficits
Peripheral vascular disease	
0	Absence of disease in past or present
1	Diagnosis of peripheral VD or aortic aneurism in the past
2	History of amputation of digits or extremities secondary to VD
	History of peripheral arterial bypass or aortic aneurism repair
	Intermittent claudication
	Recurrent cellulitis, skin infections, or toe gangrenes secondary to VD
	VD currently requiring anticoagulants
3	History of knee amputation
	Pain at rest secondary to peripheral VD
	Inoperable VD

MI, myocardial infraction; TIA, transient ischaemic attacks; VD, vascular disease.

Blood sample collection

Blood samples of 15 mL were taken before dialysis from vascular access, immediately following venipuncture, within two consecutive days for all patients. Two 5 mL ethylenediaminetetraacetic acid (EDTA) tubes were used for the cell cultures and one 5 mL tube without anticoagulant was used for the determination of cytokines, adhesion molecules and serum IgG antibodies.

C-reactive protein, serum amyloid A, serum cytokines and soluble adhesion molecule assays

Sera separated from the coagulated blood by centrifugation within 30 min after collection were transferred immediately to sterile tubes and stored at −20°C until used. The quantitative determination of high sensitivity C-reactive protein (hs-CRP), serum amyloid A (SAA) by immune-nephelometry and interleukin 1β (IL-1), interleukin-6 (IL-6), tumor necrosis factor α (TNF), interleukin-10 (IL-10), L-selectin (L-Sel), E-selectin (E-Sel), soluble intercellular adhesion molecule-1 (ICAM-1), soluble vascular cell adhesion molecule-1 (VCAM-1) were evaluated by an enzyme-linked immunosorbent assay (ELISA) (R & D System Europe Ltd, Oxon, UK). All samples and standards were assayed in duplicate.

Serum IgG against HSV-1, HSV-2, cytomegalovirus, C. *pneumoniae* and *H. Pylori*

IgG antibodies against C. *pneumoniae* were determined by indirect microimmunofluorescent assay (MIF) techniques, as previously described.[10] IgG antibodies against HSV-1, HSV-2, CMV and *H. pylori* were measured by ELISA (Institut Virion-Serion GmbH, Wurzburg, Germany). A sample was considered positive when the activities of the antibodies were higher than 30 U/mL for HSV-1, HSV-2 and *H. Pylori* and over 42 U/mL for CMV virus. All samples and standards were assayed in duplicate.

Cell cultures and subsequent PCR for detection of HSV-1, HSV-2, CMV and C. *pneumoniae* in PBMC

All microbial agents were detected from PBMC after inoculation of cell cultures with buffy coats and subsequent molecular amplification by PCR. The cell lines used for the culture were as follows: MRC-5 cells for HSV-1 and 2, as well as for CMV, and Hep-2 cells for C. *pneumoniae*.

Preparation of buffy coats

Five milliliters of EDTA whole blood from each patient was centrifugated at 3000 g for 15 min. The buffy coat was carefully aspirated with a sterile Pasteur pipette transferred into 2.5 mL cryovials and stored at −160°C (liquid nitrogen) until the day of determination.

Cell cultures

Cell cultures were performed by the shell vial technique using a commercially available kit (Vircell, SL, Granada, Spain). Cells were detached after shaking with glass beads and the resulting homogenates were purified with respect to DNA, using a commercial kit (Nucleospin Blood; Macherey-Nagel, Dueren, Germany) and used for PCR detection. Controls consisted of the following reference strains: ATCC VR-539 for HSV-1, ATCC VR-538 for CMV and ATCC VR-1355 TWAR strain 2043 for C. *pneumoniae*. Controls were run the same way as the buffy coat samples.

Polymerase chain reaction

Molecular detection of HSV-1, HSV-2 and CMV was performed by a multiplex PCR assay, as described by Markoulatos *et al.*,[11] using the following primer sets: RL-2 for HSV-1 (127 bp), UL28 for HSV-2 (227 bp) and IRL 11 for CMV (256 bp). For the detection of C. *pneumoniae* nested PCR was performed using a commercially available kit (Clonit Srl, Milano-Italy), as described previously.[10] Cell cultures with a microbial reference strain were used as positive controls and plain cultures as negative controls.

Statistics

Categorical variables were compared by χ^2. Continuous variables were analysed by ANOVA and Student's *t*-test. All biological variables measured were logarithmically transformed because of their skewed distribution (un-transformed values are shown in the tables). Pearson's correlation coefficients were calculated for the univariate analysis between parameters. Multiple logistic regression analysis was performed using atherosclerosis (or coronary or cerebrovascular or peripheral vascular disease) as the dependent variable and sex, age, time in HD, body mass index, smoking, diabetes mellitus, hypertension, HD method, dialyser membrane type, vascular access type, seropositivity to the five infectious agents, identification of the four infectious agents in PBMC and all biological variables measured as covariates with entry factors at $P < 0.05$ and with removal of factors that no longer contribute at $P > 0.10$ in a forward stepwise (like-hood ratio) fashion. A significance level of 0.05 was used for all statistical tests. Analyses were performed using SPSS version 10.0 (SPSS Inc., Chicago, IL, USA).

RESULTS

The IgG antibodies against the five infectious agents examined the viable pathogens identified in PBMC for four infectious agents (Fig. 1),[10] as well as the correlation between seropositivity or seronegativity and viable pathogens identification in PBMC, in the 122 HD patients, are shown in Table 2. Seropositivity was not correlated to the viable pathogens' presence in PBMC. In particular, seropositivity for C. *pneumoniae* was significantly higher in patients negative for this pathogen in PBMC than in positive ones.

The mean ± SD, median (range) serum levels of the biological variables determined were as follows: CRP, 8.26 ± 8.64, 5.90 (0.30–56.90) mg/L; SAA, 15.83 ±

16.54, 10.20 (1.10–93.00) mg/L; IL-1, 2.73 ± 2.65, 2.00 (0.20–20.00) pg/mL; IL-6, 8.38 ± 11.64, 5.45 (1.10–112.00) pg/mL; TNF, 13.48 ± 15.39, 10.65 (2.80–122.50) pg/mL; IL-10, 4.77 ± 7.41, 2.60 (0.30–60.00) pg/mL; ICAM-1, 608.68 ± 160.99, 576.50 (275.00–1115.5) ng/mL; VCAM-1 1203.80 ± 568.55, 994.00 (139.00–2985.00) ng/mL; and L-Sel 1217.14 ± 398.78, 1195.50 (330.50–2287.00) ng/mL. CRP was significantly correlated to SAA ($P = 0.0001$) and IL-6 ($P = 0.0001$), while SAA was significantly correlated to IL-6 ($P = 0.0001$). ICAM-1 was correlated to VCAM-1 ($P = 0.037$), L-Sel ($P = 0.009$) and E-Sel (0.001). L-Sel was significantly correlated to E-Sel ($P = 0.001$).

Forty-nine patients (40.2%) had atherosclerotic coronary, cerebrovascular or peripheral vascular disease of any grade (1, 2 or 3 according to the scoring system in Table 1), while the remaining 73 patients were free of CVD. Forty patients had signs of coronary disease (29 patients had a score of 1, 10 patients had a score of 2 and 1 patient had a score of 3), 10 patients had signs of cerebrovascular disease (eight patients had a score of 1 and two patients had a score of 2) and 15 patients had signs of peripheral vascular disease (11 patients had a score of 1, three patients had a score of 2 and 1 patient had a score of 3) according to the scoring system in Table 1.

Patients with atherosclerotic CVD had significantly higher serum levels of CRP, SAA and IL-6 than patients free of CVD (mean ± SD: 10.91 ± 10.17 *vs* 6.48 ±

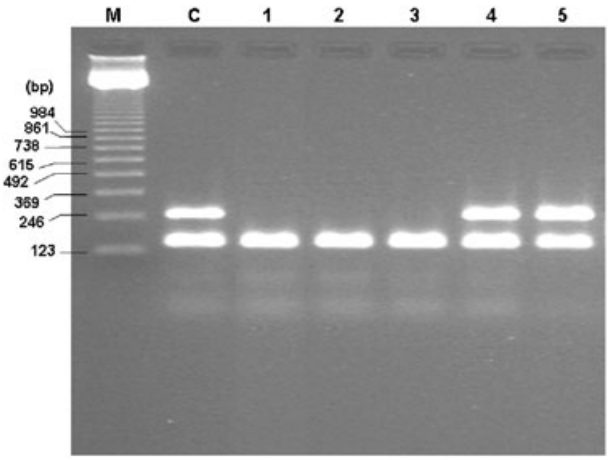

Fig. 1 Polymerase chain reaction (PCR) for herpes virus 1 (HSV-1) and cytomegalovirus (CMV) detection in DNA extracted from cell cultures. Ethidium bromide-stained agarose gel of multiplex PCR products (147 bases for HSV-1 and 256 bases for CMV) after amplification of the RL 2 and IRL 11 genome region of HSV-1 and CMV, respectively, from cultured peripheral blood mononuclear cells of haemodialysis patients (lanes 1–5). Lane M: molecular markers. Lane C: multiplex PCR products from elementary bodies of the American-type cell culture (ATCC) VR-539 of HSV-1 and ATCC VR-538 of CMV (internal positive control). Lanes 1–3: patients positive for HSV-1. Lanes 4 and 5: patients positive for HSV-1 and CMV simultaneously.

Table 2 Serum IgG antibodies and viable pathogens (identified by cell cultures and subsequent polymerase chain reaction) in PBMC for five and four infectious agents, respectively, in 122 haemodialysis patients

	IgG CMV (+)	IgG CMV (–)	No. patients (%)
CMV in PBMC (+)	38	9	47 (38.5)
CMV in PBMC (–)	68	7	75 (61.5)
	$P = 0.10$		
No. patients (%)	106 (86.9)	16 (13.1)	122 (100)
	IgG HSV-1 (+)	IgG HSV-1 (–)	No. patients (%)
HSV-1 in PBMC (+)	24	70	94 (77.1)
HSV-1 in PBMC (–)	5	23	28 (22.9)
	$P = 0.28$		
No. patients (%)	29 (23.8)	93 (76.2%)	122 (100)
	IgG HSV-2 (+)	IgG HSV-2 (–)	No. patients (%)
HSV-2 in PBMC (+)	0	0	0 (0)
HSV-2 in PBMC (–)	91	31	122 (100)
No. patients (%)	91 (74.5)	31 (25.5)	122 (100)
	IgG Cpn (+)	IgG Cpn (–)	No. patients (%)
Cpn in PBMC (+)	1	8	9 (7.4)
Cpn in PBMC (–)	59	54	113 (92.6)
	$P = 0.018$		
No. patients (%)	60 (49.2)	62 (50.8)	122 (100)
	IgG *H. pylori* (+)	IgG *H. pylori* (–)	No. patients (%)
No. patients (%)	42 (34.4%)	80 (65.6%)	122 (100)

Values represent the number of patients. CMV, cytomegalovirus; Cpn, *Chlamydia pneumoniae*; *H. pylori*, *Helicobacter pylori*; HSV-1, herpes virus-1; HSV-2, herpes virus-2; PBMC, peripheral blood mononuclear cells.

6.97 mg/L, $P = 0.002$; 21.37 ± 22.20 *vs* 12.10 ± 9.79 mg/L, $P = 0.011$; and 9.32 ± 7.35 *vs* 7.76 ± 13.80 pg/mL, $P = 0.021$, respectively).

The number or serum IgG antibodies against the five infectious agents determined per patient (serum infectious burden) and the number of viable pathogens identified in PBMC (for four agents: CMV, HSV-1, HSV-2 and C. *pneumoniae*) per patient (the PBMC infectious burden) are shown in Table 3.

Serum values of the 10 biological variables measured were not significantly higher in patients with a high serum infectious burden, as shown in Table 4; the same was valid for patients with a high PBMC infectious burden (Table 5). Patients seropositive or seronegative for each of the five infectious agents, examined separately, did not have statistically significant differences in the serum values of the 10 biological variables measured (data not shown). The same was valid in positive or negative patients for each of the four infectious agents identified in PBMC, also examined separately, with the exception of patients positive for viable C. *pneumoniae* in PBMC, as was shown previously.[12]

Atherosclerotic CVD prevalence was not found to be statistically significantly different between patients with a different serum (Table 6) or PBMC (Table 7) infectious burden. Examining atherosclerotic CVD prevalence separately in patients seronegative or seropositive in each of the five microorganisms examined was not found to have any statistically significant differences (data not shown). The same finding was valid when patients positive or negative (for viable pathogen presence in PBMC) for the four pathogens, CMV, HSV-1, HSV-2 and C. *pneumoniae*, were examined separately for each agent, except for patients positive for C. *pneumoniae* in PBMC.[10]

In multiple logistic regression analysis with atherosclerosis as the dependent factor, only male gender, increasing age and increasing log CRP were potential

Table 3 Serum infectious burden (number of positive IgG antibodies) and PBMC infectious burden (number of viable microorganisms in PBMC identified by cell cultures and PCR)

No. positive serum IgG antibodies/patient (%)†				
1	2	3	4	Total no. patients (%)
8 (6.6)	39 (32)	59 (48.4)	16 (13.1)	122 (100)
No. viable pathogens in PBMC/patient (%)‡				
0	1	2	3	Total no. patients (%)
27 (22.1)	41 (33.6)	53 (43.4)	1 (0.8)	122 (100)

†Serum pathogen burden, defined using five infectious agents: cytomegalovirus; herpes virus-1; herpes virus-2; C*hlamydia pneumoniae*; and *Helicobacter pylori*.

‡PBMC pathogen burden, defined using four infectious agents: cytomegalovirus; herpes virus-1; herpes virus-2; and C*hlamydia pneumoniae*.

PCR, polymerase chain reaction; PBMC, peripheral blood mononuclear cells.

Table 4 Inflammatory markers and adhesion molecules in 122 haemodialysis patients according to the serum infectious burden (number of serum IgG antibodies against five pathogens)

	No. serum IgG antibodies				
	1 (8 patients)	2 (39 patients)	3 (59 patients)	4 (16 patients)	*P*-value (ANOVA)
CRP mg/L	7.0 ± 5.4	9.9 ± 11.2	7.3 ± 5.4	7.9 ± 5.4	0.451
SAA mg/L	16.2 ± 8.3	15.8 ± 16.3	16.5 ± 18.9	13.0 ± 10.4	0.803
IL-1 pg/mL	2.8 ± 1.3	3.5 ± 4.1	2.5 ± 1.5	1.6 ± 1.4	0.002†
IL-6 pg/mL	5.1 ± 2.9	7.5 ± 7.4	9.5 ± 15.1	7.9 ± 7.7	0.645
TNF pg/mL	9.1 ± 3.0	12.5 ± 5.8	15.6 ± 21.3	9.7 ± 4.3	0.391
IL-10 pg/mL	7.8 ± 14.6	4.4 ± 4.7	4.6 ± 8.3	4.3 ± 3.1	0.874
ICAM-1 ng/mL	636.5 ± 171.1	609.5 ± 161.9	584.0 ± 157.5	683.7 ± 155.3	0.139
VCAM-1 ng/mL	907.8 ± 105.5	1318.6 ± 661.1	1187.7 ± 574.1	1131.0 ± 364.3	0.551
L-Sel ng/mL	1426.3 ± 303.2	1223.0 ± 378.0	1169.7 ± 384.7	1272.8 ± 519.5	0.371
E-Sel ng/mL	112.1 ± 34.8	67.6 ± 24.32	73.5 ± 32.26	83.0 ± 31.8	0.012‡

Values represent mean ± SD.

†Patients with four IgG antibodies had significantly lower IL-1 levels than patients with one, two or three IgG antibodies ($P = 0.031$, 0.001 and 0.003, respectively; *T*-test using Log IL-1).

‡Patients with one IgG antibody had significantly higher E-Sel levels than patients with two or three IgG antibodies ($P = 0.001$ and 0.006, respectively; *T*-test using Log E-Sel).

CRP, C-reactive protein; E-Sel, E-selectin; ICAM, intercellular adhesion molecule; IL, interleukin; L-Sel, L-selectin; SAA, serum amyloid A; TNF, tumor necrosis factor; VCAM, vascular cell adhesion molecule.

Table 5 Inflammatory markers and adhesion molecules in 121 haemodialysis patients according to PBMC infectious burden (number of viable pathogens in PBMC, identified by cell cultures and subsequent polymerase chain reaction for four infectious agents)

	No. viable pathogens in PBMC†			
	0 (27 patients)	1 (41 patients)	2 (53 patients)	*P*-value (Anova)
CRP mg/L	10.5 ± 13.7	6.8 ± 5.9	8.2 ± 6.8	0.628
SAA mg/L	21.2 ± 20.5	13.5 ± 11.3	14.9 ± 17.4	0.210
IL-1 pg/mL	2.5 ± 1.7	2.9 ± 1.3	2.7 ± 3.6	0.068
IL-6 pg/mL	11.6 ± 21.1	6.0 ± 5.1	8.6 ± 8.2	0.061
TNF pg/mL	12.8 ± 9.1	12.8 ± 18.0	14.3 ± 16.0	0.643
IL-10 pg/mL	4.6 ± 3.9	4.0 ± 4.3	5.5 ± 10.2	0.813
ICAM-1 ng/mL	643.2 ± 165.3	576.6 ± 150.8	617.9 ± 165.4	0.219
VCAM-1 ng/mL	1172.6 ± 599.7	1393.4 ± 670.3	1081.1 ± 422.8	0.045‡
L-Sel ng/mL	1202.4 ± 480.2	1167.5 ± 359.5	1265.0 ± 388.1	0.450
E-Sel ng/mL	65.2 ± 27.9	77.7 ± 30.7	79.7 ± 32.9	0.078

Values represent mean ± SD.

†The patient with three viable pathogens in PBMC was excluded from this analysis.

‡Patients with one viable pathogen had higher VCAM-1 levels than patients with no viable pathogen (but this difference was statistically non-significant with *T*-test using the Log VCAM-1, $P = 0.079$) and patients with two viable pathogens ($P = 0.008$, *T*-test).

CRP, C-reactive protein; E-Sel, E-selectin; ICAM, intercellular adhesion molecule; IL, interleukin; L-Sel, L-selectin; PBMC, peripheral blood mononuclear cells; SAA, serum amyloid A; TNF, tumor necrosis factor; VCAM, vascular cell adhesion molecule.

Table 6 Atherosclerosis according to the serum infectious burden (number of IgG antibodies for five infectious agents)

	No. serum IgG antibodies				
	1	2	3	4	No. patients (%)
Atherosclerosis					
Positive†	2	18	19	10	49 (40.2)
Negative	6	21	40	6	73 (59.8)
			$P = 0.101$		
No. patients (%)	8 (6.6)	39 (32.0)	59 (48.3)	16 (13.1)	122 (100)

†Coronary, cerebrovascular or peripheral vascular disease according to the scoring system used (Table 1).

Table 7 Atherosclerosis according to the PBMC infectious burden (number of viable pathogens in PBMC, identified by cell cultures and polymerase chain reaction for four infectious agents)

	No. viable pathogens in PBMC				
	0	1	2	3	No. patients (%)
Atherosclerosis					
Positive†	12	13	23	1	49 (40.2)
Negative	15	28	30	0	73 (59.8)
			$P = 0.370$		
No. patients (%)	27 (22.1)	41 (33.6)	53 (43.5)	1 (0.8)	122 (100)

†Coronary, cerebrovascular or peripheral vascular disease according to the scoring system used (Table 1).

PBMC, peripheral blood mononuclear cells.

risk factors (odds ratio, 5.128, 1.090 and 4.347; and $P = 0.001$, 0.000 and 0.024, respectively) for atherosclerotic CVD. Separate multiple logistic regression analyses for coronary disease, cerebrovascular and peripheral vascular disease revealed that the same factors remained as predictors, except for CRP, which was significant for atherosclerosis as a whole but not for specific diseases (coronary, peripheral or cerebrovascular).

DISCUSSION

Main findings

The present study showed that the infectious burden, as defined indirectly by the detection of serum antibodies against some microorganisms, does not coincide with the one defined directly by identification of the same viable

pathogens in PBMC. Although the main inflammatory indexes were increased in atherosclerotic patients, our results regarding correlation of the infectious burden were negative, as defined by both methods, either to inflammation or to atherosclerosis in HD patients at high risk of CVD.

'Direct' and 'indirect' infectious burden definition

In the present study, the infectious burden was defined by direct and indirect methodology. By applying the combination of two assays (cell culture and subsequent PCR), the viable forms of four (obligatory intracellular in eukaryotic host cells) pathogens were identified. By cell culture, viability and infectivity of these microorganisms in PBMC is proven; by PCR, a sensitive and specific molecular method, accurate identification of these pathogens is confirmed. Moreover, monocyte is a crucial cell type for an infectious-based atherosclerotic hypothesis; it is implicated in the transport of these pathogens from the infectious focus to the other tissues,[2] including the arterial wall, as well as in the atherosclerotic process itself.[13]

The discrepancy between seropositivity and identification of the viable form of the same pathogens in PBMC (Table 2), confirms similar findings in other studies, at least for C. *pneumoniae*.[14] It seems that single serum IgG determination is not sufficient to establish a diagnosis for chronic persistent infection by microorganisms explored in this study.[15] This observation, as well as the high grade of seropositivity against these pathogens in the general population,[16] might be two causes of the conflicting results in the studies,[5,6,17–19] correlating the infectious burden to atherosclerosis by using the detection of antibodies for its definition.

Infectious burden and inflammation

The serum level of the biological variables (inflammatory indexes, pro-inflammatory and anti-inflammatory cytokines and adhesion molecules) measured in the present study did not differ significantly between patients with a low or high infectious burden, as defined either by seropositivity or by identification of viable pathogens' in PBMC (Tables 4 and 5). Studies in this field, in the atherosclerotic population, have conflicting results.[17,20]

Inflammation sources in HD patients are multiple. Although renal failure itself and the HD procedure are the main ones, differences in the inflammation level commonly observed between these patients are difficult to be explained.[8] A significantly different aggregate pathogen load between these patients could be a probable cause that should explain these differences. This suggestion is not confirmed by our results. One exception is worth noting. Interleukin-1β and VCAM-1 was significantly increased in patients with viable C. *pneumoniae* in PBMC, as was found by our group in a previous study.[12]

Infectious burden and atherosclerosis

Atherosclerosis was present in a high percentage of patients included in the present study, as in other studies on HD patients.[7] These patients had significantly higher serum levels of the three indexes (CRP, SAA and IL-6) more frequently found to be increased in clinical studies correlating atherosclerosis and inflammation.[21] Neither the status of serum antibodies nor identification of viable pathogens in PBMC, for each microorganism separately or for the total infectious burden in every patient, were a determining factor for atherosclerotic CVD (Tables 6 and 7). On the contrary, as previously shown,[10] C. *pneumoniae* presence in PBMC was correlated to atherosclerosis. In multiple logistic regression analysis, only increasing age, male sex and high CRP values were found to be risk factors for atherosclerosis in HD patients included in the present study.

Although, non-traditional atherosclerotic CVD risk factors (lipoprotein-a, inflammation, oxidative stress and homocysteine) seem to play a more important role in ESRF patients than in the general population,[22] the infectious-based inflammatory atherogenous hypothesis is not supported by the findings in the present study.

Limitations of the study

Some limitations have to be noted in the present study. The first one is that a scoring system, based on clinical and medical history data and not a direct arterial wall examination, was used for the detection of atherosclerosis. With such a tool, only a late (after plaque destabilization) form of atherosclerosis can be detected. Although a recent study showed that the infectious burden is not correlated to atherosclerosis detected in a very early phase,[18] by estimation of endothelial dysfunction, this limitation has to be taken under consideration. A second limitation is that the biological variables measured were determined once, at a random point time. The variability of these indexes over time might influence our results.[8,23] Although longitudinal studies are necessary for an accurate estimation of the inflammatory status in these patients, we consider that the strict exclusion of patients with an active inflammatory process partially corrected this limitation.[10]

CONCLUSIONS

The present study showed that in the inflamed and atherosclerotic population of HD patients, the infectious burden is not correlated to inflammation or atherosclerosis.

rosis. This negative association found in the present study is probably stronger than the one in other similar studies for two reasons: first, infectious burden was defined directly, identifying the viable pathogens in PBMC, as well as indirectly by seropositivity; and second, the population examined, renal failure patients on HD, is more likely to be influenced by non-traditional atherosclerotic risk factors such as infection and inflammation than the general population. It is worth noting that the above mentioned does not seem to be valid for *C. pneumoniae*.[24]

Further studies, using similar methods for the identification of suspicious pathogens in larger populations are needed for a definite answer, negative or positive, to this interesting infectious-based atherogenous hypothesis.

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