CHLAMYDIA PNEUMONIAE INFECTION AS A RISK FACTOR FOR ACCELERATED ATHEROSCLEROSIS IN HEMODIALYSIS PATIENTS

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Atherosclerotic cardiovascular disease is the main cause of morbidity and mortality for end-stage renal disease patients undergoing chronic haemodialysis (HD). Several studies in recent years have identified *Chlamydia pneumoniae*, a respiratory pathogen, as risk factor for cardiovascular diseases in the general population. The aim of our study is to evaluate chlamydial load, in peripheral blood mononuclear cells (PBMC) of HD patients. Furthermore, the correlation between DNA chlamydial load and markers of inflammation was also examined. PBMC specimens isolated from 49 HD patients and 46 blood donors were analyzed for the presence of *C. pneumoniae* DNA by real-time PCR and *omp*A nested touchdown PCR. In HD patients, plasma levels of several inflammatory markers were also determined. A significantly higher rate of *C. pneumoniae* DNA was found in HD patients (44.9%) than in blood donors (19.6%) (p=0.016); HD patients were also more likely to have a significantly high chlamydial load (p=0.0004). HD patients with atherosclerotic cardiovascular diseases have a significantly greater chlamydial load than HD patients without cardiovascular diseases (p= 0.006). A significantly higher value of C-reactive protein, IL-6 and advanced oxidative protein products was found in HD patients with a greater chlamydial load (p<0.05). Likewise, a significantly lower monocyte HLA-DR percentage (p=0.011) as well as a lower monocyte HLA-DR expression were found in such patients (p= 0.007). In conclusion, our results show that HD patients are at high risk of *C. pneumoniae* infection correlated with chronic inflammatory response which in turn can lead to accelerated atherosclerosis and other long-term clinical complications such as myocardial infarction and stroke.

Atherosclerotic cardiovascular disease is the main cause of morbidity and mortality for end-stage renal disease (ESRD) patients undergoing chronic haemodialysis (HD), accounting for more than 50% of deaths (1).

Atherosclerosis is widely regarded as chronic inflammatory process in the artery vessel wall which is characterized by endothelial injury, accumulation of monocytic cells, and increased secretion of cytokines, indicating an active local inflammatory response. Inflammatory response in HD patients has been associated with an increase of C-reactive protein (CRP), several proinflammatory cytokines, and also a reduction of albumin synthesis (2). During the

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inflammatory process, cytokines primarily induced by macrophages and monocytes, such as interleukin-6 (IL-6), have a role in the pathophysiology of inflammation in these patients (3). CRP and IL-6 have been recognized as novel predictors for atherosclerosis, cardiovascular diseases, and poor outcome in the dialysis population (1, 4-5).

A potential link between inflammation and subsequent cardiovascular risk in HD patients may be the process of oxidative stress. Favorable conditions for the generation of oxidative stress are generally present in uremic patients on maintenance hemodialysis in whom a recurrent increased production of oxidants occurs in the face of a chronic antioxidant deficiency (6).

In addition, in patients on maintenance hemodialysis the exposure to bio-incompatible tubing and dialysis membranes, the presence of access grafts or intravenous catheter, and poor quality of dialysis water and back-filtration may chronically aggravate inflammatory processes (7). These observations collectively suggest that oxidative stress and inflammation are causally and synergistically linked to the pathogenesis of atherosclerosis and cardiovascular disease.

Over the last decade, several reports have demonstrated that *C. pneumoniae* is involved in the development of atherosclerotic process since it promotes an intense inflammatory response in the vessel wall (8-10). Indeed *C. pneumoniae* has been shown to be able to multiply in a wider array of host cell types including peripheral mononuclear cells (PBMC), endothelial cells, smooth muscle cells, mast cells and macrophages, resulting in production of proinflammatory cytokines (IL-1, IL-6), reactive oxygen species and expression of cell surface molecules that may contribute to inflammation and oxidative stress (9-15). Thus, chronic inflammatory response in HD patients results more relevant since several causes including dialysis procedure, oxidative stress, and increased susceptibility to infections are involved. In particular, *C. pneumoniae* infection has been associated with inflammatory atherogenous process by several serological and molecular studies (16-18).

In view of these facts, this study was designed to determine the association between chlamydial load and markers of inflammation in order to gain a better understanding of infectious load regulation in HD patients.

MATERIALS AND METHODS

Patients

Forty-nine HD patients from a single dialysis unit were enrolled in this study. These patients regularly received HD three times weekly and the length of time per HD procedure was 3.5-4.5 h. Data on cardiovascular diseases, smoking history, diabetes, hypertension, chlolesterol and triglycerides were collected.

Hypercholesterolemia was defined as total cholesterol level $\geq$ 220 mg/dl or lipid lowering drug administration; hypertriglyceridemia was defined as triglycerides level $\geq$ 150 mg/dl. Diabetes mellitus was defined as plasma glucose level $\geq$ 126 mg/dl or a history of current insulin therapy. Hypertension was defined as a systolic blood pressure $\geq$ 140 mmHg and/or diastolic blood pressure $\geq$ 90 mmHg or a history of current antihypertensive drug treatment.

Atherosclerotic cardiovascular disease was defined as documented past or present ischemic events involving the coronary, cerebral or peripheral circulation. Myocardial infarction was diagnosed by typical changes in the electrocardiogram and raised serum enzyme levels. Stroke was diagnosed by color flow echo Doppler imaging, cerebral scanning, transcranial Doppler scanning and peripheral arterial diseases by peripheral angiography.

The patients with other sources of inflammation such as malignancy, autoimmune disease and trauma were excluded from our study.

Baseline characteristics of HD patients are reported in Table I.

Blood samples were drawn pre-dialysis during the mid-week HD treatment. These were taken from the arterial needle immediately after needle insertion, and before any intravenous fluid was given to the patients under sterile and non-contaminated condition.

Forty-six healthy blood donors matched to patients for age (mean age 60±10 years), gender (38 males and 8 females) and body mass index (BMI) (23±4 kg/m^2) were also included in our study. At the time of blood sampling, no blood donor had cardiovascular risk factors or symptoms of respiratory infections.

The study was approved by the Medical Ethics Committee of our Institution and all the subjects gave informed consent. All the procedures followed were in accordance with institutional guidelines.

Detection of C. pneumoniae DNA

5 ml EDTA blood was collected and processed to isolate PBMC using Ficoll-Hypaque density gradient

centrifugation (Sigma) DNA was extracted from 10^6 PBMC by using a Qiagen DNA Mini-kit according to the manufacturer's instructions (Qiagen). Genomic DNA was stored at –20°C until used as template in PCR assays. In order to minimise the risk of false-positive results, negative reagent controls obtained by replacing clinical specimens with an equal volume of ultrapure water PCR grade were both included and processed throughout the whole extraction procedure.

DNA extracted from PBMC was analyzed by real-time PCR and *omp*A nested touchdown PCR. In order to assess the presence of possible PCR-inhibitors, human β-globin gene was amplified from all clinical DNA specimens with both real-time PCR and traditional PCR assays.

Real-time PCR

Detection and quantification of *C. pneumoniae* DNA were performed by real-time PCR assay, as previously described (19). Briefly, real-time PCR assay was based on FRET hybridization probes and a LightCycler instrument (Roche Diagnostics). PCR primers and probes were obtained from TIB Mobiol and target an internal 128 bp region of *C. pneumoniae* PstI species-specific fragment. LightCycler PCR reaction mixtures were carried out in a final volume of 20 µl and contained approximately 150 ng of DNA (in a volume 1-5 µl).

All PCR experiments included the melting curve analysis of hybridization probes to assess the specificity of PCR products. Only specimens with a melting temperature of 60.8°C were identified as positive. A recombinant plasmid carrying the specie-specific PstI fragment of *C. pneumoniae* was generated (by cloning the PCR fragment into pGEM T-easy vector; Promega, Madison, WI USA). Ten-fold serial dilutions (from 10^6 to 10^1 copies) of the recombinant plasmid previously characterised were used as standards in quantitative experiments (Fig.1). Each PCR-experiment contained the PCR-negative control (ultrapure water PCR grade) and the dilution series of recombinant plasmid as standard (all in duplicate), DNA specimens and the negative DNA extraction controls (all in triplicate). A calibration curve was generated from amplification of standard serial dilutions, and threshold cycle (Ct) values were determined in duplicate and plotted against plasmid copy number. A specimen was considered positive if all of three assay results were positive in the replicate test.

ompA nested touchdown PCR

Detection of *C. pneumoniae* DNA was performed by *omp*A nested touchdown PCR as previously described (20). Each clinical specimen was analyzed in triplicate. A specimen was considered positive if all of three assay results were positive in the replicate test.

Measurements of Markers of Inflammation

Highly sensitive C-reactive protein

Highly sensitive C-Reactive Protein (hspCRP) was determined by an immunoturbidimetric method (21). The plasma was reacted with specific antiserum to form a precipitate that was measured turbidimetrically at 340 nm (ADVIA 1650 Chemistry System, Bayer Corporation).

IL-6

IL-6 was determined by ELISA according to the manufacturer's instructions (R&D Systems Inc.).

Advanced Oxidative Protein Products (AOPP)

AOPP were measured by spectrophotometry on a microplate reader and calibrated with chloramine-T solutions that in the presence of potassium iodide absorb at 340 nm. 400 µl of plasma diluted 1/5 in PBS were placed on a 96-well microtiter and 40 µl of acetic acid were added. In standard wells, 20 µl of 1.16 M potassium iodide (Sigma) were added to 400 µl of chloramine-T solution (0-100 µM) followed by 40 µl of acetic acid. The absorbance of the reaction mixture was read at 340 nm against a blank containing 400 µl of PBS, 20 µl of potassium iodide, and 40 µl of acetic acid. AOPP concentrations were expressed as micromoles per liter of chloramine-T equivalents (22).

Reactive Carbonyl Compounds (RCOs)

Samples for carbonyl residues were submitted to 10 mM dinitro-phenyhydrazine in 2.5 M HCl for 1 h, followed by deproteinization with 20% TCA. The pellet was washed three times in ethanol/ethyl acetate and solubilized in 6 M guanidine. The carbonyl concentration was measured by spectrophotometry at an optical density (OD) of 370 nm with ε370=22 mM-1cm-1. RCOs concentrations were expressed as nanomoles per mg of protein (23).

Plasma-induced apoptosis on monocytes

U937 cells (ATCC CRL 1593), a human monocytic line, were cultured in RPMI 1640 medium supplemented with 10% heat inactivated fetal calf serum (FCS), 2 mM L-glutamine, 100 IU/mL penicillin, and streptomycin (100 mg/ml) (Lonza, Verviers, Belgium) and kept in a controlled atmosphere (5% CO2) incubator at 37°C.

U937 cells (10^6) were incubated with 50% RPMI 1640 medium (with 2 mM L-glutamine, 100 IU/ml penicillin, and 100 mg/ml streptomycin) and 50% patient's plasma. Apoptosis was characterized by DNA fragmentation, showing a ladder-like pattern, and nuclear fragmentation in several smaller fragments ranging in number from 2 to >20 cell.

After 96 h, cells were stained with 10 µmol/ml Hoechst

33342 (Sigma-Aldrich, St Louis, MO, USA) for 15 min at 37°C and nuclear morphology was assessed under a fluorescence microscope. Apoptosis was expressed as percent of the total cell population, counting at least 300 cells in at least 6 random selected fields (24).

Expression of HLA-DR on monocytes

Fluorescence activated cell sorter method was used to identify HLA-DR positive monocytes with monoclonal antibodies reagents. Briefly, PBMC were dual-labelled with anti-human CD14 fluorescein isothiocyanate conjugate (Becton Dickinson) and with an anti-human HLA-DR R-phycoerythrin conjugate (Becton Dickinson). Percentage of HLA-DR+ monocytes was determined by flow cytometric analysis. HLA-DR expression was measured by flow cytometric analysis (Becton-Dickinson), as medium fluorescence intensity (MFI) DR.

Statistical analysis

Statistical analysis was performed by using SPSS 13.0 (SPSS Inc., Chicago, Illinois, USA). Data are presented as mean ± standard deviation (SD) for continuous variables and proportion for categorical variables. Comparisons between two different groups were analyzed by means of χ^2 or Fischer's exact test, for categorical variables and Student's t-test for continuous variables. $p<0.05$ was considered statistically significant.

RESULTS

Detection of C. pneumoniae DNA

A total of 95 PBMC specimens, 49 obtained from HD patients and 46 from blood donors, were examined for presence of *C. pneumoniae* DNA by real-time PCR and *ompA* nested touchdown PCR. Prevalence of *C. pneumoniae* DNA and chlamydial load in PBMC of HD patients and blood donors are shown in Table II. For 50% of Chlamydia positive HD patients, *C. pneumoniae* was detected with a significantly higher chlamydial load (Average: 837 copies/10^4 PBMC) ($p< 0.0001$). By using ompA nested PCR, *C. pneumoniae* positivity rates in PBMC of HD patients and blood donors were 38.8% and 13.1% respectively ($p = 0.009$).

Comparison of real-time PCR and *omp*A nested touchdown PCR results testing PBMC of HD patients and blood donors was performed. Nineteen of 22 real-time PCR positive PBMC of HD patients were confirmed by nested PCR ($p<0.00001$) and *C. pneumoniae* DNA load ranged between 100 and 1078 copies/10^4 PBMC. Three PBMC specimens were positive by real-time PCR but negative by nested PCR and *C. pneumoniae* DNA load varied from 125 to 309 copies/10^4 PBMC.

Six of 9 real-time PCR positive PBMC of blood donors were confirmed by nested PCR ($p<0.00001$) and *C. pneumoniae* DNA load from 45 to 110 copies/10^4 PBMC. Three unconfirmed positive PBMC specimens all had a chlamydial load below 35 copies/10^4 PBMC.

All negative specimens in real-time PCR were also negative by nested PCR, indicating identical

Table I. *Baseline characteristics of HD patients.*

	Patients
N	49
Age (y)	65 ± 13
Male (%)	42 (85.7)
Months HD	69.4 ± 65.8
Mode of RRT	
Standard HD (%)	20 (40.8)
High flux HD (%)	11 (22.4)
HDF (%)	8 (16.3)
AFB (%)	3 (6.1)
HF (%)	3 (6.1)
HFR (%)	4 (8.2)
Type of membrane	
Cellulose/modified cellulose (%)	7 (14.3)
Synthetic (%)	42 (85.7)
Type of vascular access	
Central venous catheter (%)	9 (18.4)
AVF (%)	36 (73.5)
AVG (%)	4 (8.2)
Calcium x phosphate (mg^2/dL^2)	43.0 ± 10.4
BMI (kg/m^2)	24.4 ± 5.3
Albumin (g/dL)	3.9 ± 0.3
K_t/V	1.12 ± 0.24
Hypertension (%)	48 (97.9)
Smoker (%)	24 (49)
Hypercholesterolemia (%)	22 (44.9)
Hypertriglyceridemia (%)	25 (51)
Diabetes mellitus (%)	6 (12.2)
CVD (%)	24 (49)
COPD (%)	4 (8.2)
Intravenous iron medication (%)	27 (55.1)
Statin medication (%)	3 (6.1)

RRT, renal replacement therapy; HDF, hemodiafiltration; AFB, acetate free biofiltration; HF, hemofiltration; HFR, hemodiafiltration with reinfusion; AVF, arteriovenous fistula; AVG, arteriovenous graft; BMI, body mass index; K_t/V, hemodialysis dose (K, urea clearance; t dialysis time; V, body water volume); CVD, cardiovascular diseases; COPD, chronic obstructive pulmonary diseases.

Table II. *Prevalence of C. pneumoniae DNA and chlamydial load in PBMC of HD patients and blood donors by using real-time PCR.*

Patients				Blood donors			
N. Positive/total	N. Negative/total	Avg. Quantity (Copies/10^4 PMBC)	Range (Copies /10^4 PMBC)	N. Positive/total	N. Negative/total	Avg. Quantity (Copies/10^4 PMBC)	Range (Copies/10^4 PMBC)
22/49 (44.9%)	27/49 (55.1%)	521*	100-1078	9/46 (19.6%)	37/46(80.4%)	53*	15-110

*Patients vs blood donors: p=0.016; *Patients vs blood donors p=0.0004*

Table III. *Markers of inflammation in relation to chlamydial load in PBMC of HD patients.*

Inflammatory markers	Chlamydial load (Copies/10^4 PBMC)		
	< 500	>500	p
hsCRP (mg/dL)	1.03±1.11	2.28±1.49	0.036
IL-6 (pg/mL)	12.16±9.55	21.17±10.08	0.044
AOPP (μmol/L)	129±59.42	209±58.49	0.005
RCOs (nmol/mg protein)	0.95±0.44	0.98±0.15	0.848
Apo (%)	0.49±0.09	0.43±0.06	0.087
MFI DR+	121.09±44.64	67.35±45.57	0.011
HLA-DR+ (%)	97.25±3.76	91.87±4.69	0.007

hsCRP, Highly sensitive C-Reactive Protein; AOPP, Advanced Oxidative Protein Products; RCOs, Reactive Carbonyl compounds; % Apo, Apoptosis plasma on U 937 cells (96h); MFI, Medium Fluorescence intensity; HLA-DR+, % monocyte HLA- DR+.

relative specificities of both methods. Thus, the comparison of these two PCR systems showed an overall agreement of 94% both for HD patients and blood donors.

No inhibitors were detected in any of the negative PBMC when they were spiked with low concentration of *C. pneumoniae* DNA and analyzed by real-time PCR and *ompA* nested touchdown PCR.

Baseline characteristics of HD patients according to *C. pneumoniae* DNA status are shown in Fig. 2. *C. pneumoniae* positive patients were found to have atherosclerotic cardiovascular diseases (81.8%) more frequently than *C. pneumoniae* negative patients (22.2%) (p=0.0001). Patients with atherosclerotic cardiovascular diseases also had a significantly higher chlamydial load (611 copies/10^4 PBMC) than patients without cardiovascular diseases (113 copies/10^4 PBMC) (p = 0.006).

A significantly lower value of BMI was found in *C. pneumoniae* positive patients (21.2 ± 2.5 Kg/m^2) than in *C. pneumoniae* negative patients (26.2 ± 5.7 Kg/m^2) (p= 0.0004).

Correlation between chlamydial load and markers of inflammation

Data on markers of inflammation according to chlamydial load in HD patients are shown in Table III. Chlamydial quantitative findings were expressed per two subgroups: group I, below 500 copies/10^4 PBMC; group II, above 500 copies/10^4 PBMC. hsCRP, IL-6 and AOPP levels, monocyte HLA-DR percentages and monocyte HLA-DR expression

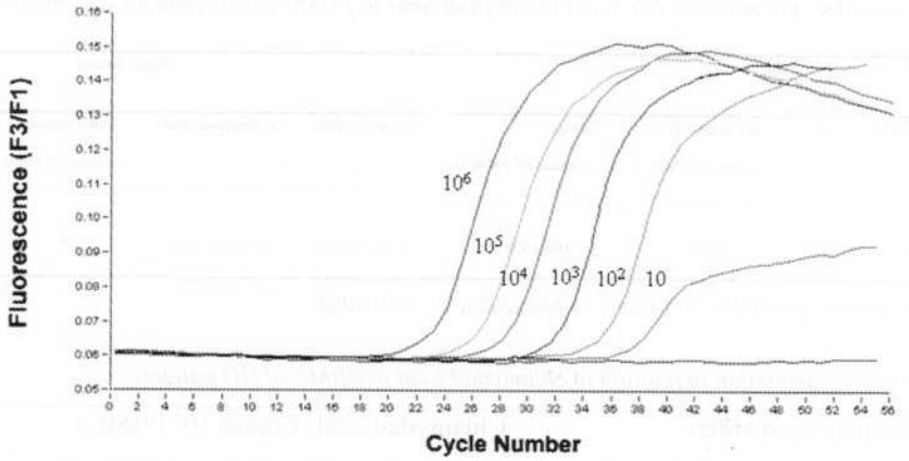

Fig.1. *Amplification plot of PstI plasmid standards using real-time PCR performed on LightCycler instrument and detected with FRET probes. 10-fold dilution series of plasmid carrying the species-specific PstI fragment of C. pneumoniae (from 106 to 10 copies) were analyzed as described in Materials and Methods. Negative control (H2O, dotted) were included in each run.*

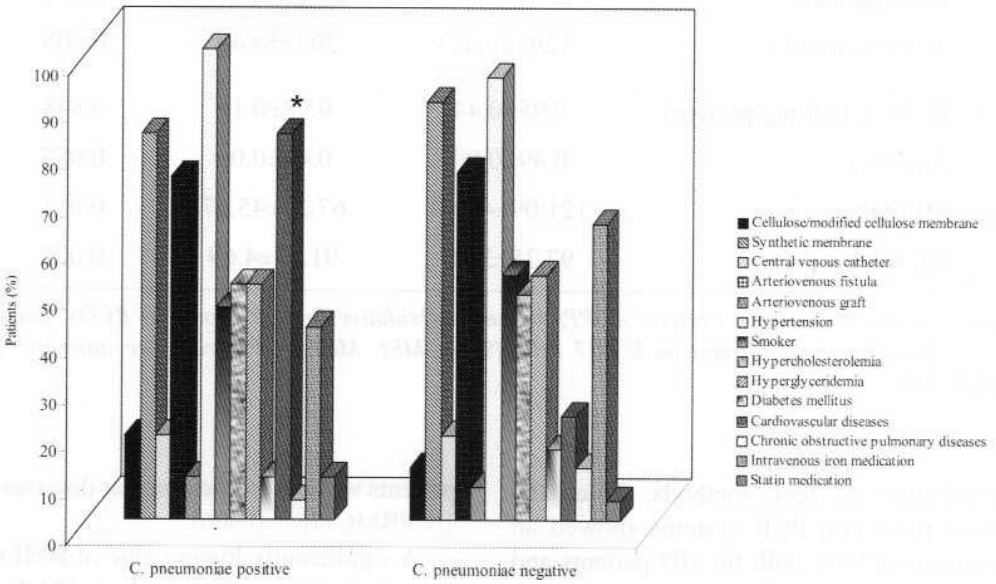

Fig. 2. *Baseline characteristics of HD patients in according to C. pneumoniae DNA status detected by real-time PCR. Histograms displaying a significant correlation between C. pneumoniae DNA and cardiovascular diseases (* p= 0.0001).*

were significantly different between the two groups classified by chlamydial load ($p< 0.05$).

DISCUSSION

Our findings further confirm that real-time PCR is specific and more sensitive than nested PCR since a higher number of PBMC positive to *C. pneumoniae* was detected by real-time PCR. In fact, the prevalence of *C. pneumoniae* DNA, by real-time PCR was 44.9% in HD patients and 19.6% in blood donors ($p= 0.016$) while the positivity rates by *omp*A nested touchdown PCR were 38.8% and 13.1% respectively ($p= 0.009$). A significantly

higher chlamydial load was found in HD patients (Average: 521 copies/10^4 PBMC) than in blood donors (Average 53 copies/10^4 PBMC) (p= 0.0004).

Our study demonstrates that chlamydial load might have predictive value in HD patients for risk of *C. pneumoniae*-associated atherosclerotic cardiovascular diseases. Indeed, in HD patients, we found a significant correlation between *C. pneumoniae* DNA in PBMC and atherosclerotic cardiovascular disease (p=0.0001). In particular, patients with cardiovascular diseases showed a significantly higher chlamydial load (Average: 611 copies/10^4 PBMC) than patients without cardiovascular diseases (113 copies/10^4 PBMC) (p= 0.006). This finding, together with results of our earlier study (19), suggests that *C. pneumoniae* infection may trigger the destabilization of artery atherosclerotic plaques leading to acute cardiovascular and/or cerebrovascular events.

As regards characteristics of HD patients, a significant association was found between *C. pneumoniae* DNA in PBMC and BMI; *C. pneumoniae* positive patients showed a significantly lower BMI (21.2 Kg/m^2) than *C. pneumoniae* negative patients (26.2 kg/m^2) (p= 0.0004).

Overweight (BMI= 25-30 Kg/m^2) and obesity (BMI > 30 Kg/m^2) have become mass phenomena with a pronounced upward trend in prevalence in western countries and have been associated with increased cardiovascular risk and poor survival in the general population. However, in ESRD patients undergoing maintenance hemodialysis an "obesity paradox" has been consistently reported i.e., a high BMI is incrementally associated with better survival (25-28).

Several possible causes have been hypothesized to clarify the aetiology of this inverse association in HD patients; Beddhu (29) has suggested that the association of low BMI with high mortality may be explained with greater inflammation and progression of atherosclerosis. Our results support such hypothesis since 82% of patients with high chlamydial load had low BMI and atherosclerotic cardiovascular diseases, clinical outcome of atherosclerotic process.

As regards inflammatory markers, we found a significant association between chlamydial load and hsCRP, IL-6 and AOPP levels suggesting that in HD patients chronic inflammatory state is exacerbated by the presence of *C. pneumoniae*. Indeed, inflammation and oxidative stress, as evidenced by increased levels of hsCRP, IL-6 and AOPP are well-known factors predisposing to acceleration of atherosclerosis and cardiovascular complications in HD patients (30-35).

Therefore, *C. pneumoniae* infection together with chronic inflammation induced by the dialysis procedure could be responsible for accelerated atherosclerosis.

Of particular concern is also the evidence that immune status imbalance observed in HD patients, characterized by a significant decrease in monocyte HLA-DR percentage and monocyte HLA-DR expression, could be related to chlamydial load. Indeed, ESRD patients undergoing HD show an impaired immune response with a high prevalence of infectious complications (36). Several factors are likely to depress the immune system of these patients and thus make them more susceptible to infection. Impaired monocyte function has been characterized by inadequate respiratory burst, activation, antigen presentation and lowered expression of human leukocyte antigen-DR (HLA-DR) (36). Expression of HLA-DR by monocytes is essential for the processing and presentation of peptides, derived from ingested pathogens, to CD4-positive T cells, and for triggering a specific immune response (37).

In conclusion, our results show that HD patients are at high risk of *C. pneumoniae* infection correlated with chronic inflammatory response which, in turn, can lead to accelerated atherosclerosis and other long-term clinical complications such as myocardial infarction and stroke.

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