Anti-*Chlamydophila pneumoniae* Antibodies as Associated Factor for Carotid Atherosclerosis in Patients with AIDS

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Abstract: Atherosclerosis is a multifactor disease. Lately, infectious factors such as *C. pneumoniae* have been found to be involved. To determine whether the infection by *C. pneumoniae* is a risk factor for atherosclerosis in patients with AIDS. Case-control study on 43 patients with AIDS under HAART (16 cases and 27 controls). To document atherosclerosis, a carotid and transcranial Doppler ultrasound was performed. Anti-*C pneumoniae* antibodies were searched using a micro-immunofluorescence test for IgM and IgG levels. To study the associations with risk of atherosclerosis, Odds Ratios were calculated for each IgG anti-*C. pneumoniae* antibody titre. A titre of 1:64 significantly increased the risk of atherosclerosis. These results suggest that hypertriglyceridemia and *C. pneumoniae* infection coexistence significantly increases the risk of atherosclerosis. The inverse geometric average of the antibodies titre against *C. pneumoniae* in individuals with atheromatous plaque fell to 64, two titres above the controls. This difference turned out to be statistically significant. Exposure to *C. pneumoniae* with antibodies (IgG) should be considered in any HIV diagnosed patient as a risk factor for atherosclerosis, having found that the inverse geometric averages of antibodies titre are significantly different comparing cases and controls, especially in patients with dyslipidemia, hypertriglyceridemia or in patients whose treatments could cause these conditions. In patients with concomitant hypertriglyceridemia, the association increases up to three times. It is advisable that AIDS patients take a serological test to determine exposure to *C. pneumoniae*, and to assess treatment options.

Keywords: *Chlamydophila pneumoniae*, risk factor, atherosclerosis, HIV-AIDS.

INTRODUCTION

The HIV epidemic is a significant public health problem [1, 2]. Patient survival has increased through antiretroviral therapy which entails, in the long run, added pathologies such as dyslipidemia, lipodystrorphy, Diabetes Mellitus type 2 (DM2) and ischemic cardiopathy, all involved in atherogenesis [3].

Diabetes Mellitus 2, high blood pressure (HBP), dyslipidemia, hyperuricemia, obesity, nicotine addiction, sedentarism, and high density lipoprotein (HDL) concentrations and lipoprotein-a reduction are some of the known factors associated with atherosclerosis. Lately, other infectious factors have been identified, such as the infection by *Chlamydophila pneumoniae* (*C. pneumoniae*) [3].

In 1999, after the taxonomy of the *Chlamydiaceae* was revised on the basis of genomic studies, its family was divided into two genera: *Chlamydia* and *Chlamydophila*. *Chlamydia trachomatis* was retained in the *Chlamydia* genus, while *Chlamydia psittaci* and *Chlamydia pneumoniae* were considered part of the new *Chlamydophila* genus [4].

Seroepidemiologic studies have shown associations between *C. pneumoniae,* and coronary arteries and carotid atherosclerotic injuries, and aneurisms of the abdominal aorta and aortic valves [5]. Recently other risk factors have been studied including inflammation markers, infections, and homocysteine [6,7].

Other studies included patients with cerebrovascular disease. Patient's IgG, IgM and IgA were measured using microimmunofluorescence (MIF), finding increased IgA titles suggesting a persistent infection [8]. However, literature includes studies that did not find significant associations between anti-*C. pneumoniae* antibodies titres and the presence or severity of aortic atherosclerosis [9]. An increase in protein C - Reactive levels in patients with chronic *C. pneumoniae* infection has been associated with higher mortality by cardiovascular illnesses [10]. Apolipoprotein B levels were significantly higher in patients with positive DNA samples [11]. Further studies found a high prevalence of *C. pneumoniae* infection which relates independently and significantly to systemic arterial hypertension and an increase of TNF-alpha and IL-6 circulating levels [12]. The impact of antibiotic treatment on early atherosclerosis evolution in patients with *anti-Chlamydophila* antibodies has also been investigated [13].

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1570-162X/08 $55.00+.00

There are several methodologies for detecting *C. pneumoniae* chronic infection. Studies have been conducted to analyse the sensitivity of molecular diagnosis methods, such as PCR, shelled PCR, and immunohistochemical [14, 15]. Our group conducted two previous studies to identify IgG and IgM antibodies against *C. psittaci, C. trachomatis* and *C. pneumoniae* using MIF [16, 17].

Given the strong evidence regarding the presence of *C pneumoniae* infection and atherosclerosis in its diverse clinical manifestations - cerebrovascular disease, coronary disease and aortic atheromatous plaques presence in postmortem studies – the need was felt to investigate how *C. pneumoniae* and atherosclerosis related in HIV-AIDS patients. Although this relation has been studied in patients with other risk factors such as DM2, HBP and dyslipidemia, we found no information on HIV-AIDS population. Due to the multiplicity of risk factors coexisting in these patients, associated with the adverse effects of highly active antiretroviral therapy (HAART), it was important to investigate if atherosclerosis was associated with the presence of anti-*Chlamydophila pneumoniae* antibodies.

METHODS

A case-control study was carried out at the Infectology Hospital and at the Immunology and Infectology Medical Research Unit of the National Medical Centre "La Raza". Patients with HIV-AIDS under HAART above 18 years of age were included, regardless of the combination of therapeutic families they received: Non Nucleoside Reverse Transcriptase Inhibitors (nNRTI), Nucleoside/Nucleotide Reverse Transcriptase Inhibitors (NRTI) and Protease Inhibitors (PI). Information was collected from October 2004 to July 2005 Out of 52 selected patients, 43 accepted to participate in the study after signing an informed consent form. An institutional ethics committee reviewed and approved the protocol. Patients were examined to obtain general information and their records reviewed to learn about their previous laboratory results. Furthermore, their weight, size, vital signs, lipid profile, glycaemia, CD4+, viral load, and serology for *C. pneumoniae, C. trachomatis* and *C. psittaci* were measured using MIF. HIV-AIDS carriers under HAART with carotid and/or cerebral atherosclerosis were defined as cases. HIV-AIDS carriers under HAART with no carotid nor cerebral atherosclerosis were defined as controls. Atherosclerosis was defined as the accumulation of atherosclerotic plaques in carotid arteries, with plaque growth, lumen narrowing and compensatory external diameter enlargement. Presence of atherosclerosis by ultrasound tracking with carotid and transcranial Doppler was documented (SONOLINE VERSA-PLUS with a 7,5 megahertz transducer) with real time gray scale images. Common carotid artery proximity and proximal part of the bilateral internal and external carotids were explored. Intimae-media thickness was measured (a thickness of >=1mm was considered carotid atherosclerosis). Presence or absence of the atheroma plaque was also taken into consideration as well as the percent stenosis of the passageway. To measure transcranial atherosclerosis, the temporomandibular joint capsule was located, for measuring speed flow, systolic peak and median right and left cerebral artery resistance index. To determine the presence of anti-*C. pneumoniae* antibodies, a MIF test was performed to detect IgM and IgG against *C. pneumoniae* (Labsystems Valaner). Dilutions of serum until 1:512 were made from each patient. Then, a 10uL dilution from the patients' serum was added and a drop of undiluted control serum was placed into the wells of the plates. IgG plates were kept in an incubator at 37ºC for 30 minutes; the IgM for 3 hours. The plates were washed and dried at 37ºC. The conjugated was added to each well, incubating again at 37ºC for 30min, and later washed and dried.

Assembly fluid was added and observed through an epifluorescence microscope. A positive reaction was noted as the elementary bodies were covered by specific antibodies resembling "coins" or "rings". IgG and IgM cut value was greater or equal to 1:16 titre [18]. Other confounding variables such as PI treatment, DM2, HBP, nicotine addiction, alcoholism, dyslipidemia and obesity, overweight and ischemic cardiopathy were taken into consideration.

Definition's criteria for DM2 and HBP were taken from the American Diabetes Association [19], the Joint National Committee on Detection, Evaluation and Treatment of High Blood Pressure (JNC-V) [20] and from the diagnosis of dyslipidemia from the Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults (ATP III) [21].

Obesity and overweight conditions were estimated according to the body mass index (BMI). A BMI higher than 25% was considered overweight, while obesity was assumed for a BMI higher than 30% [17, 22].

Presence of different types of ischemic cardiopathies was investigated, from stable and unstable angina to acute myocardial infarction. Diagnosis of lipodistrophy was based on criteria issued by Carr *et al.* [23].

STATISTICAL METHODOLOGY

The sample size was calculated allowing one control per case, a 95% confidence level and 5% reliability, taking into account a 94.3% prevalence of anti-*C. pneumoniae* antibodies for the group with atheroma plaque and 37% for the group without atheroma plaque [24]. The X^2 Test, Fisher's Exact Test, and Student T-Test were used to verify covariation. The association between different variables and the risk of atherosclerosis was calculated using the odds ratio, and 95% confidence intervals (CI). Spearman's test was used to assess the correlation between atherosclerosis and different variables. A univariate analysis was performed to identify risk factors associated with atherosclerosis. A multivariate analysis was carried out to determine the main associations. Geometric averages and standard geometric deviations for *C-pneumoniae* antibodies titres were calculated. A base-2 log-normal distribution was used, since the analysis of the results was based on the dilution factor. A Student's T- test was used for independent samples.

RESULTS

Out of the 16 cases 13 were men and 3 were women. The 27 controls were constituted by 20 men and 7 women. The average age of the cases was 38.4 years (range 28-48) and of the controls was 39.0 years (range 29-48). Cases had an 8.0 years average with a HIV diagnosis and controls 8.2 years (Table 1). Some 6% (1/16) of the cases had a family history of systemic arterial hypertension *vs* 30% (8/27) of the con-

trols; of the cases, 37% (6/16) had a family history of DM2 *vs* 19% (5/27) of the controls; some 6% (1/16) of the cases had a family history of both hypertension and diabetes *vs* 22% (6/7) of the controls. Tobacco addiction and alcoholism were both found in 56% (9/16) of the cases and 41% (11/27) of the controls. Co-morbidities were identified: Hypertriglyceridemia in 25% of the cases, 66% of the controls; hypercholesterolemia in 13% (2/16) of the cases, 4% (1/27) of the controls; mixed dyslipidemia in 37% (6/16) of cases, 7% (2/27) of the controls. Diabetes was detected in 19% (3/16) of the cases *vs* 0% of the controls.

Table 1. Cases and Controls General Characteristics

Variable	Cases n= 16 No. (%)	Controls n=27 No. (%)	P
Men	13 (81)	20 (74)	0.86*
Women	3 (19)	7 (26)	0.86**
Genetic Load HAS	1 (6)	8 (30)	0.12**
Genetic Load DM2	6 (37)	5 (19)	0.30*
Genetic Load HAS and DM2	1 (6)	6 (22)	0.22**
Tobacco addiction	9 (56)	11 (41)	0.50*
Alcoholism	9 (56)	11 (41)	0.50*
Hypertriglyceridemia	4 (25)	18 (66)	0.01**
Mixed Dyslipidemia	6 (37)	2 (7)	0.03**
Diabetes Mellitus 2	3 (19)	0 (0)	0.04**

*Chi-square, **Fisher's Exact test.

In average, the cases had been under HAART for about 79 months and the controls 92 months. No statistically significant differences between groups were found in terms of age, sex distribution, heredofamilial history, presence of hypercholesterolemia, lipodistrophy, tabaquism, alcoholism, HBP, obesity, NRTIs treatment, duration of HIV, BMI, total cholesterol, triglycerides, viral load, CD4+ cell count (Figs. 1 and 2), or months under antiretroviral therapy.

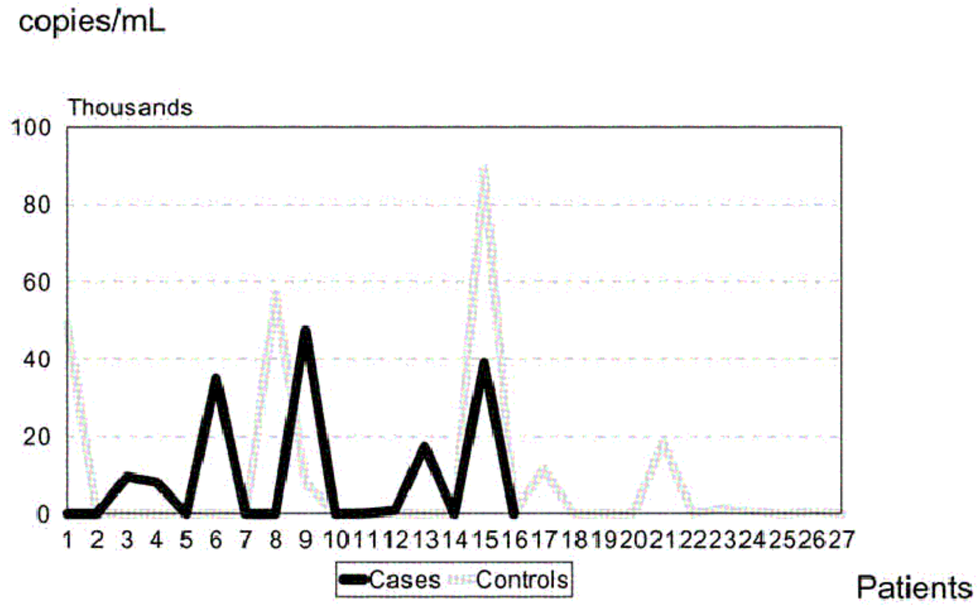

Fig. (1). Viral load in cases and controls.

Regarding antiretroviral therapy, the most common PI among the cases were Indinavir (18,8%), Amprenavir (6,2%), Ritonavir (6,2%) and Lopinavir/Ritonavir (6,2%); controls mostly used Nelfinavir (18,4%), Ritonavir (11,2%) and Saquinavir (11, 2%).

As for, nNRTIs 50% (4/16)) of the cases took Nevirapine and 31.2% Efavirenz (5/16); 40.8% of the controls took Nevirapine (11/27), none took Efavirenz. Otherwise 25% of the cases received NRTIs, 6.2% (1/16) Stavudine; 37.5% (6/16), Zidovudine; and 37.5% (6/16), Lamivudine and 18.8% (3/16), Didanosine; while 11.2% (3/27) of the controls received Stavudine; 48.1% (13/27), Zidovudine; and 33.3 (9/27); Lamivudine.

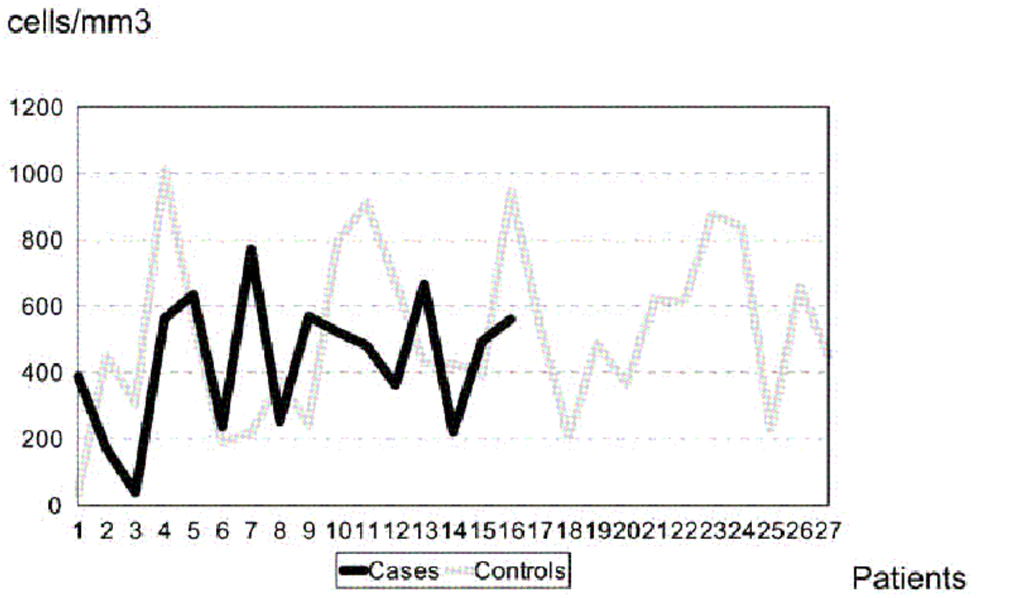

Fig. (2). CD4 counts in cases and controls.

Most patients, both cases and controls (16/27) with *C. pneumoniae* had IgG type antibodies, showing a high level of exposure to this microorganism: 23 out of 43 patients, cases and controls, were IgG positive for *C. trachomatis*. Only 5 out of 43 patients, cases and controls, were IgG positive for *C. psittaci*. Neither cases nor controls were IgM positive for any species of *Chlamydia* (Table 2).

Table 2. IgG anti-*Chlamydia* Titres in Both Groups

Microorganism	Cases		Controls	
	Positive	Negative	Positive	Negative
C. pneumoniae	13	3	21	6
C. trachomatis	9	7	14	13
C. psittaci	0	16	5	22

Regarding Spearman correlation coefficients only two variables showed statistical significance: a correlation between triglycerides and atherosclerosis of 0.30 with p<=0.05; and a correlation between glycaemia and atherosclerosis of 0.32 with p<=0.05. To assess the risk of developing atherosclerosis from *C. pneumoniae* infection ORs were calculated for each IgG anti-*C. pneumoniae* titre. A 1:32 titre had an OR=1.61 (95% CI 1, 27 - 2, 04) and a 1:64 titre had anOR=3.05 (95% Cl calculate the 95% intervals). Consequently, an IgG anti-*C. pneumoniae* titre of 1:64 was quite significant since it substantially increased atherosclerosis risk. However, we did not find a gradient exposure relation, considering thata titre of 1:254 did not increase the risk of atherosclerosis. This result might be due to the small size of the sample (Table 3).

Other variables were found to the significantly associated with the development of atherosclerosis. Mixed dyslipidemia resulted in an OR of 7.5 (95% Cl 1.29 – 43.6) while DM2 had an OR of 3.07 (95% Cl 1.96 – 4.8). This is consistent

with the already well-known atherosclerosis multicausality emphasising, however, the importance of dyslipidemias as a major risk factor.

Table 3. Atherosclerosis Risk by IgG *C. pneumoniae* Titres

Titre	OR	IC 95%
1:8	0.80	0.17 – 3.8
1:16	0.15	0.18 – 1.41
1:32	1.61	1.27 – 2.04
1:64	3.05	2.15-15.9*
1:256	1.32	0.25 -6.86
Risk Estimation for Atherosclerosis According to Antecedents		
Antecedents	OR	IC 95%
Mixed Dyslipidemia	7.5	1.29-43.60
Diabetes Mellitus 2	3.07	1.96-4.80
Positive IgG for C. pneumoniae1: 64	3.05	2.15-15.9*

*This IC was calculated at 95%.

Through multivariate analysis we found that the factors with a stronger association with the development of atherosclerosis were hypertriglyceridemia and mixed dyslipidemia; thus, a simultaneous analysis was carried out separating the group with hypertriglyceridemia from the group without hypertriglyceridemia, while keeping anti-*C. pneumoniae* antibodies presence as a major assessment value. In the patients group without hypertriglyceridemia but with positive antibodies we found that the OR for a >1:64 titre was 7.5 (95% Cl 3.15-23.33). For patients with hypertriglyceridemia and positive antibodies, for a >1:64 titre, the OR was 25.5 (p=0.02; 95% Cl 1.21 - 1.217) (Table 4).

Table 4. Anti *C. pneumoniae* Antibodies Relation with Atherosclerosis

Hypertriglyceridemia	Titre	OR	IC 95%	P
YES	>1: 64 *vs* <1: 64	25.5	1.21 – 1,217	0.02
NO	>1: 64 *vs* <1: 64	7.50	3.15-23.33	0.02

Considering the risk of atherosclerosis in patients with mixed dyslipidemia and IgG anti-*C. pneumoniae*, we consider this association to be weak because only eight patients were found to be mixed dyslipidemia carriers. Finally, a multivariate analysis was carried out to evaluate the relation between IgG anti-*C. pneumoniae* titres and atherosclerosis. Hypertriglyceridemia (f=6.65, with p=0.01), treatment with PI (f=4.74, with p=0.03), and mixed dyslipidemia (f=7.89, with p=0.007) were found to be significant factors (Table 5). The serological behaviour of IgG against *C. pneumoniae* presented a geometric average (Xg) + 2 standard deviations of the inverse of the titre of 53,81 + 12.22 for the cases, and 19,16 + 4.46 for the controls, being statistically significant with p<0.001.

Table 5. Multivariate Analysis of Predicting Variables

Variable	F	P
PI Treatment	4.74	0.03
Hypertriglyceridemia	6.65	0.01
Mixed Dyslipidemia	7.94	0.01

DISCUSSION

The relation between *C. pneumoniae* as a risk factor for the development of atherosclerosis has been widely investigated. Most studies found that 1:32 and 1:64 titres are significant cut-off points, but did not take into account confounding factors associated with atherosclerosis. The articles we reviewed showed that a 1:32 titre can increase the risk of atherosclerosis up to three or four times. This risk is similar to that associated with diabetes and hypertension [25-29]. Our research group found that although most of the general characteristics were similar, significant differences were found between the groups studied which were the bases for later analysis. Such differences were particularty evident in terms of confounding variables - hypertriglyceridemia, mixed dyslipidemia, diabetes, nITRAN treatment and PI - thus many patients already showed the collateral symptoms of antiretroviral treatment. Mixed dyslipidemia, hypercholesterolemia and DM2 are the three main risk factors associated with atherosclerosis in our analysis *(univariate analysis cannot by any means have anything to do with risk factors, which are a measure of association between two variables!!)* The analysis based on the presence of antibodies (IgG) showed a significant association between exposure to *C. pneumoniae* and atherosclerosis, increasing up to three fold in patients with concomitant hypertriglyceridemia. It is important to consider that HIV patients under PI or Efavirenz treatment show an increase of the triglycerides level as an adverse side effect of medication. The adding up of an infection by *C. pneumoniae* can consequently increase the risk of atherosclerosis.

The use of MIF has been widely accepted as a serological standard [30]. It is important to point out that the literature also includes studies that have not found an association between anti-*C. pneumoniae* antibodies titres and the presence aortic atherosclerosis among the general population [9], nor the presence of carotid plaques in patients with HIV -1 [29, 31]. The titre accepted in literature as a positive cut value is 1:16 [18]. However our study found that the titre that more significantly increases the risk of atherosclerosis in patients with HIV is 1: 64. Even in patients not suffering from any type of dyslipidemia, an exposure to *C. pneumoniae* results in an OR of 3.0 of developing atherosclerosis. Patients with high IgG anti-*C. pneumoniae* titres (1:256) probably have a shorter exposure time than patients with positive but lower titres (1:64). Damage to the endothelium and genesis of the atherosclerosis plaque could be explained by the duration of exposure to this chronic infection.

To prevent development of atherosclerosis and all associated cardiovascular diseases it is important to keep cholesterol, triglycerides and glycemia under control. Lifestyle changes in terms of eating habits, exercise, treatment of alcoholism and nicotine abuse should also be encouraged, as well as the use of

medication such as fibrates, biguanides and statins, although there are potential drug-drug interactions between PI and some statins. Because the shared metabolism via the cytochrome P450 3A4 (CYP3A4) system, there are concerns about drug-drug interactions and an increased risk of skeletal muscle toxicity in patients taking PI and statins in combination. This is a rare but well-recognized adverse effect of statins [32].

The present report has a limitation, it suffers from a small sample size. The series presented is, indeed, too thin to let any reliable, not casualty-bound, interpretation be set up. This shortcoming makes the results weak and hardly comparable.

As a conclusion, about the inverse geometric average of the antibodies against *C. pneumoniae* titre in individuals with atheromatous plaque fell to 64, two titres above the controls. This difference turned out to be statistically significant. Exposure to *C. pneumoniae* with antibodies (IgG) titre should be considered in any HIV diagnosed patient as a risk factor for atherosclerosis, having found that the inverse geometric averages of antibodies titre are significantly different comparing cases and controls, especially in patients with dyslipidemia, hypertriglyceridemia or in patients whose treatments could cause these conditions. An assessment of the therapy against *C. pneumoniae* and HAART should always be carried out. In patients with concomitant hypertriglyceridemia, the association increases up to three times. It is advisable that AIDS patients take a serological test to determine exposure to *C. pneumoniae*, and to assess treatment options.

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Received: February 25, 2008 Revised: March 17, 2008 Accepted: March 18, 2008