Lack of association of *Chlamydia pneumoniae* with cardiovascular diseases in virologically suppressed HIV patients

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**SUMMARY**

Cardiovascular disease (CVD) is a major public health problem in developed countries with over 17 million deaths per year. In the last decade, several infectious agents rather than any single pathogen, including *Chlamydia pneumoniae* and human immunodeficiency virus (HIV), have been shown to contribute to the development of atherosclerosis and subsequent cardiovascular events by inducing systemic inflammation and/or acting directly on the vascular wall.

For the first time, we evaluated *C. pneumonia* DNA in peripheral blood mononuclear cells from HIV patients by real-time polymerase chain reaction in order to shed light on *C. pneumonia* as a co-factor with HIV in the development of CVDs. *C. pneumonia* DNA was not detected in our virologically suppressed HIV patients (<37 copies/mL). This finding may be related to high CD4+T cell count (>500 cells/μl) found in HIV patients suggesting functional cell-mediated immunity as a fundamental mechanism for the clearance of chlamydial infection in this population. Larger studies are needed to confirm this hypothesis.

**INTRODUCTION**

Cardiovascular disease (CVD) is a major public health problem in developed countries with over 17 million deaths per year and the main pathological process underlying this disease is the atherosclerosis, a chronic inflammatory syndrome (Mendis *et al.*, 2011). The atherosclerotic process typically begins with foam cell formation followed by fibrous cap and thrombus formation in the advanced plaque, leading to cardiovascular events, such as myocardial infarction and stroke.

Nowadays, it is widely accepted that the increased risk of CVDs is probably the result of a high prevalence of both traditional cardiovascular risk factors, such as hypertension, diabetes, dyslipidemia and smoking, and nontraditional risk factors including inflammation, oxidative stress and infectious agents. In the last decade, infectious agents have acquired a growing importance, since they are able to induce inflammation and/or oxidative stress (Di Pietro *et al.*, 2013; Campbell and Ronsefeld, 2015; Filardo *et al.*, 2015).

*C. pneumoniae*, an intracellular obligate pathogen responsible for respiratory infections, has been considered as the most plausible additional risk factor for atherosclerosis since it is the sole viable pathogen detected in atherosclerotic plaque. Further evidence that *C. pneumoniae* might play a role in CVDs came from studies in which the microorganism was detected in atherosclerotic lesions of coronary and carotid arteries and abdominal aorta aneurysm but seldom in healthy arteries (Joshi *et al.*, 2013).

Even more important are in vivo studies demonstrating the ability of *C. pneumoniae* to systematically disseminate from the lungs through peripheral blood mononuclear cells (PBMCs) and localize in several extra-pulmonary tissues including the vasculature (Sessa *et al.*, 2007; Di Pietro *et al.*, 2013; Joshi *et al.*, 2013). In fact, circulating infected PBMCs have been considered a means by which *C. pneumoniae* can induce a chronic systemic inflammation, as evidenced by high sensitive C-reactive protein (hsCRP) levels, contributing to the development and progression of CVDs (Roivainen *et al.*, 2000; Rosenfeld and Campbell, 2011).

Interestingly, *C. pneumoniae* may also act directly on the vascular wall since it has been shown to infect vascular cells inducing the production of reactive oxygen species, cytokines, growth factors and cellular adhesion molecules - all responsible for the typical pathological changes in atherosclerotic plaque (Di Pietro *et al.*, 2013; Campbell and Rosenfeld, 2015).

In recent years, several studies have provided evidence that several infectious agents, labelled the “infectious burden”, rather than any single pathogen, may be involved in the development of atherosclerosis and the subsequent...
cardiovascular events (Rosenfeld and Campbell, 2011; Sessa et al., 2014). The human immunodeficiency virus (HIV) has been involved in the pathogenesis of CVDs since a growing body of evidence has reported that HIV patients have a higher overall risk of CVDs as compared to general population (COHERE, 2012; Hunt, 2014). The pathogenesis of CVDs in HIV patients is the result of the complex interplay of traditional risk factors and infection-related factors. In fact, HIV infection is well known to induce chronic systemic inflammation and to activate the innate immune system that, in turn, stimulates the immune cells, such as monocytes and macrophages, involved in the atherosclerotic process (d’Ettorre et al., 2016; Vos et al., 2016). To date, few studies have investigated the infectious burden of C. pneumoniae and HIV in the development of CVDs, and these are sero-epidemiological reports (Maggi et al., 2006; Gaona-Flores et al., 2008). Therefore, the aim of our study was to evaluate the presence of C. pneumoniae in PBMCs from HIV patients by real-time polymerase chain reaction (PCR), in order to shed light on C. pneumoniae as a co-factor with HIV in the development of CVDs.

MATERIALS AND METHODS

Study population
Fifty-six peripheral blood samples were collected from HIV-1 consecutive patients on combined antiretroviral therapy (cART) admitted to the Umberto I “Sapienza” University Hospital in Rome for routine controls. This study enrolled patients with CVDs, defined as documented ischemic events involving coronary and cerebral circulation, and a control group including patients without CVDs, matched for age and gender. Patients with past or current respiratory tract infections were excluded. The study was approved by the hospital ethics committee. All study patients gave written informed consent and the research was conducted in accordance with the Helsinki Declaration of 1975 and revised in 2000. A questionnaire was compiled for each subject including patient history, life-style and risk factors for CVDs such as smoking, diabetes, dyslipidemia (abnormal total cholesterol, high-density lipoprotein cholesterol and triglycerides) and hypertension. HIV-RNA levels and nadir CD4+ T cell count were also recorded. Current CD4+ T cell count was determined by FACScalibur flow cytometer (Becton Dickinson, San Jose, CA, USA). Glucose, total cholesterol, high-density lipoprotein (HDL) cholesterol and triglycerides were also measured.

HIV-1 RNA levels
HIV-1 RNA levels in plasma were quantified using the VERsant® HIV-1 RNA 1.0 kPCR molecular system (Siemens Healthcare Diagnostics, Tarrytown, NY, USA) according to the manufacturer’s instructions. It is an automated amplification assay based on reverse transcription and kinetic PCR technology. It is composed of a sample preparation module, used to extract RNA from plasma with magnetic silica beads, and an amplification detection module. The RT-PCR step uses primers and probes targeting the highly conserved integrase region of the HIV-1 pol gene. A linear response is claimed between 37 and 11,000,000 copies/mL of plasma.

Isolation of PBMCs and C. pneumoniae detection
PBMCs were isolated from peripheral blood samples by Ficoll-Hypaque density gradient centrifugation (Sigma-Aldrich, St. Louis, MO, USA) and dry pellets of 10⁶ PBMCs were stored at -20°C. Total DNA was extracted from PBMCs using a QiAamp DNA mini kit (QIAGEN, Hilden, Germany), according to the manufacturer's instructions. Extracted DNA was stored at -20°C until used for amplification. Detection and quantification of C. pneumoniae DNA were performed by real-time PCR targeting MOMP gene using the Primerdesign™ genesig® Kit according to the manufacturer’s instructions. Each PCR-run contained the PCR-negative control (ultrapure water PCR grade) and positive and negative extraction controls. DNA sample, positive and negative extraction controls were analyzed in triplicate. The sample was considered positive if all three assay results were positive in the replicate test. The detection limit of the assay was 2 genomic copies/mL.

hsCRP measurements
Plasma concentration of hsCRP was measured using enzyme-linked immunosorbent assay kit (BioVendor GmbH, Heidelberg, Germany).

Statistical analysis
Statistical analysis was performed using SPSS 13.0 (SPSS Inc. Chicago, IL, USA). Differences in demographic and

<table>
<thead>
<tr>
<th>HIV patients with CVDs (n=37)</th>
<th>HIV patients without CVDs (n=19)</th>
<th>P-values</th>
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</thead>
<tbody>
<tr>
<td>Age, years</td>
<td>59.5±9.2</td>
<td>56.7±5.4</td>
</tr>
<tr>
<td>Gender, n male (%)</td>
<td>34 (91.9)</td>
<td>17 (89.5)</td>
</tr>
<tr>
<td>Duration of HIV infection, years</td>
<td>20 (15-32)</td>
<td>18 (14-24)</td>
</tr>
<tr>
<td>Time on antiretroviral therapy, years</td>
<td>18 (12-26)</td>
<td>16 (12-20)</td>
</tr>
<tr>
<td>Smoking, n (%)</td>
<td>9 (24.3)</td>
<td>4 (21.1)</td>
</tr>
<tr>
<td>Hypertension, n (%)</td>
<td>18 (48.6)</td>
<td>0</td>
</tr>
<tr>
<td>Diabetes, n (%)</td>
<td>8 (21.6)</td>
<td>0</td>
</tr>
<tr>
<td>Hypertriglyceridemia, n (%)</td>
<td>15 (40.5)</td>
<td>2 (10.5)</td>
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<tr>
<td>Hypercholesterolemia, n (%)</td>
<td>14 (37.8)</td>
<td>3 (15.8)</td>
</tr>
<tr>
<td>Hyperlipidemia, n (%)</td>
<td>23 (62.2)</td>
<td>7 (36.8)</td>
</tr>
</tbody>
</table>
C. pneumoniae and cardiovascular diseases in HIV patients

Clinical characteristics between HIV patients with and without CVDs were assessed using the chi-squared or Fisher’s exact test. Comparison of means was performed by using a two-tailed Student’s t-test. A value of p<0.05 was considered statistically significant.

RESULTS

A total of 56 HIV patients on cART were screened for the presence of C. pneumoniae DNA. The baseline characteristics of our patients are shown in Table 1. Thirty-seven HIV patients had CVDs, whereas 19 HIV patients did not. HIV patients with CVDs had an increased prevalence of hypertension, diabetes and hypertriglyceridaemia compared to HIV patients without CVDs (P<0.05).

All patients had undetectable plasma HIV-1 RNA levels (<37 copies/mL) and were on cART regimens based on integrase inhibitors (INIs) or nucleoside reverse transcriptase inhibitors (NRTIs) plus protease inhibitors (PIs). C. pneumoniae DNA was undetectable in PBMCs isolated from HIV patients or below the detection limit of the applied real-time PCR.

As shown in Figure 1, HIV patients with CVDs showed higher levels of hsCRP and a significantly lower nadir CD4+ T cell count compared to HIV patients without CVDs (P<0.01 and P<0.05 respectively). Conversely, the CD4+ T cell count was similar in both groups. Concerning the lipid profile, HIV patients with CVDs had significantly higher levels of triglycerides than HIV patients without CVDs (P<0.01). On the contrary, cholesterol, HDL and LDL levels were comparable in both groups (Figure 2).

Figure 1 - hsCRP levels, nadir CD4 and CD4+ T cell count in HIV patients with and without CVDs.

Figure 2 - Lipid profile in HIV patients with and without CVDs.
DISCUSSION

This is the first study to evaluate the presence of C. pneumoniae DNA by real-time PCR in PBMCs from HIV patients in order to investigate the involvement of C. pneumoniae in the pathogenesis of CVDs in this population. C. pneumoniae DNA was not detected in either HIV patients with CVDs or in the control group, including HIV patients without CVDs. This result may be explained by the fact that all of our patients were virologically suppressed (<37 copies/mL) at the time of enrollment, maintaining a CD4+ T cell count above 500 cells/µl, representative of good immune system health (HRSa, 2014). Therefore, a functional cell-mediated immunity seems to be of the utmost importance for the clearance of C. pneumoniae infection in HIV patients. In addition, since C. pneumoniae infection has been associated with both low CD4+ T cell count and high HIV load (Tositti et al., 2005), its prevalence could be higher in patients who do not respond to cART. Specifically, although cART usually results in diminished viral replication and in increased CD4+ T cell counts, approximately 20% of all HIV-infected patients do not achieve optimal immune reconstitution despite suppression of viral replication (Gaardbo et al., 2012, Dronda et al., 2002).

It is well known that the natural history of HIV disease has evolved over the years. Opportunistic infections due to the compromised immune system were once predominant, whereas since the mid-1990s, the introduction of cART has improved the cell-mediated immune response and led to an expected life-span comparable to that of the general population. As a consequence, a new set of complications, usually most common in the elderly (e.g. over 65 years old), have begun to affect relatively young HIV patients (e.g. over 50 years old), including coronary heart disease, stroke and myocardial infarction (Nasi et al., 2016). Our HIV patients with CVDs had an average age of less than 60 years and exhibited a significantly increased prevalence of cardiovascular risk factors such as diabetes, hypertension and hypertrierylglyceridemia. The higher prevalence of traditional cardiovascular risk factors in HIV patients has also been related to cART. Indeed, regimens based on PIs and certain NRTI may contribute to modifications in lipid composition and function, exacerbating the risk of developing CVDs (Lambert et al., 2016; da Cunha et al., 2016). Modification of lipid profile, in turn, seems to increase the prevalence of hypertension in the HIV population (Martin-Iguacel et al., 2016).

A particularly interesting finding of our study was the significantly lower nadir CD4+ T cell count observed in HIV patients with CVDs, leading to the compelling hypothesis that nadir CD4 may be associated with cardiovascular outcomes in individuals living with HIV, in spite of the efficacy of treatment. In fact, nadir CD4, considered a biomarker for the degree of HIV-associated immunosuppression and surrogate for the intensity of the ongoing immune activation and chronic inflammation, has been shown to contribute to the initiation and progression of atherosclerosis in HIV patients (Okeke et al., 2016; Nasi et al., 2014). Not surprisingly, our HIV patients with CVDs had increased inflammation, as evidenced by high circulating levels of hsCRP.

In conclusion, our results do not support the involvement of C. pneumoniae in the pathogenesis of CVDs in HIV patients, but we cannot rule out its contributing role in HIV patients. On the one hand, genomic copies of C. pneumoniae present in PBMCs may be below the limit of detection of the real-time PCR used. On the other hand, it was recently suggested that only chlamydial antigens may be carried by PBMCs to the vasculature where they become targets of attack by antigen-specific immune cells, exacerbating the atherosclerotic process (Zafiratos et al., 2015). In this respect, several chlamydial antigens such as effector proteins of type III secretion system have recently been described (Lutter et al., 2012; Mueller et al., 2014) and may be considered targets for developing novel diagnostic methods.

In the future, further studies on larger HIV populations, including immunological non-responders and virologically unsuppressed patients, and analyzing new targets, such as chlamydial antigens, may be helpful to clarify the interplay between the host immune response and C. pneumoniae in HIV patients and its involvement in the pathogenesis of CVDs.

References


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tension is a key feature of the metabolic syndrome in subjects aging with HIV. Curr. Hypertens. Rep. 18, 46.


