Infectious agents and atherosclerosis: current perspectives and unsolved issues

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The large amount of data accumulated in recent years has reinforced the idea that infectious agents may play a significant role in the pathogenesis of atherosclerosis and in the clinical manifestations of vascular disease. Seroepidemiological and experimental data linking *Herpesviridae* and *Chlamydia pneumoniae* to atherosclerosis appear to be confirmed by a number of studies, while the available evidence regarding *Helicobacter pylori* is more conflicting, partly due to the fact that the interest in this agent is more recent. Infectious agents may influence atherogenesis through a number of mechanisms, ranging from cell lysis to the stimulation of adhesion molecule expression and cytokine production by infected cells. The development of atherosclerosis after an acute infection seems unlikely. Rather, it appears that a chronic, persistent form of infection, especially with *Chlamydia pneumoniae*, may favor those structural and proinflammatory changes in the vascular wall which are necessary for the formation of an atheroma. A persistent chlamydial infection is accompanied by an increased production of microbial heat shock protein 60, which may induce antigenic mimicry and a chronic inflammatory reaction in the vascular wall. Pharmacological trials have yielded conflicting indications regarding the hypothesis that treatment with macrolide antibiotics may limit the progression of vascular disease and the recurrence of cardiovascular events, although in a limited number of cases. However, antimicrobial drugs do not act specifically against a single infectious agent and more specific therapeutic agents would be needed in order to test a causative link between a single infectious agent and vascular disease.

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Introduction

Atherosclerosis is considered an inflammatory disease[1]. However, traditional risk factors have not been able to fully explain the development of this disease and its variable clinical expressions. In particular, hyperlipidemia has been considered for a long time by many as the obvious therapeutic target. Trials with hypolipidemic drugs have yielded extraordinary results in terms of morbidity and mortality reduction in patients with cardiovascular disease, both in primary and secondary prevention[2,3]. However, not all patients with atherosclerosis have dyslipidemia and not all dyslipidemics develop atherosclerosis. Therefore, recent years have witnessed an increasing search for non-traditional risk factors. Among these, infectious agents have attracted considerable interest. Over the years, seroepidemiological and experimental research have mostly focused on *Herpesviridae* (Herpes simplex virus-HSV and *Cytomegalovirus*-CMV), *Chlamydia pneumoniae* (*C. pneumoniae*) and, more recently, on *Helicobacter pylori* (*H. pylori*). In addition, the preliminary results of pharmacological trials with antimicrobial drugs, particularly macrolides, have suggested the possibility of new forms of therapeutic intervention in the secondary prevention of cardiovascular diseases. This article will review the current knowledge on the possible involvement of infectious agents in the development of atherosclerosis, the mechanisms by which they may trigger its clinical manifestations and the results of pharmacological trials.

Seroepidemiology and experimental background

Herpesviridae. Infections with HSV and CMV in the general population are mostly chronic and asymptomatic. About 80-90% of the adult population has positive antibody titers against HSV-1[4] and more than 60% of adults > 65 years have serologic evidence of previous exposure to CMV[5]. Older studies have demonstrated a higher incidence of seropositivity to CMV in patients with carotid atherosclerosis than in control subjects matched for cholesterol levels and other traditional risk factors[6]. In addition,

high immunoglobulin G titers against CMV have been advocated as an independent risk factor for the development of restenosis 6 months after percutaneous directional coronary atherectomy: seropositive patients had a 5-fold greater incidence of restenosis compared with seronegative individuals[7].

The first experimental evidence of a possible direct role of *Herpesviridae* in the pathogenesis of vascular disease was the induction of atherosclerosis in chickens by infection with Marek's disease virus, an avian herpesvirus[8]. Over the years, the advances in molecular biology techniques have permitted the detection of CMV DNA in the vascular tree, for example in 90% of femoral and abdominal arterial samples from patients undergoing vascular surgery versus 53% in a control group[9]. Since there is no evidence of an active CMV infection in the human vascular tree and since CMV DNA is widely distributed in the vascular tree[10], it has been hypothesized that the vascular wall may harbor a latent CMV infection which might induce chronic alterations and damage leading to the development of atherosclerotic lesions[11]. As a matter of fact, *Herpesviridae* genomic sequences and antigens may be found in the initial atherosclerotic lesions of the coronary arteries of young trauma victims[12].

Chlamydia pneumoniae. As for *Herpesviridae*, serologic evidence of previous exposure to *C. pneumoniae* increases with age reaching about 40-50% in the middle-aged population[13]. Patients affected by coronary artery disease and with acute myocardial infarction have higher antibody titers and specific circulating immune complexes against *C. pneumoniae* than control subjects[14,15]. Serologic evidence of previous *C. pneumoniae* infection appears to be associated with an approximately 2-fold increase in the risk of coronary artery disease[16]. In addition, elevated antibody titers or immune complexes containing chlamydial lipopoly-saccharide have been proposed as an independent risk factor for the development of acute myocardial infarction[17].

C. pneumoniae DNA may be found in about 50 to 80% of atherosclerotic coronary artery specimens[18,19]. However, the positivity of tissue samples does not necessarily correlate with the antibody titers, raising some doubts about the real significance of seroepidemiological data. As already described for CMV, *C. pneumoniae* DNA appears to be widely distributed in the human vascular tree[20]. In addition, *C. pneumoniae* DNA has been detected in the coronary arteries of young adults who have died of non-cardiac causes[21]. Therefore, the concept that the vascular wall may harbor a latent infection which might induce chronic alterations and damage leading to the development of atherosclerotic lesions might well also apply to *C. pneumoniae*. For example, a chronic infection with *C. pneumoniae* results in a constant production of chlamydial heat shock protein (HSP) 60, which may stimulate the formation of autoantibodies against human HSP60, thus provoking an autoimmune cascade, local and systemic inflammation and the clinical manifestations of ischemic heart disease[22,23].

Helicobacter pylori. Again, about 40-50% of the general population has serologic evidence of *H. pylori* infection[24,25]. Although the seroepidemiology linking *Herpesviridae* and *C. pneumoniae* to atherosclerosis is well accepted, the data regarding *H. pylori* are more conflicting. Some studies have reported a significant prevalence of *H. pylori* infection (about 65%) in patients with coronary artery disease[24,25] or previous myocardial infarction[26]. However, the ARIC study, a prospective investigation over a median follow-up period of about 3 years, has reported a negative association between *H. pylori* infection and clinical coronary heart disease events[27].

H. pylori DNA has been found in about 52% of the atherosclerotic plaques obtained from carotid endarterectomy specimens, while it has not been found in any of autopsy control atherosclerosis-free carotid samples[28]. In the same study, half of the DNA-positive samples tested positive for morphological and immunohistochemical evidence of *H. pylori* infection. In another study, evidence of *H. pylori* DNA has been found in 37% of carotid endarterectomy specimens versus none of the controls[29], while an older study has reported negative results[30]. In addition, *H. pylori* specific DNA may also be found in human coronary artery specimens[31]. Owing to the fact that the interest in *H. pylori* is more recent, the presence of this agent in the vascular tree has not been studied as extensively as that of *Herpesviridae* and *C. pneumoniae*. However, it may be hypothesized that also *H. pylori* DNA is widely distributed in the vascular tree. Since *H. pylori* may cause a chronic infection of the gastrointestinal tract and since there is no evidence of an active infection in the human vascular tree, the concept that the vascular wall may harbor a latent infection which might induce chronic alterations and damage leading to the development of atherosclerotic lesions may also apply to *H. pylori*. As a matter of fact, a recent prospective study has correlated the evolution of carotid plaques to chronic infections with a virulent strain of *H. pylori* (CagA-positive), while no association has been found with non-virulent strains (CagA-negative) infections[32]. In addition, a cross-reactivity of anti-CagA antibodies with vascular wall antigens has been recently described, raising speculations about a possible role of antigenic mimicry and autoimmunity in the relationship between *H. pylori* infection and atherosclerosis[33].

Infections and clinical expressions of atherosclerosis

Infectious agents as a cause of atherosclerosis. Despite the substantial amount of seroepidemiological and experimental data, the role and the mechanisms by

which infectious agents are involved in the development and clinical manifestations of vascular disease are far from being completely understood. The first question is whether infectious agents may be considered as a direct cause of atherosclerosis. The original studies reporting the development of atherosclerosis in chickens infected with Marek's disease virus[8], generated the illusion that a direct viral infection could induce vascular disease. However, the fibrous lesions developed in this experimental situation required the administration of a cholesterol-rich diet in order to resemble a true atheroma. In recent years, some animal studies conducted with *C. pneumoniae* have attempted to define the possible role of a direct infection with this agent. One study was conducted in New Zealand White rabbits on a normal diet and infected by means of intranasal inoculation. This study showed the development of lesions resembling an initial atheroma (fatty streaks) in a minority of cases, while non-infected animals exhibited no lesions[34]. Another study was conducted with the same method of infection in C57BL/6J mice[35]. In this study, apolipoprotein E-deficient mice, which spontaneously develop atherosclerosis, showed *C. pneumoniae* in the atherosclerotic lesions for up to 20 weeks after infection, while wild-type mice, which do not develop atherosclerosis, showed *C. pneumoniae* in the aortic wall only in a minority of cases. However, this study did not include a wild-type group on an atherogenic diet or a group with non-infected apolipoprotein E-deficient mice. A more recent study has conducted this comparison and has shown no difference in the atherosclerotic lesions between infected and non-infected apolipoprotein E-deficient mice[36]. Recently, also *H. pylori* has been investigated as a possible direct cause of atherosclerosis. One such study has been conducted in wild-type C57BL/6J mice and LDL-deficient mice, which develop atherosclerosis only when fed a cholesterol-rich diet[37]. This study showed no difference between the atherosclerotic lesions induced in cholesterol-fed LDL-deficient mice that were either infected or non-infected with *H. pylori*. In the light of these data, it is unlikely that infectious agents may be considered *per se* as a direct cause of atherosclerosis. However, it may be conceived that a permissive hyperlipidemic environment may be necessary to promote the full expression of vascular disease, whose development and clinical expression may be influenced by infection of the vascular wall. In this regard, some recent clinical studies suggest that individuals with serologic evidence of chronic *C. pneumoniae* or *H. pylori* infection tend to have a more atherogenic lipid profile, such as lower HDL levels and a decreased HDL-to-total cholesterol ratio[38,39].

There is a substantial amount of data indicating that infectious agents, although not a direct cause of atherosclerosis, may influence the progression and clinical expression of vascular disease through infection of vascular cells. Cell lysis may be a first effect of infection. Endothelial cells and smooth muscle cells, the main constituents of a normal vessel wall and the infection and lysis of which may represent the first step in the alteration of the vascular wall homeostasis, may be infected by either *Herpesviridae*[40,41] or *C. pneumoniae*[42]. The latter may also directly infect macrophages[42]. In particular, the lysis of endothelial cells may allow the migration of leukocytes into the vessel wall and the initiation of an inflammatory process. Infectious agents may modulate the activity of vascular cells toward the production of proinflammatory and procoagulant mediators. Endothelial cells produce interleukin (IL)-6 in response to CMV infection[43] and IL-6 and IL-8 in response to *H. pylori* infection[44]. Monocytes produce tumor necrosis factor (TNF)-α in response to HSV-1 infection[45] and interferon (IFN)-γ, TNF-α, IL-1β and IL-6 in response to *C. pneumoniae* infection[46]. Macrophages produce TNF-α, IL-1β and colony stimulating factor-1 in response to CMV infection[47]. Leukocyte-endothelial contact is considered a fundamental step in the initiation of the inflammatory process of atherogenesis and is mediated by adhesion molecules expressed on endothelial cells. Endothelial P-selectin, one of such adhesion molecules, is induced by infection with *Herpesviridae*[48]. Infection of endothelial cells with *C. pneumoniae* induces the expression of endothelial leukocyte adhesion molecule-1, intercellular adhesion molecule (ICAM)-1 and vascular cell adhesion molecule (VCAM)-1[49], while in these same cells up-regulation of VCAM-1, ICAM-1 and E-selectin is stimulated by infection with *H. pylori*[44]. A procoagulant state and the development of a coronary thrombus are considered the mainstay of acute coronary syndromes. It has been shown that *Herpesviridae* and *C. pneumoniae* modulate the procoagulant activity of infected endothelium. Infection with HSV-1 augments tissue factor expression[50] and thrombin generation with increased platelet binding[51], while thrombomodulin expression and plasminogen activator inhibitor activity are reduced[50,52]. *C. pneumoniae* induces tissue factor expression in endothelial cells[53]. In addition, systemic infection may induce acute-phase reactants (C-reactive protein, fibrinogen), which could precipitate thrombosis on a preexistent atherosclerotic plaque, whereas bacterial endotoxins and cytokines released during bacteremia might activate the vascular cells and leukocytes resident within the atheroma, induce local cytokine production, or recruit additional leukocytes[22,54].

The effects of a chronic latent infection with *Chlamydia pneumoniae*. As already stated, atherosclerosis is largely viewed as a chronic inflammatory disease[1] and it seems unlikely that an acute infection may generate inflammation within the vascular wall so extensive as to be capable of triggering atherogenesis. Rather, it appears more reasonable that a chronic form of infection may alter the vascular biology, promoting cytokine production and adhesion molecule expres-

sion, inducing a chronic local inflammatory state and thereby atherogenesis. Since there is no evidence of an active infection, but the DNA of microbial pathogens is widely distributed in the vascular tree[10,20], it has been hypothesized that the vascular wall may harbor a latent infection which may induce chronic alterations and damage leading to the development of atherosclerotic lesions.

The life cycle of chlamydiae has been extensively studied and it appears that these organisms can achieve a state of chronic, persistent infection, in which they are metabolically quiescent and do not replicate, are not identifiable in cultures, but are still viable (Fig. 1)[22,23,55]. This state of chronic, persistent infection may be induced by a number of conditions, including exposure to IFN-γ, an immune mediator produced by activated T cells present within the atheroma[56]. During this state of infection, chlamydiae express large quantities of HSP60. HSPs are a ubiquitous family of intracellular highly conserved proteins and, in an attempt to stabilize cellular proteins, their expression is increased during a variety of conditions such as heat shock, nutrient deprivation, infections, and inflammatory reactions[57]. Although *C. pneumoniae* can infect most cells present in an atheroma[42], within human coronary artery lesions it has been detected mostly in macrophages[58]. The mediators produced by these phagocytic leukocytes probably contribute importantly to atherogenesis, plaque instability and thrombosis. Chlamydial HSP60, together with human HSP60, is localized in the macrophages of human atheromas and induces macrophage secretion of TNF-α and matrix metalloproteinases, functions relevant to arterial inflammation and to the complications of atherosclerosis[59] (Fig. 2). In addition, chlamydial and human HSP60 activate endothelial cells, smooth muscle cells and macro-phages by inducing IL-6 production, and they also stimulate E-selectin, ICAM-1 and VCAM-1 expression on endothelial cells[60]. Interestingly, *C. pneumoniae* infection induces foam cell formation through its lipopolysaccharide[61] and it appears that HSP60 activates monocytes, the precursors of macrophages, through CD14, the same receptor utilized by the lipopolysaccharide[62].

Since HSPs share a high degree of homology across different species and since chlamydial and human HSP60 have been both detected in atherosclerotic lesions[59], chlamydial HSP60, a product of chronic, persistent infection with *C. pneumoniae*, rather than acting as a protein with protective cellular functions, may induce antigenic mimicry, complement fixation and the generation of a local inflammatory response, thereby influencing atheroma formation and evolution[63]. Older studies have shown that atherosclerosis may be induced in normocholesterolemic rabbits by immunization with mycobacterial HSP65[64], and increased anti-HSP65 serum antibody titers have been found in patients with atherosclerotic lesions of the carotid arteries[65]. More recently, serum antibodies against HSP65/60 obtained from subjects with atherosclerosis have been found to cross-react with human and chlamydial HSP60 and al-

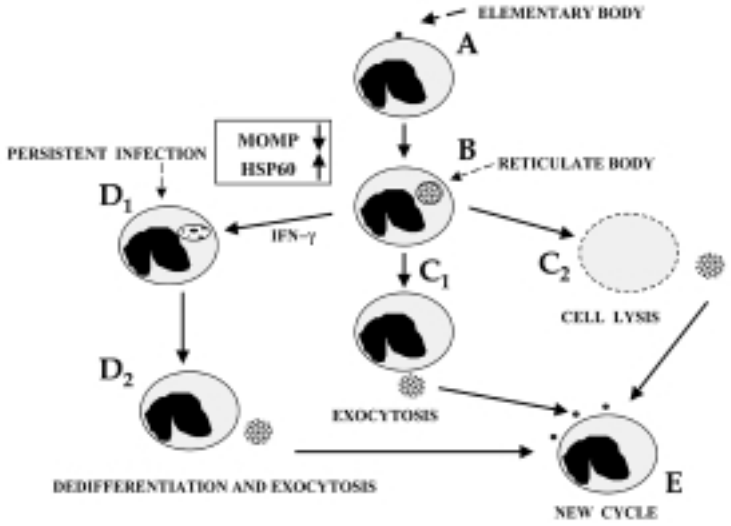

Figure 1. *The life cycle of Chlamydiae. Chlamydiae are obligate intracellular pathogens. The chlamydial life cycle begins when its infectious form, the elementary body, enters a host cell, for example by phagocytosis (A). The elementary body, once inside the cell, enlarges to the metabolically active form, the reticulate body (B). The chlamydial life cycle can then follow two different pathways ($C_{1,2}$ and $D_{1,2}$): 1) the resulting reticulate bodies, after undergoing a number of replication cycles, can assume the form of elementary bodies, and exit the cell by exocytosis (C_1) or through cell lysis (C_2), thereby initiating the next cycle in a new host cell (E); 2) high levels of interferon (IFN)-γ, antibiotics such as penicillin, or the lack of nutrients such as cysteine may inhibit the replication of reticulate bodies, promoting conversion to larger atypical metabolically inactive chlamydial forms which are viable, but which are not identifiable in cultures (persistent infection) (D_1); upon removal of IFN-γ and antibiotics, and upon the restoration of normal levels of cysteine, the atypical forms can dedifferentiate to reticulate bodies and complete the life cycle (D_2), becoming ready to begin the next cycle in a new cell (E). During a persistent infection, chlamydiae reduce the expression of the major outer membrane protein (MOMP) and increase the levels of heat shock protein (HSP) 60. Since antibodies to MOMP appear to neutralize Chlamydia infectivity, while HSP60 elicits a local inflammatory response, such a persistent infection may explain why viable Chlamydiae have not been cultured from atherosclerotic tissue and still provide a mechanism for a potential link between atherogenesis and chronic latent infections. From Kol and Libby[22], with permission.*

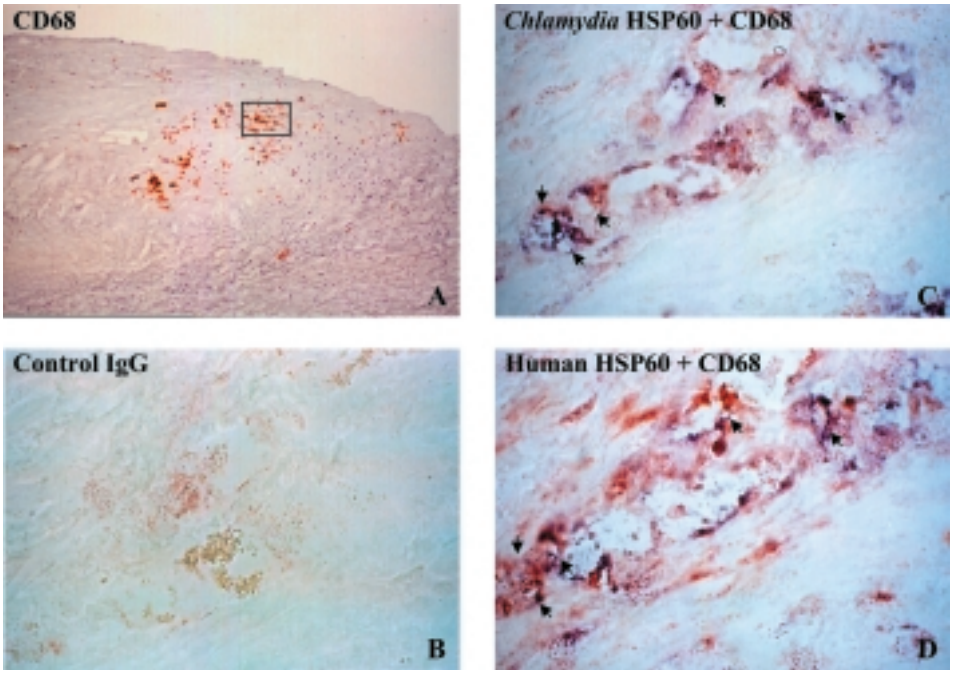

Figure 2. *Chlamydial heat shock protein (HSP) 60 co-localizes with human HSP60 within atherosclerotic plaque macrophages. Human atherosclerotic plaque (×100) stained for macrophages (CD68, red). The* rectangle *indicates the macrophage-rich region (intimal plaque shoulder) sampled in high power views (×400, B, C and D) of serial sections adjacent to the one depicted in* A. B: *the section stained with mouse immunoglobulins (Ig) G as negative control yielded no staining.* C: *double staining for chlamydial HSP60 (red) and macrophages (CD68, blue).* D: *double staining for human HSP60 (red) and macrophages (CD68, blue). The* arrowheads *in* C *and* D *indicate macrophages (CD68+) that stain positively for both chlamydial and human HSP60. Analysis of adjacent sections showed that both human and chlamydial HSP60 are localized within macrophage clusters. The lumen of the artery is at the top of each photomicrograph. Analysis of 7 of 9 samples (77%) showed similar results. From Kol et al.[59], with permission.*

so mediate endothelial cytotoxicity[66]. Human HSP60 itself, when expressed by heat-shocked endothelial cells, can provoke an autoimmune reaction mediating endothelial cytotoxicity[67]. In addition, the serum levels of soluble HSP60 seem to be significantly elevated in subjects with carotid atherosclerosis and seem to correlate with the thickness of the common carotid artery intima/media[68]. A recent report has shown that high levels of antibodies against chlamydial HSP60 are present in patients with acute coronary syndromes[69]. Others have shown that only high levels of antibodies against human HSP60, but not against chlamydial HSP60, especially in the presence of increased C-reactive protein levels, are able to predict coronary events with a 7-fold increased risk[70,71]. A possible explanation is that a chronic infection with *C. pneumoniae* results in a constant production of chlamydial HSP60[22,23], which would stimulate the formation of autoantibodies against human HSP60, thus provoking an autoimmune cascade, local and systemic inflammation and the clinical manifestations of ischemic heart disease.

Antimicrobial therapy and coronary events

The large amount of seroepidemiological and experimental data linking infectious agents, particularly *C. pneumoniae*, to the development of atheromas and to the precipitation of acute vascular events, has raised many hopes that antimicrobial therapy might modify the prognosis of ischemic heart disease.

Infections with chlamydiae are generally treated with a class of antibiotics called macrolides. The ROXIS trial has been the study that has pioneered the use of macrolides, particularly roxithromycin, in the secondary prevention of acute coronary events and ischemic death[72]. This study has showed a significant reduction of ischemic events in unstable angina patients after 1 month of treatment with roxithromycin and these data were confirmed after 3 and 6 months of follow-up[73]. Almost contemporarily to the ROXIS trial, a different group[74] has published similar encouraging data obtained with another macrolide, azithromycin. In this study, *C. pneumoniae* seropositive survivors of myocardial infarction were treated for 3 or 6 days and followed up for a mean period of 18 ± 4 months. The risk for adverse cardiovascular events in seropositive treated patients decreased to the level of seronegative patients. The enthusiasm aroused by these studies, however, has been somewhat deflated by the results of further studies. In the ACADEMIC trial, seropositive patients affected by coronary artery disease were treated with azithromycin (300 patients) or placebo (60 patients) for 3 months and followed up for 2 years. Unfortunately, no significant difference was observed in the rate of cardiovascular events between the two groups[75]. Larger trials with a longer follow-up have unfortunately confirmed these negative results. The

AZACS trial has enrolled 1439 patients with unstable angina or acute myocardial infarction: treatment with azithromycin for 5 days was not found to have any benefit after 6 months of follow-up[76]. The recently completed WIZARD trial, which has enrolled 7747 patients with a prior myocardial infarction randomized to a 3-month course of azithromycin or placebo, has also shown no benefit after a median follow-up of 14 months[77,78].

Although the data from pharmacological trials appear contradictory, it has to be remembered that macrolides do not act specifically on *C. pneumoniae*, but may target a number of different infectious agents. Interestingly, in the ROXIS trial there were no changes in the titers of anti-*C. pneumoniae* antibodies between the treated and the placebo group, while C-reactive protein levels decreased significantly in the treatment arm[73]. These findings raise doubts about the specific effect of roxithromycin on *C. pneumoniae*, while a general decrease in an inflammatory trigger seems plausible. Since endothelial and smooth muscle cells infected with *C. pneumoniae* produce adhesion molecules and proinflammatory cytokines[60], the interference of antibiotic treatment on this proinflammatory state may explain, at least in part, the beneficial effects of macrolides observed in some preliminary trials. Although *C. pneumoniae* has been detected mainly in the macrophages of human atheromas[58,59], it appears that neither oral azithromycin nor rifampin, an antimicrobial agent of a different class, are capable of eradicating infection from circulating monocytes, the precursors of macrophages[79]. In this setting, the lack of susceptibility of *C. pneumoniae* to antibiotic treatment may render treatment useless and thus explain the negative results of the larger pharmacological trials. Nonetheless, a different study has shown that *C. pneumoniae* may be eradicated from the coronary artery endothelium and smooth muscle cells after treatment with macrolides, roxithromycin being the most effective, rifampin or quinolones[80]. In addition, the endothelial function in seropositive ischemic patients appears to improve after treatment with azithromycin[81].

Conclusions

On the basis of the considerable amount of data accumulated over the recent years, a possible role of infectious agents, especially *Herpesviridae*, *C. pneumoniae* and *H. pylori,* in the pathogenesis of atherosclerosis and its clinical expressions would now seem acceptable. However, the nature of this association remains elusive and a number of issues remain unsolved. It seems unlikely that an acute infection may generate atheromas or an acute coronary syndrome. Rather, since microbial DNA has been found in the early atherosclerotic lesions of young subjects who have died of non-cardiac causes[12,21], and since there is no clear evidence of any active infection in the human vascular tree, it appears more plausible that a chronic infection might alter the vascular wall homeostasis through a number of mechanisms, ranging from cell lysis to the stimulation of adhesion molecule expression and cytokine production. For example, the increased production of microbial HSP60 during a chronic, persistent infection with *C. pneumoniae*, may induce antigenic mimicry and vascular inflammation[22,23]. In addition, atherosclerosis may be influenced by more infectious agents acting together on the same individual. It appears that the higher the infectious burden, represented by the number of infectious agents to which an individual shows seropositivity, the higher the extent of atherosclerosis and the cardiovascular mortality rate[82,83]. Preliminary studies with azithromycin or roxithromycin have generated considerable enthusiasm and expectations about the possibility that the prognosis of patients with acute coronary syndromes may be ameliorated by these antibiotics[73,74]. However, the negative results of large-scale trials prompt us towards a more skeptical approach[76,78]. In addition, antimicrobial drugs do not act specifically against a single infectious agent and more specific therapeutic agents would be needed. Future experimental studies and pharmacological trials will need to address the many unsolved issues regarding the link between infectious agents and vascular disease.

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