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Infection and Atherosclerosis Development

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Abstract

Atherosclerosis is a chronic disease hallmarked by chronic inflammation, endothelial dysfunction and lipid accumulation in the vasculature. Although lipid modification and deposition are thought to be a major source of the continuous inflammatory stimulus, a large body of evidence suggests that infectious agents may contribute to atherosclerotic processes. This could occur by either direct effects through infection of vascular cells and/or through indirect effects by induction of cytokine and acute phase reactant proteins by infection at other sites. Multiple bacterial and viral pathogens have been associated with atherosclerosis by seroepidemiological studies, identification of the infectious agent in human atherosclerotic tissue, and experimental studies demonstrating an acceleration of atherosclerosis following infection in animal models of atherosclerosis. This review will focus on those infectious agents for which biological plausibility has been demonstrated in animal models and on the challenges of proving a role of infection in human atherosclerotic disease.

Keywords

Atherosclerosis; Infectious agents; Chlamydia pneumoniae; Periodontal pathogens; Antibiotic trials

Introduction

Atherosclerosis is a disease of chronic inflammation, which ultimately results in tissue damage and fibrosis. The ongoing stimulus that promotes chronic inflammation remains unclear; however, lipid deposition and modification are thought to be key components. Multiple risk factors for atherosclerosis have been defined including smoking, hypercholesterolemia, hypertension, hyperglycemia and genetic factors. However, cumulatively, these factors do not account for the incidence of atherosclerosis, and

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cardiovascular disease (CVD) can develop in the absence of such risk factors (1). Considerable evidence has emerged indicating that infection is also a risk factor of atherosclerosis and contributes to chronic inflammatory processes either through direct or indirect effects (2). The association of infectious agents with atherosclerosis has been guided by seroepidemiological evidence demonstrating an increased risk of cardiovascular disease with the presence of antibodies against specific infectious agents as well as the demonstration of pathogens in atherosclerotic lesions. However, the burden of proof for a causative role of infectious agents lies in demonstrating biological plausibility that the organism contributes to atherosclerotic processes and ultimately, that therapeutic intervention or preventive strategies impact disease progression. The direct and indirect mechanisms through which infectious agents contribute to chronic inflammation, the infectious agents that have been associated with CVD, evidence indicating biological plausibility, and the challenges of proving causality are discussed herein.

Direct vs. Indirect Mechanisms

Infectious agents may contribute to chronic inflammatory processes by direct mechanisms or indirect mechanisms (2). A direct effect would be indicated by the ability of the organisms to infect vascular cells, demonstration of the organism within the atherosclerotic plaque and acceleration of lesion development following infection in animal models of atherosclerosis. Alternatively, an indirect effect of infectious agents resulting from infection at a non-vascular site would be supported by increases in cytokines and other acute phase proteins resulting in the acceleration of atherosclerosis in experimental models. Based on these criteria, the strength of data in supporting a role of individual infectious agents in contributing to atherogenesis ranges from strong (where the cumulative data suggest evidence of both direct and indirect mechanisms) to weaker suggesting that the association of infection with cardiovascular disease is only through an indirect mechanism (2). Although a wide variety of bacterial and viral pathogens have been detected in human atherosclerotic plaques or have been associated with CVD by other methods, the main focus of this review will be on those agents for which indirect or direct mechanisms have been supported through demonstration of an effect on atherosclerotic processes in experimental models of atherosclerosis.

Evidence of Infection of Atherosclerotic Plaques—A wide range of pathogens have been identified by nucleic acid or antigen detection methods in human atherosclerotic plaque (2). Examples of bacterial pathogens include *Chlamydia pneumoniae* (*C. pneumoniae*), *Mycoplasma pneumoniae*, *Helicobacter pylori*, *Enterobacter hormaechei*, and multiple periodontal organisms (e.g., *Poryphyromonas gingivalis*, *Aggregatibacter actinomycetemcomitans*, *Prevotella intermedia*, *Tanerella forsythia*, *Fusobacterium nucleatum*, *Streptococcus sanguis, and Streptococcus mutans)* (3–8). Examples of viral pathogens include: cytomegalovirus, hepatitis C virus, human immunodeficiency virus, herpes simplex viruses, Epstein-Barr Virus, enteroviruses, and parvovirus (14,15,18–34). Several studies have reported the presence of more than one infectious agent in the atheromatous tissue (6–8,17,18,21,22,25,26,29,33). However, culture of these infectious agents from human atheromas is rare, and has been reported only for *C. pneumoniae* and *E. hormaechei* (35–38). *C. pneumoniae* has been cultured from the coronary artery of a patient

with coronary atherosclerosis, a carotid endarterectomy specimen, and atherosclerotic specimens obtained during myocardial revascularization (35–37). *E. hormachei* was isolated from femoral atherosclerotic plaque (38). Although attempts to isolate *P. gingivalis* and *A. actinomycetemcomitans* from atheromatous tissue have been unsuccessful, using homogenates of human atherosclerotic plaque that had DNA evidence of both organisms in cell invasion assays of ECV-304 cells, the organisms were detected within the cells. Because viable organisms of these two species are required for invasion of non-phagocytic cells, these investigators concluded the atheromatous tissue contained viable organisms, although they were noncultivable (39).

Evidence in Animal Models that Infection Can Affect Atherosclerotic Processes—Although the notion of an infectious basis for atherosclerosis was proposed over a century ago, the first experimental evidence that infection could induce atherosclerotic changes was demonstrated by Fabricant et al. who reported that Marek's disease virus (MDV), a chicken herpes virus, could induce atherosclerosis in chickens (40). Significantly, vaccination prior to challenge prevented this induction (41). Since these initial studies, other infectious agents, including respiratory pathogens (*C. pneumoniae* and influenza viruses), periodontal pathogens (*P. gingivalis*, *A. actinomycetemcomitans)*, a gastric pathogen (*H. pylori)*, and cytomegalovirus, a common cause of congenital and perinatal infections, have been reported to accelerate atherosclerotic lesion progression in animal models providing evidence of a biological role in the pathology of atherosclerosis.

Chlamydia pneumoniae—*C. pneumoniae* is an etiology of acute respiratory disease in humans. Infection is ubiquitous; everyone is infected by age 14 and reinfection is common (42). Intranasal inoculation of mice simulates human respiratory tract infection with mice developing pneumonitis (43). In hyperlipidemic animal models, *C. pneumoniae* infection disseminates to and establishes persistent infection of the aorta, is detected in foam cells within the lesion, and accelerates lesion progression, indicating a direct effect of infection on atherosclerosis. These animal models include diet-induced hyperlipidemic mice (C57BL/6J and low-density lipoprotein receptor deficient [$LDLR^{-/-}$]) mice, apolipoprotein E deficient ($apoE^{-/-}$) mice that develop atherosclerosis spontaneously in the absence of an atherogenic diet, and cholesterol-fed New Zealand white rabbits (44–47). Whereas the aforementioned studies were done using repeated infection to simulate chronic infection, a recent study demonstrated in $apoE^{-/-}$ mice that a single intranasal inoculation with *C. pneumoniae* followed by administration of a high-fat diet also exacerbated atherosclerosis (48). This increased lipid accumulation in the aortic sinus was associated with a significant increase in the presence of activated myeloid dendritic cells (DCs) as well as plasmatoid DCs, suggesting that a role of these cell types in *C. pneumoniae* induced acceleration of atherosclerosis (49). In contrast, repeated *C. pneumoniae* infection of normolipidemic mice (C57BL/6J or $LDLR^{-/-}$ mice fed a chow diet) did not initiate atherosclerosis, although infection of New Zealand white rabbits fed a noncholesterol diet induced atherosclerotic-like changes in the aorta (45,49,50). Significantly, acceleration of diet-induced atherosclerosis in mice was dependent on the timing of administration of the atherogenic diet. Although initiation of an atherogenic diet simultaneously with the first of three intranasal inoculations with *C. pneumoniae* augmented lesion development, infection prior to the initiation of an

atherogenic effect did not (51). In ApoE3-Leiden mice fed an atherogenic diet, *C. pneumoniae* infection increased T-cell influx into the atherosclerotic lesion and enhanced complex lesion formation at earlier time points in comparison to uninfected animals (52). Collectively, these studies suggest that *C. pneumoniae* is a co-risk factor for atherosclerosis in conjunction with hyperlipidemia.

Several studies have addressed the effect of mice deficient in pathways known to play an independent role in atherosclerotic lesion development on *C. pneumoniae* accelerated atherosclerosis. The effect of *C. pneumoniae* infection is abrogated in hyperlipidemic TNF-α p55 receptor, IL-17 A, Toll-like receptor (TLR) 2, TLR4, and MyD88 knockouts (53–56). In contrast, infection of mice deficient in the constitutively expressed endothelial nitric oxide synthase (eNOS), which maintains endothelial function/tone, had no effect while knockout of inducible NOS (iNOS) enhanced *C. pneumoniae* accelerated atherosclerosis (57). This enhancement was presumably due to an increased bacterial load of the organism resulting from the absence of the bactericidal effects of iNOS derived nitric oxide. Another potential mechanism by which *C. pneumoniae* could contribute directly to atherosclerotic processes is through activation of the lectin-like oxidized LDL receptor (LOX-1), the major scavenger receptor on endothelial cells for uptake of oxidized LDL (oxLDL) that is also found on macrophages and smooth muscle cells (58–61). Activation of this receptor results in the upregulation of various pro-atherogenic factors including adhesion molecules, matrix metalloproteinases, and monocyte chemoattractant protein-1 (MCP-1) (62–64). *C. pneumoniae* has been demonstrated to bind to and activate this receptor *in vitro* and upregulate the expression of LOX-1 *in vivo* (65–68). Preliminary studies in hyperlipidemic LOX-1 knockout mice suggest that *C. pneumoniae* acceleration of atherosclerosis is ablated (unpublished data).

In addition to a direct effect of infection on atherosclerotic plaque progression, *C. pneumoniae* respiratory tract infection of C57BL/6 or apoE$^{-/-}$ mice results in significant increases in plasma cytokines, aortic cytokines, and acute phase reactant proteins (54,69–71). *C. pneumoniae* infection of mice also significantly decreases the anti-inflammatory properties of HDL, which occurs concomitantly with an increase in plasma serum amyloid A, an acute phase reactant protein that associates with HDL and alters the protective functions of HDL (69,72).

Several studies have been done using animal models to determine whether the acceleration of atherosclerosis by *C. pneumoniae* infection could be prevented by treatment with antibiotics. Two separate studies were done in ApoE$^{-/-}$ mice to determine whether azithromycin had any beneficial effects. One study inoculated mice with *C. pneumoniae* twice, 2 weeks apart and treated animals with azithromycin after each inoculation. The other inoculated mice three times with *C. pneumoniae*, 1 week apart, followed by a 6-week course of azithromycin. Both studies used a dose comparable to that given to humans for chlamydial respiratory infection (73,74). Neither study found any differences in lesion size in infected mice treated with azithromycin in comparison with untreated infected controls. Importantly, Rothstein et al. also detected *C. pneumoniae* DNA in the lung, heart and aorta in 50% of mice at the endpoint of the study (26 weeks of age) regardless of whether or not mice were treated with azithromycin (74). In contrast, treatment of infected New Zealand

white rabbits with azithromycin decreased *C. pneumoniae* accelerated intimal thickening. However, *C. pneumoniae* antigen was still detected in similar frequency in rabbits regardless of whether or not they were treated with azithromycin (75). In hyperlipidemic rabbits infected three times, 2 weeks apart, Fong et al. found that early initiation of clarithromycin treatment (after the first inoculation) resulted in a statistically significantly reduction in the number of rabbits that developed early atherosclerotic lesions as well as the number of animals in which *C. pneumoniae* antigen was detected in atherosclerotic tissues (76). If treatment was delayed until after the third inoculation, statistically significant reductions were not observed for either parameter.

Older hyperlipidemic mice with advanced atherosclerosis have been investigated to determine the effect of *C. pneumoniae* infection on plaque destabilization. *C. pneumoniae* infection of LDLR$^{-/-}$ apoE$^{-/-}$ double knockout mice with advanced atherosclerosis did not further accelerate lesion progression. However, increased production of matrix metalloproteinases (MMP-2 and MMP-9), which are known to contribute to plaque destabilization, were noted as well as a reduction in fibrous cap area (77). In older apoE$^{-/-}$ mice (1–2 years of age), *C. pneumoniae* infection resulted in an increased frequency of intra-plaque hemorrhage, a marker of plaque disruption, in the innominate artery in comparison to mice inoculated with heat killed *C. pneumoniae* or sham inoculated with PBS (69).

Endothelial dysfunction (impaired vasodilation and increased vasoconstriction) contributes to the pathogenesis of acute coronary syndrome (78). *C. pneumoniae* infection of apoE$^{-/-}$ mice resulted in endothelial dysfunction, which occurs through the nitric oxide pathway (79). In apoE$^{-/-}$ mice infected with both *C. pneumoniae* and *Helicobacter pylori*, statistically significant decreases in the relaxation response and plasma levels of nitrite/nitrate were observed in comparison to mice infected with either pathogen alone. The additive effect of dual infection on endothelial dysfunction also provides support to the hypothesis that pathogen burden can augment atherosclerotic processes (80). *C. pneumoniae* infection of pigs has also been found to cause endothelial dysfunction in the coronary vessels through the nitric oxide pathway (81). In addition, an increased systemic level of fibrinogen, which favors a procoagulant state and enhances the risk for vascular thrombosis, was also observed (81).

Periodontal Pathogens—Periodontal disease is a chronic inflammatory disease resulting from a chronic multibacterial infection of tissues surrounding the teeth. Periodontal disease begins as gingivitis and can progress to periodontitis, resulting in connective tissue destruction and alveolar bone loss (82). Periodontal bacteria as well as their components can gain access to the vasculature via bleeding periodontal pockets. As with *C. pneumoniae*, several studies have demonstrated that *P. gingivalis* as well as *A. actinomycetemcomitans* could contribute to atherogenesis via both direct and indirect mechanisms.

In mouse and rabbit models, *P. gingivalis* infection induces periodontal disease as well as enhancing atherosclerotic processes. An initial report demonstrated that intravenous inoculation of *P. gingivalis* for 10, 14, or 24 consecutive weeks resulted in earlier atherosclerotic lesion formation and accelerated lesion progression in apoE$^{-/-}$ fed either a

chow diet or a high-fat diet (83). Significant differences in serum levels of SAA and IL-1B were noted in the 14 and 24 week groups. Although bacteria were not detected in heart tissue at the earlier time points, all mice were PCR positive following 24 weeks of challenge with the organism (83). Using the natural route of infection, oral infection of hyperlipidemic apoE-null mice resulted in elevated levels of plasma IL-6, detection *of P. gingivalis* in aortic tissue, increased aortic expression of vascular cell adhesion molecule-1 and tissue factor, and acceleration of early atherosclerosis in the aortic sinus (84). No gender differences were observed in the ability of *P. gingivalis* infection to induce increased levels of SAA and atherosclerotic lesion formation in young male and female mice (85). In infected mice treated with doxycycline or metronidazole, an antibiotic effective against anaerobic organisms, atherosclerotic lesion formation as well as plasma IL-6 and SAA were decreased in comparison to untreated, infected mice (86,87). In addition, nasal immunization of apoE deficient spontaneously hyperlipidemic mice ($apoE^{shl}$) with the *P. gingivalis* 40-kDa outer membrane protein, a virulence factor involved in coaggregation and hemagglutination, prior to IV challenge with *P. gingivalis*, significantly reduced atherosclerotic lesions in the aortic sinus as well as serum levels of pro-inflammatory cytokines and chemokines (88). Using $apoE^{-/-}$ mice to assess the effect of *P. gingivalis* on restenosis after balloon angioplasty, infection resulted in an increased monocyte invasion and increased atherosclerotic plaque growth. These effects were significantly reduced by treatment with a broad spectrum chemokine-binding protein (m-T7), suggesting a key role of chemokine-mediated inflammation (89).

In New Zealand white rabbits fed a high fat diet, application of *P. gingivalis* to animals that had received ligatures around their mandibular premolars to induce periodontitis, had significant increases in atherosclerotic lesion formation in the aorta, in comparison to the control group of rabbits without periodontal disease (90). Additionally, the severity of periodontal disease correlated with the extent of lipid deposits. Because *P. gingivalis* was not detected in the aortic tissue from any of the infected rabbits, the investigators concluded that infection had an indirect effect on atherosclerosis through stimulation of chronic inflammatory responses in the oral cavity that affected innate immune responses at other sites (90). Interestingly, oral treatment with Resolvin E1, a potent mediator of inflammation resolution, significantly decreased atherosclerotic lesion formation following *P. gingivalis* induced periodontitis (91).

It has been suggested that the Nod-like receptor family, pyrin domain containing 3 (NLRP3) inflammasome plays a role in the development of inflammation of the vasculature and atherosclerosis, as activation of proinflammatory cytokines such as IL-1B, and IL18 are NLRP3 inflammasome-dependent (92). Activation of the NLRP3 inflammasome by microorganisms results in caspase-1 dependent processing and secretion of IL-1B (93). A recent study compared atherosclerosis development in $apoE^{-/-}$ mice infected with wild type (WT) *P. gingivalis*, and two mutants deficient in virulence factors (gingipain and fimbriae) known to activate proinflammatory immune responses and signaling pathways (94). Oral infection with WT *P. gingivalis* significantly increased lesion area in the aortic sinus, production of IL-1B, IL-18 and TNF-alpha in peritoneal macrophages and aortic gene expression of NLRP3, proIL-1B, pro-IL-18 and procaspase 1 (94,95). *P. gingivalis* was also

more frequently detected in the aorta of mice infected with WT *P. gingivalis* than with the mutant strains.

To determine whether different proatherogenic stimuli affected atherosclerosis development via the same mechanisms, Kramer et al. compared gene expression patterns in aortic tissue of apoE$^{-/-}$ mice that were infected with *C. pneumoniae*, *P. gingivalis*, or fed a Western diet (96). Each pro-atherogenic stimulus had distinct gene signatures and pathways. Thus, although all three stimuli promote vascular inflammation and dysfunction, the pathways leading to these outcomes differ mechanistically. The observed expression patterns suggested that *P. gingivalis* may promote atherosclerosis by contributing to mitochondrial dysfunction and decreased exit of lipid from the vessel wall, whereas *C. pneumoniae* may contribute to atherosclerotic processes by augmenting lipid uptake and metabolism (96).

Intravenous inoculation with *A. actinomycetemcomitans*, another periodontal pathogen, has also been shown to accelerate atherosclerosis in hyperlipidemic Apoe^{shl} mice (97). *A. actinomycetemcomitans* increased serum levels of C-reactive protein, LPS, IL-6, IL-8, TNF-α, and MCP-1. Bacterial DNA was detected in the heart and increased levels of expression of various proatherogenic factors in the aorta were observed including TLR2, TLR4, adhesion molecules, LOX-1, HSP60 and MCP-1. Although live organisms had the largest effect on accelerating atherosclerotic lesion development, heat-killed organisms or *A. actinomycetemcomitans* LPS, also had a significant effect (97). All treatment groups demonstrated enhanced expression of Toll and nucleotide oligomeric domain like receptors and oxidation of LDL, which could play a role in lesion progression (97). *A. actinomycetemcomitans* also promoted induction of Th17 cells in the spleen, increased serum levels of IL-17, IL-6, IL-10 and IL-1B, enhanced mRNA expression of Th17 related molecules as well as inflammasome-related mRNA expression (AIM-2, Mincle, and NLRP3) in the aorta, and increased the development of atherosclerosis in the aortic sinus (98). Th17 cells are characterized by their production of the inflammatory cytokine 1L-17 and have been shown to promote chronic inflammatory responses although their role in atherosclerotic processes remains controversial (99,100).

Another periodontal organism that results in connective tissue destruction and alveolar bone resorption in periodontal disease and has been detected in atherosclerotic plaque is *T. forsythia*. Paradoxically, although repeated oral infection of mice resulted in increased SAA, decreased serum nitric oxide, and increased serum lipoproteins in comparison to uninfected mice, a decreased aortic lesion size was noted in infected animals (101).

H. pylori—*H. pylori* is a common cause of chronic gastritis and considered a risk factor for the development of gastric and duodenal ulcers as well as gastric cancer (102). This organism has also been identified in human atherosclerotic lesions. Only a few studies have reported the effects of *H. pylori* on atherosclerotic processes in mouse models, which had differing results. *H. pylori* infection had no effect in normolipidemic C57BL/6J mice but enhanced atherosclerosis in hyperlipidemic C57BL/6J mice fed an atherogenic diet (103). In contrast, *H. pylori* infection had no effect on atherosclerosis progression, lipid deposition or lesion cellularity in C57BL/6J mice or LDL-receptor deficient congenic mice fed a high cholesterol diet (104). Ayada et al. demonstrated that *H. pylori* infection enhanced

atherosclerosis in heterozygous apoE$^{+/-}$ x LDLR$^{+/-}$ mice, which appeared to be associated with an elevated Th-1 immune response directed against the *H. pylori* HSP 60 along with increased transendothelial T-cell migration (105). These effects could be abrogated by immunization with *H. pylori* HSP60 or by treatment with anti-*H. pylor*i antibiotics (106).

Cytomegalovirus—CMV infection is frequent in humans and usually asymptomatic in immunocompetent individuals (107,108). In immunocompromised patients undergoing heart transplantation, CMV infection has been associated with heart transplant vasculopathy and transplant rejection (109,110). There have been multiple reports indicating evidence of CMV in atherosclerotic plaques (19–24,26,111); however, the virus is also found in nonatherosclerotic tissue (19,111) In several studies, CMV has been shown to increase lesion size in hyperlipidemic models of infection and to increase levels of IFN-γ and TNF-α (112–115). An early study suggested that mouse CMV (MCMV) had both direct and indirect effects on atherosclerosis based on enhanced cytokine expression both systemically and locally within the aortic arch (115). However, a subsequent study by the same group showed that regardless of whether infectious virus or UV-inactivated virus was injected into mice, atherosclerotic lesion size was augmented and increased numbers of T cells were observed in atherosclerotic lesions (114,115). Because the virus was detected in the lesion when infectious MCMV was injected, but not UV-inactivated virus, an indirect effect of the virus on atherosclerotic processes was indicated (114). In a study to address the effect of pathogen burden on atherosclerotic lesion progression, dual infection of apoE knockout mice with MCMV and *C. pneumoniae* was examined. Although infections with mouse MCMV or *C. pneumoniae* alone or both significantly increased lesion size, the percent increase in plaque was less (45% increase) with combined infection in comparison to either *C. pneumoniae* (80% increase) or MCMV alone (74% increase) (112). In this study, the authors noted a significant increase in serum IFN-γ by MCMV, which they proposed had a dual effect in contributing to the acceleration of lesion formation by MCMV and decreasing the acceleration of atherosclerosis by *C. pneumoniae* due to its chlamydiacidal effect because MCMV infection preceded infection with *C. pneumoniae* (112).

Analysis of differential gene expression in aortas of apoE knockout mice provided insight into one mechanism by which CMV could potentiate atherosclerotic lesion formation. In MCMV infected mice, atherosclerotic lesion size was increased and demonstrated more advanced disease in comparison with uninfected mice (116). The investigators found that 60% of genes in the MAPK pathway were upregulated in infected mice, including p38 and ERK1/2 MAPK genes (116). Levels of VCAM-1, ICAM-1 and MCP-1 were also upregulated. Use of an inhibitor of p38 resulted in a decreased viral load in the aorta as well as lower levels of pro-atherogenic molecules leading to the conclusion that upregulation of p38 by MCMV infection might be the driver of MCMV-induced acceleration (116).

Influenza A—Influenza infection is associated with acute coronary syndrome and fatal myocardial infarction (117). Following intranasal inoculation of normolipidemic and hyperlipidemic mice with influenza virus and to address the short-term effects of infection, the virus was cultured at 7 days postinoculation from the lung, aorta and heart, but not from other organs (e.g., liver, kidney, spleen) and, somewhat surprisingly, not the blood (118).

Further investigation in influenza infected apoE$^{-/-}$ mice demonstrated a concomitant increase in the aorta of various chemokines (MCP-1, Rantes, IP10, and KC) and cytokines (e.g. IL-6, IL-1B) as well as an increase in expression of genes for macrophage markers. Notably, a decrease in eNOS expression was observed in infected mouse aortas in comparison to uninfected aortas. Influenza infection also significantly increased plasma levels of pro-inflammatory cytokines (MCP-1, Rantes, and IL-6, IL-1A, and Il-1B, respectively, as well as cytokines mediating monocyte differentiation GM-CSF- and G-CSF) (118). Influenza A infection in mice also results in the loss of the anti-inflammatory properties of HDL, providing an additional mechanism by which the virus could have an indirect effect on atherosclerotic lesion progression (72). Thus, these studies suggest potential direct effects through infection of the vascular wall and indirect effects by increasing circulating levels of proinflammatory cytokines. Although the virus has been detected in the vascular wall in mice, whether influenza infection accelerates atherosclerotic lesion progression in this animal model has yet to be investigated. However, it should be noted that influenza infection in humans is generally thought to be restricted to the pulmonary system and there have not been any reports of detection of the organism in human atherosclerotic plaques. Thus, evidence of a direct effect of influenza viruses in the pathogenesis of atherosclerosis in humans is not supported

Several studies have indicated that acute respiratory infections can serve as an inducer of myocardial infarction (119,120). Thus, the hypothesis was tested that influenza infection could play a role in the complications of atherosclerosis by infecting older apoE$^{-/-}$ mice with established atherosclerosis and determining whether infection induced histopathological changes in the atherosclerotic lesion in the aorta, at 3, 5, and 10 days post infection (121). Mice were infected IN with 1 LD50 of the virus. Infected apoE$^{-/-}$ mice exhibited substantially increased subendothelial cellular infiltrates in comparison to uninfected apoE$^{-/-}$ mice as well as infected C57BL/6 normolipidemic mice. The infiltrates consisted of smooth muscle cells, macrophages, and T lymphocytes. Significantly, in one infected mouse, a subocclusive platelet and fibrin-rich thrombus was observed (121). Notably, no histopathological changes were observed in nonatherosclerotic segment of the infected aorta.

The Burden of Proof—Based on cumulative evidence demonstrating the prevalence of *C. pneumoniae* in atherosclerotic lesions in humans and experimental evidence in hyperlipidemic animal models that pulmonary infection with this organism accelerated atherosclerosis, several small clinical trials determined if treatment with antibiotics effective against *Chlamydia* would prove beneficial in secondary prevention of atherosclerotic disease (122). Eight of these early studies focused on treatment of patients with coronary heart disease with macrolides from 3 days to 3 months and half demonstrated beneficial effects. In addition, in four small trials treating with patients with vascular disease with doxycycline (3 months) or roxithromycin (28–30 days) positive benefits were noted by measuring either aortic aneurysm growth, carotid artery thickness, or peripheral vascular disease symptoms (122). Overall, these studies engendered enthusiasm for the potential therapeutic intervention in atherosclerotic cardiovascular disease and the expectation that large scale trials, if successful, would provide additional validation of the infectious hypothesis.

Subsequently, four large clinical trials were completed using antibiotics with efficacy against *C. pneumoniae*. Collectively, these studies enrolled over 20,000 patients with stable coronary artery disease (WIZARD, ACES, and CLARICOR) or acute coronary syndrome (PROVE-IT-TIMI) (123–126). Additional clinical trials were done in patients with peripheral artery disease treating with roxithromycin or rifalazil (PROVIDENCE-1) (127,128). Overall, none of the trials demonstrated any long term benefit of antibiotic treatment.

As emphasized prior to the end of trials and revisited in a recent publication in which the authors conclude that the etiology of *C. pneumoniae* in cornary heart disease is a hypothesis that remains untested, the antibiotic trials were not designed as etiological studies (122,129). Thus, regardless as to whether benefical effects were observed or not, any conclusion with regard to a causal role of *C. pneumoniae* in atherosclerotic disease was precluded (122,130). However, the negative outcome of these clinical trials led some to conclude that *C. pneumoniae* has no etiological role and overall, diminished research on the role of infectious agents in atherosclerosis. Other investigators have noted additional considerations in interpretation of the trials (2,47,131–134). These include a) antibiotic treatment was given to patients late in the course of atherosclerotic disease that is unlikely to be altered; b) most of the patients had previously experienced a myocardial infarction and the pathogenesis of myocardial infarction differs from that of atherosclerosis; c) antibiotics may be ineffective in treatment of persistent chlamydial infection (122,134,135); d) a combination therapy may be more effective than monotherapy in treatment as noted in patients with chronic *Chlamydia*-induced reactive arthritis (136); and e) antibiotic treatment might be ineffective due to pathogen burden and differing antibiotic susceptibilities of other infectious agents contributing to atherosclerosis (2,137). Thus, there are significant challenges in designing studies to prove or disprove an etiologic role of individual infectious agents in human atherosclerotic disease let alone addressing the concept of pathogen burden in which complex interactions of more than one infectious agent contributes to atherosclerosis.

Although *H. pylori* has been identified in atherosclerotic plaque, it has not been cultured and, as mentioned above, only a few studies reported the effect of *H. pylori* on atherosclerotic processes in mouse models, which had opposing results. However, two small clinical studies have demonstrated that eradication of *H. pylori* infection in patients had beneficial effects in human atherosclerotic disease. In the first study, treatment of *H. pylori* positive patients undergoing PCTA with antibiotic therapy that eradicated *H. pylori* resulted in a significant reduction in the loss of coronary artery lumen in comparison to the placebo-treated group, as well as a reduction in plasma cytokines including TNF-α, IL-1β and IL-8 (12). In the second study, patients with recent acute coronary symptoms, who were positive for *H. pylori* infection, were treated with either an antibiotic regimen having efficacy against *H. pylori* or with placebo. The treated group had a significant reduction in subsequent coronary events at 6 and 12 months post treatment (138).

In early discussions on the feasibility of vaccine development for *C. pneumoniae*, which was fueled by the association of the organism with cardiovascular disease, a group report noted that the demonstration of efficacy of a *C. pneumoniae* vaccine in the treatment or prevention of cardiovascular disease would provide the best evidence of causality due to the specificity

of the intervention, which would not impact other risk factors or other infectious agents (139). However, clinical trials to demonstrate any effect of vaccination on prevention of atherosclerosis in humans, would take decades to complete. Although trials and surveillance testing a vaccine effective in preventing respiratory tract infection could be conducted in a shorter time frame and yield potential information as to the role of an agent in atherosclerosis, the fundamental issue remained as to whether there would be sufficient benefits to children to justify vaccination because of the mild clinical symptoms of *C. pneumoniae* respiratory infection in this age group (139). To date, the only putative vaccine candidate that has been tested in animal models has been a heptavalent cytotoxic T cell epitope DNA mini-gene vaccine. Interestingly, although the vaccine provided protection against respiratory *C. pneumoniae* infection in both young and older C57Bl/6J mice, the vaccine was partially effective in preventing *C. pneumoniae* dissemination to the cardiovascular system in young mice, but did not impact dissemination in aged mice (140).

The only infectious agent associated with atherosclerosis in which the effect of vaccination on cardiovascular disease has been studied in humans has been Influenza. Individuals with chronic diseases such as CVD are more likely to have severe complications occurring from influenza infections (141–143). Moreover, deaths resulting from influenza infection occur more frequently in patients with cardiovascular disease in comparison to other chronic diseases (141). Thus, various studies have assessed the effects of immunization against influenza on cardiovascular events and several have demonstrated a correlation between influenza vaccination and a reduction in cardiovascular events (143–149). A recent meta-analysis polled data from five randomized controlled trials assessing the efficacy of influenza vaccination in preventing cardiovascular morbidity and mortality with over 292,000 patients; the endpoints studied were myocardial infarction, all-cause mortality and/or major adverse cardiovascular events (150). Overall, influenza vaccination was associated with significant reduction in each endpoint. In a systematic review and meta-analysis of three databases (ranging from 1946–2013) for randomized clinical trials that compared influenza vaccine vs placebo or no vaccination in patients with varying degrees of cardiovascular disease that reported cardiovascular outcome, 6735 patients were included. Influenza vaccination was associated with a lower risk of major adverse cardiovascular events and the greatest association was observed among patients with recent acute coronary syndrome in comparison to patients with stable coronary artery disease (151). As emphasized by the authors, these collective findings have significant public health impact based on the number of individuals that go unvaccinated, the high number of cardiovascular complications associated with respiratory tract infection, and the beneficial effect of vaccination on individuals with high cardiovascular risk (151). The molecular mechanisms by which influenza vaccination exerts the protective effect have not been defined but have been suggested to occur either through avoiding atherosclerotic plaque rupture or other cardiac injury. Alternatively, others have suggested that the protective effect of vaccination occurs by preventing influenza infection, which is known to promote a procoagulant state (152). Interestingly, a study designed to assess the effect of vaccination on atherosclerotic plaque development in hyperlipidemic mice demonstrated that mice receiving influenza vaccine developed smaller lesions with lower lipid content but had increased smooth muscle cells and collagen deposition in comparison to animals receiving a pneumococcus vaccine or

no vaccine (153). Additionally, decreased levels of pro-inflammatory cytokines (IFN-γ, TNF-α, IL10) and higher levels of the anti-inflammatory cytokine IL-4 were noted in the influenza vaccinated group (153). Thus, the authors concluded that influenza vaccination may exert a protective effect by promoting stable atherosclerotic plaques and induction of atheroprotective immune responses. However, this did not negate the concept that protective effects were related to protection against the flu or on vaccine modulation of immune responses (153). An alternative hypothesis that has been considered is that the immune response cross-reacts with a host protein(s) that could affect cardiovascular homeostasis. Evidence has suggested that one potential candidate was the B2 bradykinin receptor (154). In all of these scenarios, why natural infection would not elicit similar responses as vaccination remains to be defined.

Conclusions

Research on the role of infection in atherosclerosis is at a crossroad. Despite the fact that a variety of infectious agents have been found within human atherosclerotic plaques and that causation with regard to atherosclerotic plaque progression has been demonstrated in animal models, there are numerous questions that remain unanswered as to whether infectious agents can be considered as risk factors for cardiovascular disease. These include the following:

1. How can we convincingly demonstrate that the indirect effects of infection contribute to atherosclerosis progression?
2. Why have there been so few examples of successful isolation of viable organisms from atherosclerotic plaques?
3. How can we model the combined effects of independent and simultaneous infections with multiple infectious agents?
4. How do infectious agents contribute to plaque instability and clinical events?
5. Can we fully explain experimentally why the clinical trials with antibiotics failed to protect against cardiovascular disease?

It is hoped that by addressing these and additional questions we can build on the wealth of published data outlined in this review and ultimately help reduce and treat atherosclerosis, the number one cause of mortality worldwide.

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