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# Chlamydia pneumoniae in Cardiovascular Diseases: Risk Factor or Incidental Finding?

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**By**

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**Abstract:** Cardiovascular disease (CVD) is a multifactorial disease that involves the interplay of many risk factors. Traditional risk factors as defined by the American Heart Association fail to account for all instances of atherosclerosis and CVD. Only within the last 100 years have infectious diseases gained prominence as potential risk factors for cardiovascular illnesses. Within the last 50 years, *Chlamydiae pneumoniae,* a pervasive intracellular pathogen, has been implicated in the development of atherosclerosis and CVD. This investigation aimed to clarify the relationship between *Chlamydia pneumoniae* (*C. pneumoniae*) and CVD. This task was accomplished through in-depth research within the literature of PubMed, using Koch's postulates as guidelines to determine the role of *C. pneumoniae* in cardiovascular disease. Findings from this investigation suggest a causative relationship between *C. pneumoniae* and CVD. In addition to traditional risk factors, this investigation found *C. pneumoniae* to be a risk factor in the development of atherosclerosis and ensuing cardiovascular disease.

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## Introduction

According to the American Heart Association, as of 2017, cardiovascular disease remains the number one cause of death within the United States, and includes death by stroke, heart disease, and other cardiovascular illnesses. The American Heart Association reports an astonishing 85.6 million people suffer from ≥1 type of cardiovascular disease, and in 2013, 800,237 deaths in the United States were the direct result of complications due to cardiovascular disease. The 2017 update by the American Heart Association regarding cardiovascular disease in America estimates a financial burden of 316.6 billion dollars[1]. In addition to being the number one cause of death in the United States, cardiovascular disease remains the number one cause of global morbidity[2]. Traditional risk factors for cardiovascular disease include tobacco use, physical inactivity, family history and genetics, metabolic syndrome, diabetes, obesity, high blood pressure, abnormal blood lipid levels, poor nutrition, and chronic kidney disease[1]. Atherosclerosis is a chronic, inflammatory process characterized by constriction of arteries and veins within the cardiovascular system and is the primary pathological mechanism of cardiovascular disease development. Numerous studies have validated traditional risk factors as contributors to atherosclerosis and cardiovascular illness. Within the last 50 years, numerous infectious diseases including Epstein-Barr virus, Cytomegalovirus, *Porphyromonas gingivalis* and *Chlamydia pneumoniae* have

been implicated in the development of atherosclerosis and cardiovascular disease[3].

***Cardiovascular Disease:***

Broadly defined, cardiovascular disease entails diseases of the heart and blood vessels and includes, but is not limited to: coronary artery disease, cerebrovascular disease, peripheral artery disease, rheumatic heart disease, deep vein thrombosis and pulmonary embolism, and congenital heart disease. Globally, cardiovascular disease (CVD) is the number one cause of death, with an estimated 17.9 million deaths per year directly resulting from CVD[4]. Cardiovascular disease is listed as the underlying cause of death in approximately one out of every three deaths within the United States. Additionally, the global financial burden of CVD in 2011 was estimated at $863 billion, and is expected to rise to 1,044 billion dollars by 2030[4]. Cardiovascular diseases are multifactorial, progressive diseases frequently warranting medical treatment when lifestyle changes fail to improve cardiovascular health adequately.

***Heart Attack (myocardial infarction):***

A heart attack occurs when constriction and blockage within the coronary arteries stop the delivery of oxygen-rich blood to the heart muscle, causing death of heart muscle tissue. Myocardial infarction is a life-threatening acute condition requiring immediate hospitalization and medical intervention. There are an estimated 720,000 new cases of heart attack annually in the United States

alongside 335,000 recurrent heart attack episodes. Coronary artery disease (CAD) is defined as the narrowing of the primary arteries that supply the heart with blood. The progressive narrowing of such arteries can lead to a heart attack; every 40 seconds an American will suffer a heart attack. Heart attack and other complications caused by coronary artery disease account for 1 in 7 deaths within the United States[1]. In 2015, heart attacks claimed the lives of 114,023 Americans, with an annual associated treatment cost exceeding 12 billion dollars; making heart attack the most expensive condition to treat within the American health care system[1].

***Stroke:***

Stroke occurs when arteries that supply blood to the brain become obstructed or rupture. Stroke is a leading cause of disability within the United States and kills nearly 133,000 people per year. Similarly, an American will suffer a stroke every 40 seconds, will die of a stroke every 3 minutes and 45 seconds, and in 2015 stroke accounted for 11.8% of the total deaths worldwide[1].

***Hypertension:***

Hypertension is a state of chronic high blood pressure. High blood pressure increases the workload on the heart to push blood through the arteries. The compensatory force can cause damage to the endothelium within the arteries, resulting in a cascade of events that propagates atherosclerosis, the buildup of plaque on the endothelial surfaces, and subsequent constriction of the arteries and veins. Hypertension affects more than 100 million Americans and is

a major contributor to cardiovascular health decline and disease[1]. The estimated direct and indirect costs of treatment in the US for hypertension is 73.4 billion dollars[5]. With such a high cost of life and substantial financial burden, understanding, treating and preventing cardiovascular disease is of paramount importance.

**Figure 1:** Annual death rate per 100,000 in the United States resulting from coronary heart disease, stroke, and total cardiovascular diseases within the United States[1].

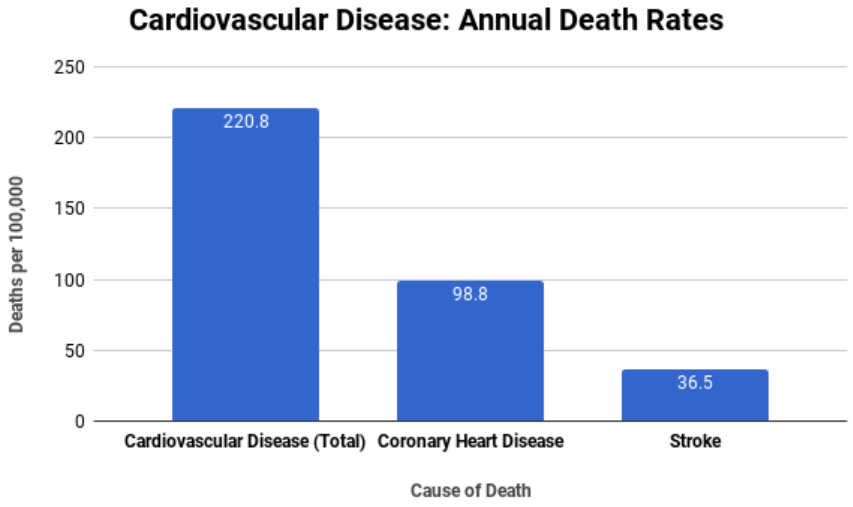
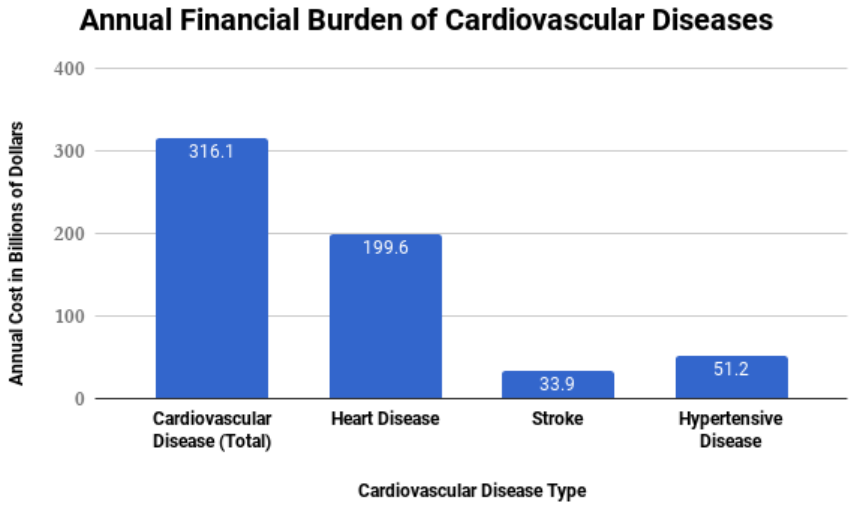



**Figure 2:** Total cost in billions of dollars to treat the major cardiovascular disease types within the United States[1].



### *Ideal Cardiovascular Health and Prevention:*

In 2009, the American Heart Association (AHA) established a set of recommendations, known as impact goals, to reduce the prevalence of cardiovascular disease by 20% before 2020. Additionally, such impact goals aim to reduce deaths caused by cardiovascular diseases and stroke by 2020. Instrumental in the realization of the 2020 impact goals are seven key factors, identified as mediators of cardiovascular health. Such factors are used as guidelines set by the AHA to model ideal cardiovascular health. Each factor is assigned a range of values that are considered the optimal range for cardiovascular health as defined by the American Heart Association. The seven key factors are blood pressure, physical activity, fasting cholesterol levels, diet, weight, tobacco use, and fasting blood glucose levels.

**Table 1:** Seven factors and associated ranges for cardiovascular health as defined by the American Heart Association in conjugation with the Impact Goals of 2020[6]. All data represented are reflective of parameters outlined for adults greater than 20 years of age.

| AHA Metric | Ideal Cardiovascular Health Definition |
|---|---|
| **Tobacco Use** | Never tried or quit >12 months ago |
| **Body Mass Index** | < 25kg/$m^2$ |
| **Physical Activity** | ≥ 150min per week moderate intensity or ≥ 75min per week vigorous intensity or combination |
| **Health Diet Score** | 1) Fruits and Vegetables: ≥ 4.5 cups per day<br>2) Fish: ≥ 3.5 oz per week (oily fish)<br>3) Fiber Rich Whole Grains: ≥ 1.1 g of fiber per 10g of carbohydrates ≥ 3 1oz servings per day<br>4) Sodium: < 1500 mg per day<br>5) Sugar-sweetened beverages: ≤ 450 kcal (36oz) per week |
| **Total Cholesterol (LDL+HDL)** | < 200mg/dl |
| **Blood Pressure** | < 120/ < 80 mmHg |
| **Fasting Plasma Glucose** | < 100 mg/dl |

***Atherosclerosis: The Mechanism of CVD:***

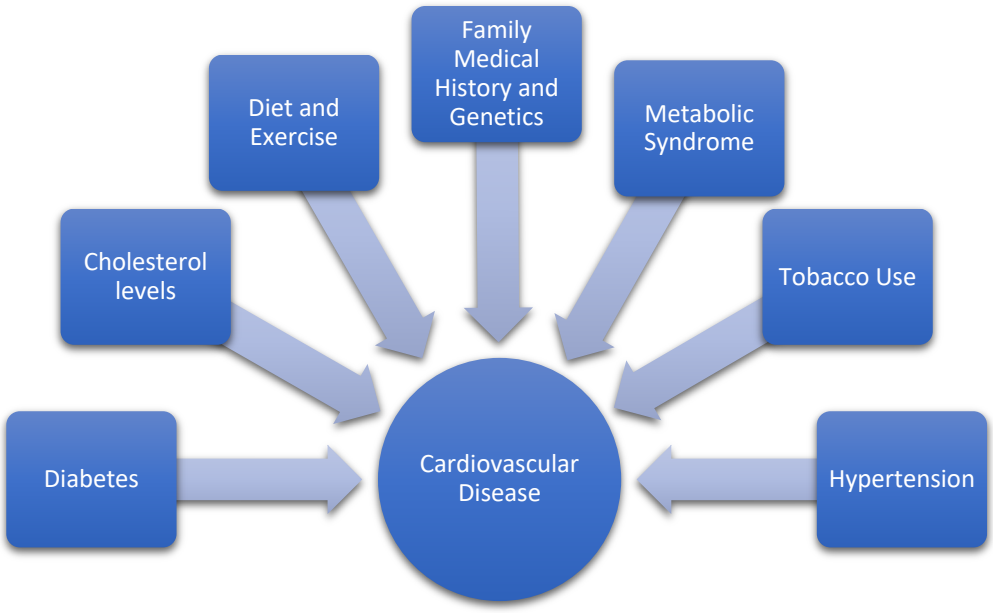
Atherosclerosis is the thickening of arterial walls causing partial or complete blockage of blood flow. Atherogenesis is initiated in response to endothelial damage within the artery, leading to a lesion formation within the endothelium known as a fatty streak. Establishment of a fatty streak is indicative of early-stage atherosclerotic disease, consisting primarily of monocyte-derived macrophages and T-lymphocytes [7]. Cells within the developing lesion release chemical mediators that induce proliferation of smooth muscle cells and formation of an extracellular tissue matrix, initiating a progressive inflammatory response[8, 9]. Oxidized, circulating lipids are internalized via scavenger receptors by macrophages within the lesion, leading to the formation of foam cells[7]. The development of such foam cells promotes recruitment and influx of circulating monocyte-derived macrophages that release cytokines, growth factors and hydrolytic enzymes that further damage the endothelium. The continued proliferation of smooth muscle cells and the evolution of fibrous tissues within the lesion are characteristic of a chronic, more advanced intermediate lesion. The continued release of cytokines, prostaglandins, and other inflammatory mediators further aggravate the endothelium. The end result is a cyclical process of endothelial damage, influx of leukocytes, cytokine and chemical mediator release, foam cell proliferation, lipid accumulation, further endothelial damage and increased inflammation. Over time, chronic inflammation facilitates fibrous tissue formation and the advancement of a large, complex-lesion that

encroaches upon the lumen of the endothelium, initiating the development of cardiovascular disease.

***Cardiovascular Disease and the Current Model of Understanding:***

Risk factors for CVD include diabetes, hypertension, metabolic syndrome, tobacco use, diet and exercise parameters, genetic abnormalities, and infectious diseases [1, 10-12]. Atherosclerosis is an inflammatory disease and the primary pathophysiological mechanism of cardiovascular dysregulation. The current model of the genesis and progression of CVD involves an interplay of independent and associated risk factors. Although the aforementioned traditional risk factors are associated with the development of CVD, they do not account for the total incidence of cardiovascular diseases, generating the need for a broader, more comprehensive understanding[3, 12-14].

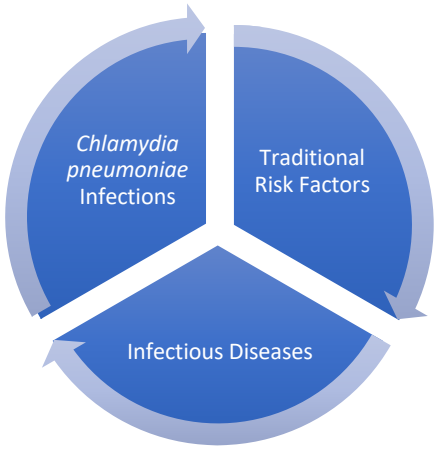
**Figure 3:** Tradition risk factors associated with the development of atherosclerosis and subsequent cardiovascular disease. Such traditional risk factors do not account for the total incidence of cardiac disease[15-18].



***Infectious Diseases and Chlamydia pneumoniae:***

Infectious diseases were first recognized as potential risk factors for the development of atherosclerosis in 1978 when Marek's disease herpesvirus was shown to cause atherosclerosis in chickens[15]. Since that time, numerous bacteria and viruses including *Helicobacter pylori Cytomegalovirus, Porphyromonas gingivalis,* and *Chlamydia pneumoniae (C. pneumoniae)* have been associated with the development or progression of atherosclerosis and CVD[11]. However, such associations are rather loose, and the mechanisms by which such infectious agents play a role in the development, and progression of atherosclerosis and CVD remains unclear. *Chlamydia pneumoniae* was first established as a potential risk factor for CVD when the bacteria were detected in coronary arterial fatty streaks and lesions[16, 17]. Since that time, the organism has garnered considerable attention as another potential risk factor for the development of atherosclerosis and CVD. This paper hypothesizes that there is sufficient evidence within the literature to establish an association between *Chlamydia pneumoniae* and the genesis, progression, sustainment or aggravation of atherosclerosis and subsequent cardiovascular disease.

**Figure 4:** Proposed interplay between traditional risk factors, infectious diseases, and *Chlamydia pneumoniae* infections as independent and associated risk factors contributing synergistically in the development of cardiovascular disease.




## Methods

A systematic and in-depth review of the literature will be conducted within PubMed, regarding *C. pneumoniae* and any important links to cardiovascular disease. This paper will explore known mechanisms involved in CVD, infectious diseases implicated in CVD, Koch's postulates regarding *C. pneumoniae* and CVD. This paper intends to establish and understand the role, if any, played by the infectious disease *C. pneumoniae* in CVD, and demonstrate how such an understanding may lead to potential therapeutic targets and better medical outcomes regarding cardiovascular diseases.

## Literature Review

### *Infectious Disease and Cardiovascular Disease:*

Human cytomegalovirus was one of the first infectious diseases garnering attention as a potential contributor to atherosclerosis when Fabricant et al.

established a link between Herpes virus infections and atherosclerosis via experimental animal models[18]. Further corroboration of the link between human cytomegalovirus (CMV) and atherosclerosis was elucidated through a serological study that demonstrated higher titer concentrations of CMV, herpes simplex virus type-1 (HSV1), and herpes simplex virus type-2 (HSV2) antibodies present in patients undergoing vascular surgery for atherosclerosis[19] than in control patients. The presence of CMV, HSV1 and HSV2 antibodies is consistent with chronic infections that induce inflammatory processes within the vasculature, leading to atherosclerosis. Multiple studies solidified the link between virally induced atherosclerosis and cardiovascular disease[20, 21].

*Helicobacter pylori* is a gram-negative bacterium and the causative agent of gastric ulcers. *H. pylori* has only recently come to light as a possible contributor to the development of cardiovascular disease, as one study indicated a drastic reduction in heart attacks paralleled the reduction of duodenal ulcers caused by *H.pylori*[22]. Serological evidence found a positive association between the presence of *H. pylori* antibodies (HSP) or antibodies respondent to CagA, a virulence factor of *H. pylori*, and an increased risk of the development of atherosclerosis and coronary artery disease. Such studies determined that *H. pylori* infection is an independent risk factor for coronary artery disease [23, 24]. However, such a conclusion has been disputed by experimental results that yielded no such association[25, 26]. The relationship between *H. pylori* infectious burden and cardiovascular disease remains ambiguous.

Oral pathogens have also been linked to cardiovascular diseases, primarily affecting immunocompromised patients. Periodontal disease, an infection in the gums that may result in tooth and bone loss, is a condition fostered by poor oral hygiene and microbial overgrowth. *Actinobacillus actinomycetemcomitans, tannerella forsythensis, Porphyromona gingivalis, Prevotella intermedia,* and *Treponema denticola* are pathogenic organisms that cause periodontal disease[27, 28]. One study by Spahr et al. found that total pathogen burden in periodontal disease was a predictor for a coronary event. Spahr et al. concluded that pathogenic burden (total amount of microbes present) is an independent risk factor for cardiovascular disease. Authors of the study also postulated that the number of *A. actinomycetemcomitans* microbes present within periodontal pockets was also a statistically significant predictor of coronary heart disease[27]. *Porphyromonas gingivalis* is another periodontal pathogen linked to cardiovascular disease. A study conducted by Ghizoni et al. found increased levels of *P. gingivalis* within the gingival pockets of 20 stroke patients, supporting a relationship between *P. gingivalis* and cerebral infarction[28]. Collectively, the majority of studies regarding periodontal disease and cardiovascular illness assert that pathogenic burden within periodontal disease is an independent risk factor for cardiovascular disease[27, 28].

### *Koch's Postulates:*

Koch's postulates are a set of guidelines used to establish a causative relationship between a microorganism and a disease. They are summarized in the table below.

**Table 2:** Definitions of Koch's Postulates.

| **Postulate** | Definition |
|---|---|
| **Koch's 1st Postulate** | Microorganisms should be found in heavy abundance in all disease hosts, and absent in healthy organisms. |
| **Koch's 2nd Postulate** | Microorganism must be isolated from diseased host, and grown in pure culture. |
| **Koch's 3rd Postulate** | Microorganism must cause associated disease when introduced into healthy organism. |
| **Koch's 4th Postulate** | Microorganism must be re-isolated from diseased, experimental host and determined to be identical to original causative agent. |

### *Chlamydia pneumoniae: Infection, Isolation, and Detection:*

*Chlamydia pneumonia* was first isolated from the conjunctiva of a Taiwanese child, and later on in another case, isolated as a respiratory sample from a patient with pharyngitis[29, 30]. The newly acquired pathogenic strain was labeled TWAR, in reference to the first two clinical isolates and was established as the third Chlamydial species[31]. *Chlamydia pneumoniae* is a gram-negative intracellular bacteria responsible for an average of 10% of community-acquired

pneumonia[31].Transmission is believed to occur through direct contact with respiratory secretions from infected hosts. Clinical manifestations of *C. pneumoniae* infection mimic that of a sinus infection and include, malaise, coughing, pharyngitis and slow recovery time[31]; more severe and systemic infections are unusual, but have been documented [32]. Prior to invasion of host cells, *C. pneumoniae* exists as a smaller, biologically inactive extracellular form called an elementary body (EB). Elementary bodies are phagocytosed by host cells and undergo transformation into a larger, replicating form known as an inclusion body[31]. Elementary bodies are pear-shaped when viewed under a microscope and are indicative of *C. pneumoniae* infection. Observation of either elementary bodies via microscopy, or inclusions bodies conjugated with fluorescein, is an effective method of *C. pneumoniae* detection within clinical isolates[31]. Isolation and growth of *C. pneumoniae* as a pure culture has been only mildly successful. Such limited success could be due in part to the rapid decay in viability of *C. pneumoniae* at room temperatiure[33]. Culturing of C. *pneumoniae* has been successful within chicken eggs, and more fastidious strains of *C. pneumoniae* have been grown within HL cells and HEp-2 cell monolayers[31, 34, 35]. The incorporation of cycloheximide, a eukaryotic protein synthesis inhibitor, into culture media has been utilized to inhibit host cell activity and increase *C. pneumoniae* isolation success[31]. Since culturing and isolation of *C. pneumoniae* often fails, other methods for the detection of *C. pneumoniae* have been implemented. The primary serological test for the detection of *C.*

*pneumoniae* is the microimmunofluorescence test (MIF test). The MIF test is able to detect IgM and IgG antibodies specific to *C. pneumoniae,* and is considered positive when there is a fourfold increase in IgA or IgM titers, compared to noninfected individuals. The appearance of IgM and IgG antibodies are slow and generally take 3-4 weeks to be detected[31]. Species-specific antigen detection that uses monoclonal antibodies coupled with fluorescent labels (immunohistochemistry) has been used to detect *C. pneumoniae* within cell culture [31]. Additionally, other antibody detection methods have been applied (ELISA) to detect the presence of *C. pneumoniae*. Polymerase chain reaction (PCR ), utilizing *C. pneumoniae* specific DNA probes, is also used as to detect *C. pneumoniae* from clinical samples and is frequently coupled with serological testing. Multiple DNA probes can replicate rRNA sequences, gene sequences unique to *C. pneumoniae* (MOMP and Omp1 genes), and a 60 kDa cysteine-rich protein of *C. pneumoniae*. Collectively, serological testing (MIF), antigen detection (ELISA), PCR , and the observation of elementary/inclusion bodies are the primary means of detection for *C. pneumoniae*[31]. This investigation will try to oblige by strict parameters outlined by Koch's postulate, however, since isolation of *C. pneumoniae* in pure culture is so difficult, detection with the aforementioned techniques should be considered sufficient when trying to establish Koch's postulates and the relationship between *C. pneumoniae* and atherosclerosis.

***Chlamydia pneumoniae, CVD, and Koch's 1st Postulate:***

To determine if there is enough evidence to satisfy Koch's first postulate, studies were reviewed within this investigation that utilized PCR, immunohistochemistry or microimmunofluorescence as primary detection methods; as such methods are taken to be the most reliable in detecting the presence of *C. pneumoniae* within atherosclerotic lesions and host serum. Davidson et al. conducted a study in which coronary artery tissues were harvested post-mortem from 60 indigenous Alaska Natives and examined for the presence of *C pneumonia*. Subjects were labeled as low-risk for coronary artery disease, and 97% of subjects had died from non-cardiovascular causes[36]. Of note, all of the samples harvested from subjects contained some atherosclerotic tissue, and polymerase chain reaction or ICC detected *Chlamydia pneumonia* within coronary fibro-lipid atheromas in 15 of 60 harvested samples. Additionally, 7 of 60 samples tested positive for the bacterium within early flat lesions of coronary artery tissues. The odds ratio, a quantitative measure of association between exposure and outcome, for *C. pneumonia* in tissues with raised atheroma lesions was assigned a value of 6.1, which asserts that the presence of *C. pneumoniae* is statistically associated with that of raised atheromatous lesions. From the detection of *C. pneumoniae* within atheromas via PCR and ICC, investigators concluded that *C. pneumoniae* present in serum precedes coronary atherosclerosis[36].

Kuo et al. autopsied 36 South African individuals 8-36 hours after death and harvested coronary artery tissues. Samples were collected from individuals who had died from traumatic accidents. Atherosclerotic lesions from coronary samples were analyzed for the presence of *C. pneumoniae* using PCR that amplified 16sribosomal RNA sequences unique to *C .pneumoniae*. Additionally, immunohistochemistry was used to detect *C. pneumoniae* in coronary artery atheromas. *Chlamydia pneumonia* was detected within atheromas by immunohistochemistry in 15 of 36 cases and by PCR in 13 of 30 cases. Further confirmation was attained when six atheroma lesions were analyzed using electron microscopy, and visual confirmation was attained for *C. pneumoniae*-like organisms; such organisms are described as pear shaped[16].

In a compelling study, 105 Iranian patients had undergone coronary bypass grafting and provided coronary artery samples to be analyzed by PCR for the presence of *C. pneumoniae*. Of those patients, 53 non-atherosclerotic mammillary arteries, were also collected and tested for *C. pneumoniae* as controls. Of the 105 patients, 23 patients tested positive for the presence of *C. pneumoniae* in atherosclerotic plaques within coronary samples. Importantly, PCR results were negative in all 53 control mammillary arteries for the presence of *C.pnuemoniae*[37].

Haider et al. searched for *C. pneumoniae* in 63 patients diagnosed with concurrent angina and myocardial infarction. An additional 40 subjects were assessed for the presence of *C. pneumoniae* as healthy controls. Polymerase

Chain Reaction for 16 SrRNA was utilized to detect *C. pneumoniae* within blood samples. Serum samples were analyzed via ELISA for species-specific IgA antibodies as an indirect detection of *C. pneumoniae*. Immunoglobulin-A antibodies were detected in 66.66% of cardiovascular disease patients compared to just 41.37% in control groups. Of 40 cardiovascular disease patients, 32.5% tested positive for the presence of *C. pneumoniae* within the serum using PCR detection analyses, 76.92% of which also tested positive for *C. pneumoniae* IgA antibodies during serological testing[38].

A cross-sectional study conducted in 2015 looked at 85 patients referred for coronary bypass grafting for the presence of *C. pneumoniae*. In this investigation, nesting PCR for Pst1 fragment was utilized as a metric for the presence of *C. pneumoniae*. Importantly, 25 out of 85 patients tested positive using the methods mentioned above for the presence of *C. pneumoniae* within atherosclerotic plaques; this is in comparison to only 5 out of 85 control group patients that were referred for thoracic biopsy[39].

A 2005 study involving 303 persons aimed to determine if *C. pneumoniae* was present more frequently in patients with atherosclerosis compared to healthy adults. Serum samples were obtained from 188 patients with diagnosed atherosclerosis and 115 healthy patients. Samples were analyzed via ELISA for the presence of IgA and IgG *C. pneumoniae* antibodies. For atheroma patients, 63.8% and 49.9% tested positive for IgA and IgG. In non-atheroma patients, 46.9% and 54.8% tested positive for IgG and IgA respectively. A statistically

significant difference was found between the prevalence of C. pneumonia antibodies within atherosclerotic, and non-atherosclerotic patients[40].

In 2004 Sessa et al. utilized semi-nested PCR to determine if *C. pneumoniae* was present in both atherosclerotic and non-atherosclerotic tissues of the coronary arteries harvested at autopsy[41]. Three arterial segments were collected from each of 60 patients, totaling 180 samples. Additionally, 30 healthy coronary arteries were taken as control samples. *Chlamydia pneumoniae* was detectable by PCR in atherosclerotic coronary arteries in 19 of 60 samples examined, and not detectable in any of the 30 non-atherosclerotic [41].

Despite promising results to satisfy Koch's first postulate, there have been numerous studies that have been unable to detect *C. pneumoniae* in atherosclerotic patients. Agmon et al. conducted a study looking at 385 subjects, with atherosclerosis, undergoing transesophageal echocardiography, to evaluate the presence of *C. pneumoniae* IgG antibodies in blood samples [42]. Of the 385 samples taken, IgG specific *C. pneumoniae* antibodies were detected within the serum of only 287 subjects. Fifty-eight patients were found to have low antibody titers, 144 had intermediate antibody titers, and high antibody titers were found in only 85 subjects. After statistical adjustments for confounding variables, it was determined that *C. pneumoniae* antibody titers were not associated with the presence of aortic plaques[42].

Altman et al. assayed *C. pneumoniae* specific IgG antibodies utilizing indirect immunofluorescence detection methods in the serum of 159 (arterial

group) patients with severe arterial disease. Additionally, a second group of 203 patients with heart valve prostheses (valvular group) and no signs of coronary heart disease (CHD) were also included in the study and assayed for *C. pneumoniae* antibodies[43]. Patients of both groups were monitored over the course of 2 years for fatal, non-fatal and systemic thrombotic events. One hundred and seven patients within the arterial group (67.3%), and 120 (59.1%) in the valvular group tested positive for *C. pneumoniae* antibodies. Atman et al. reported 23 patients within the arterial group had fatal or non-fatal vascular events, and only two patients within the valvular group had fatal, or non-fatal vascular events. The prevalence of *C. pneumoniae* antibody was nearly equal in arterial/valvular groups; importantly, the number of clinical events in positive patients (227) and *C. pneumoniae* negative patients (135) was found to be the same. Altman et al. concluded that *C. pneumoniae* infection is not an independent risk factor for arterial disease[43].

Hagiwara et al. tested for the presence of *C. pneumoniae* in atherosclerotic plaques from 50 Japanese patients that had undergone carotid endarterectomy, using ELISA and immunohistochemistry for *C. pneumoniae* [44]. Detection of antibodies IgG or IgA was also determined via blood samples. *Chlamydia pneumoniae* IgG antibodies were detected in 24 (48%) patients within the study. *Chlamydia pneumoniae* IgA antibodies were detected in 29 (58%). Additionally, 8 (16%) patients were immunocytochemically positive for a monoclonal antibody specific for *C. pneumoniae*. Despite seropositivity and

immunohistochemical detection, frequency of stenosis, ulcerative plaques, or symptomatic plaques was not increased in these patients[44]. Hagiwara et al. concluded that *C. pneumoniae* is not a contributor to carotid atherosclerosis within the Japanese population.

***Chlamydia pneumoniae, CVD, and Koch's 2nd Postulate:***

*Chlamydia pneumoniae* is an obligate-intracellular pathogen that infects eukaryotic cells, and a fastidious microbe difficult to isolate and maintain in cell culture[45]. Pharyngeal swabs of *C. pneumoniae* must be transferred immediately to a specialized Chlamydial transport medium and refrigerated, as the cells deteriorate at room temperature[31]. In addition to being a highly fastidious and slow-growing organism, *C. pneumoniae* form smaller inclusion bodies than other Chlamydial species, making culture and identification all the more difficult[31]. Despite these challenges, *C. pneumoniae* has been grown and passed successfully within HEp-2 cells lines.[45] During the course of this review, it has been noted that detection of *C. pneumoniae* in patients with cardiovascular disease has relied exclusively on serological testing, immunohistochemistry, electron microscopy, amplification of 16sRNA, MOMP gene, or other gene sequences specific to *C. pneumoniae* via nested, or semi-nested PCR. Few studies have attempted to directly culture *C. pneumoniae* from the atherosclerotic tissue of patients with cardiovascular disease. This result is most likely due to the challenges of culturing and isolating *C. pneumoniae* in the laboratory. Despite an extensive search within the literature of Pubmed, only two studies reported

successful isolation of viable *C. pneumoniae* directly from atherosclerotic tissues in patients suffering cardiovascular disease[46, 47].

The study by Ramirez et al. aimed to link atherosclerosis and *C. pneumoniae* by asserting, that if a causative relationship between *C. pneumoniae* and atherosclerosis does exist, viable bacteria should be found within atherosclerotic tissue [47]. Ramirez and colleagues collected coronary arteries from explanted hearts of twelve patients undergoing heart transplant surgery. Segments of the coronary arteries were collected and transferred with care via Chlamydial transfer buffer for isolation at three separate laboratories. They report the HEp-2 monolayers were successfully infected with *C. pneumoniae*. Polymerase chain reaction, immunohistochemistry, in situ hybridization, electron microscopy, and serological testing were performed for detection and identification of *C. pneumoniae* from the samples collected. Twelve patients participated in the study, of which ten patients were diagnosed with atherosclerosis of coronary arteries[47]. Importantly, *C. pneumoniae* was isolated from a *single* patient (patient three) using HEp-2 cells as described by Roblin et al[45]. Three culturing sites/laboratories successfully isolated *C. pneumoniae* from samples harvested from the same patient. The isolated bacterium was confirmed as *C. pneumoniae* through species-specific monoclonal antibody detection, detection of inclusion bodies in HEp-2 cells via electron microscopy, and sequencing of the omp1 gene for comparison to published sequences of *C. pneumoniae*.

Jackson et al. accomplished the second successful isolation of *C. pneumoniae* from atherosclerotic tissue[46]. *Chlamydia pneumoniae* was isolated from patients undergoing carotid endarterectomy, a procedure involving removal of large amounts of atheromatous tissue[46]. The study included 25 patients who had undergone carotid endarterectomy, and similar to the findings of Rameriz et al., only a *single* isolation of *C. pneumoniae* was successful from the harvested atherosclerotic tissue. The culture was isolated after six days of incubation and was confirmed as *C. pneumoniae* through species specific-monoclonal antibody reactivity. Successive passage of *C. pneumoniae* in HL cells yielded characteristic inclusion bodies, and further identification was provided via observation of a pear-shaped morphology under electron-microscopy[46]. *Chlamydia pneumoniae* isolated from atherosclerotic plaques by Jackson et al. was confirmed to be identical to clinical respiratory *C. pneumoniae* isolates by DNA hybridization and PCR. The results of the experiment by Jackson et al. further establishes an association of *C. pneumoniae* and cardiovascular disease by demonstrating the presence of viable bacteria within atherosclerotic tissue and assists in satisfying Koch's second postulate.

***Chlamydia pneumoniae, CVD, and Koch's 3rd Postulate:***

Many studies have satisfied a modified version of Koch's third postulate; meaning microbes confirmed as *C. pneumoniae* through microbiological testing, have been inoculated into animal models and demonstrated to induce atherosclerotic lesions. The most significant studies aiming to solidify the

relationship between *C. pneumoniae* and atherosclerosis, and satisfy Koch's 3rd postulate are described below.

Muhlestein et al. utilized direct intravascular inoculation of *C. pneumoniae* into the aortas of rabbits via a drug delivery catheter to test the potential for *C. pneumoniae* to cause atherosclerosis. [48] Inclusion-forming units of *C. pneumoniae* were directly injected into the abdominal wall of the aorta and rabbits were subsequently fed a normal diet and monitored for two months until aortic dissection. Aortas of experimental animals were examined histologically for the presence of *C. pneumoniae* coupled with immunofluorescent antibodies specific to *C. pneumoniae*[48]. Minor, atherosclerotic lesions were observed at the site of inoculation, and in the thoracic aorta. Researchers concluded from these results that *C. pneumoniae* infections are capable of initiating lesions within the aorta[48].

Moazed et al. investigated the role of *C. pneumoniae* in atherogenesis using apolipoprotein (apo) E-deficient transgenic mice that develop atherosclerosis spontaneously, as well as C57BL/6J mice which will do so only when fed an atherogenic diet[49]. Apo transgenic mice are considered an ideal model for investigating the causative role of infectious agents in atherosclerotic development, given that such mice undergo all biological processes observed in atherosclerotic development in the absence of high fat and high cholesterol diets. Sixty-eight male homozygous apoE-deficient mice were intranasally inoculated with *C. pneumoniae* at 8, 10 and 12 weeks of age with standardized volumes of

inoculum. Histological examination of the abdominal aorta at two weeks post-inoculation revealed macrophage adherence to the endothelium, indicative of early-stage atherosclerosis[49]. Histological examination of tissues mentioned above at 4, 6 and 8 weeks post-inoculation revealed lesion progression from fatty streak to early atheromas. C57BL mice, a genetically engineered strain of mice that develop atherosclerosis *only* under an atherogenic diet, were treated under similar experimental conditions. Immunohistochemistry, PCR, and isolation of *C. pneumoniae* at different tissue sites were used to determine the affinity of *C. pneumoniae* for atheromatous lesions. Moazed et al. determined that long-term persistence of *C. pneumoniae* in the developing lesions within aortas of apoE-deficient mice, and not in C57BL mice, is evidence of a strong affinity between *C. pneumoniae* and atheromatous lesions.[49]

Moazed et al. conducted an additional experiment to confirm the results found in the experiment mentioned above. Again, the ability *Chlamydia pneumoniae* to contribute to atherosclerosis in an apoE-deficient mouse was investigated[50]. Twenty-one experimental animals were inoculated intranasally with standardized inclusion forming units of *C. pneumoniae* 3 times, at 8, 9 and 10 weeks of age. Eighteen control animals were identically inoculated using sterile PBS. Atherosclerotic lesions were observable along the inner curvature of the aortic arch in both experimental and control animals. In the experimental group, lesions were observed to be 2.4-fold greater in size 8 weeks after the first inoculation and 1.6-fold greater in size at 20 weeks compared to control

animals[50]. Moazed et al. asserted that such results clearly show that *C. pneumoniae* contributes to the progression of atherosclerosis in apoE-deficient mouse models[50].

A study conducted by Campbell et al. reported similar results using apoE-deficient mice inoculated with *C. pneumoniae* [51]. Mice of the C57BL/6J line were fed a normal diet and inoculated with sterile SPG medium for comparison as control animals. Mice were inoculated with *C. pneumoniae* intranasally using standardized volumes of inclusion-forming units either singly, or at multiple time points. Animals within the multi-inoculation experimental group received inoculations at 8, 10 and 12 weeks, this same schedule was maintained for C57BL/6J control animals receiving inoculations of sterile SPG medium. Computer-assisted morphometric analysis was utilized to assess the size of observed atherosclerotic lesions. Lesions within the aorta of repeat inoculation treatment in apo-E deficient mice were observed to be 140% larger 6 weeks post-inoculation, compared to apo-E deficient mice receiving a single inoculation. No atherosclerotic lesions were detected within C57BL/6J controls.

Fong et al. conducted an experiment that inoculated twelve New Zealand White rabbits via the nasopharynx with *C. pneumoniae* and five control rabbits with carrier buffer[52]. Ten of 11 experimental animals exhibited serological evidence of infection, in contrast with control animals that showed no seroconversion. Experimental animals were sacrificed on days 7,14, 21 and 28 where controls animals were sacrificed on days 7 and 14. Importantly,

histological examination of two rabbits within the experimental group showed early signs of atherosclerosis. The first animal was sacrificed day seven post inoculation and showed accumulation of foamy macrophages in the aortic arch[52]; such findings are consistent with an early-stage atherosclerotic lesion in the form of a fatty streak. The second experimental animal showing signs of atherosclerosis was sacrificed day 14 post-inoculation and showed spindle cell proliferation of smooth muscle cells, indicative of intermediate lesion formation. All histopathological assessments were reported as negative for such lesions within control animals[52].

In a separate study, Fong et al. showed the ability of *C. pneumoniae* to induce atherosclerosis in rabbits suffering from respiratory infections of *C. pneumoniae*[53]. In this experiment, 23 animals were infected with *C. pneumoniae* through the nasopharynx using a catheter and standardized test inoculums. A total of thirty-six control animals were included in the study and inoculated with sucrose phosphate glutamic acid (SPG) buffer (n=24) or HEp-2 cells and SPG buffer (n= 12)[53]. Of interest, atherogenic changes were observed in the aortas of 6 of 23 experimental animals just three months after initial inoculation. A second experimental group (n=24) was inoculated with *C. pneumoniae*, as described above, three times within six weeks. It was noted upon histological examination via light microscopy that within six weeks-time, the multiple inoculation experimental group had grade III atherosclerotic lesions in 8 of 23 rabbits. Atherosclerotic changes within controls animals inoculated with HEp-2 cells and

carrier broth were not observed after three months of inoculation. Fong et al. concluded from this result that *C. pneumoniae* may be important in the pathogenesis of atherosclerosis in humans[53].

Despite the overwhelming majority of experimental results indicating a positive association between *C. pneumoniae* and atherosclerosis, a few studies have been unable to replicate such findings. Caligiuri et al. experimented using ApoE-KO mice, and C57BL/6J mice as controls, infecting both groups intranasally with live *C. pneumoniae* and looking at the downstream effects on arterial atherosclerosis [54]. Thirteen ApoE-KO mice were infected once at age eight weeks (n=13), and another subgroup of mice was inoculated a second time (n=14) at age 16 weeks. Control C57BL6/J mice were inoculated once at age 8 weeks (n=8). All animals were fed a normal chow diet for 22 weeks after primary infection. Polymerase chain reaction and ELISA were utilized to confirm successful infection and a humoral immune response[54]. Aortas of all animals were harvested, dissected, and atherosclerotic lesions were analyzed using blinded lesion quantification as described[55]. Comparative analyses of atherosclerotic lesions within the aortas between ApoE-KO groups and control C57BL6/J group revealed no statistically significant differences[54]. It was also noted that *C. pneumoniae* infection within wild-type C57BL6/J mice was unable to produce significant atherosclerotic changes. Caligiuri et al. determined from these results that *C. pneumoniae* does not induce atherosclerosis in wild-type mice, nor is it capable of accelerating atherosclerosis in ApoE-KO mice[54].

Aalto-Setala et al. conducted a similar experiment using an ApoE-deficient mouse, fed low or high-fat diets, inoculated intranasally with *C. pneumoniae* 3 times at one-week intervals [56]. Ten control animals and ten experimental animals inoculated with *C. pneumoniae* were kept on a regular chow diet. Additionally, six control and four *C. pneumoniae* inoculated animals were fed a high-fat diet. Control and experimental animals from both high-fat and low-fat diet groups were sacrificed at 10 weeks post-initial inoculation. Aortic lesions from all diets, experimental groups, and control groups samples were histologically characterized for size. Importantly, it was noted that there was no observable or significant difference in aortic lesion size in *C. pneumoniae* infected or noninfected control mice from either low fat or high fat diets[56]. Aalto-Setala et al. concluded that *C. pneumoniae* infection does not accelerate atherogenic changes in experimental ApoE-deficient mouse models[56].

***Chlamydia pneumoniae, CVD, and Koch's 4th Postulate:***

Koch's 4th postulate involves re-isolating the organism suspected of causing disease, from an experimentally infected animal inoculated with the suspect organism, manifesting the associated disease state. However, detection via PCR, immunohistochemistry, serological testing, or ELISA, as opposed to isolation, within the atherosclerotic tissues was observed in the majority of the aforementioned studies [16, 36-43]. Despite an extensive search, there are no currently published studies that truly satisfy Koch's 4th postulate in regards to *C. pneumoniae* being the causative agent of atherosclerosis.

## Results

### *Chlamydia pneumoniae, CVD, and Koch's 1st postulate:*

Koch's 1st postulate is one of the most logical steps in confirming a suspect organism as the causative agent of a particular disease. During this investigation, and an attempt to satisfy Koch's 1st postulate, ten primary investigations found within Pubmed were read in-depth and analyzed for relevant information. The studies encompass a broad range of research parameters and techniques, but similar in the attempts to detect *C. pneumoniae* within atherosclerotic tissue. The results of these investigations is summarized in the table below:

**Table 3:** Summarized findings of studies showing detection of *C. pneumoniae* in atherosclerotic tissue and/or serum. Of note, studies that utilized PCR to detect *C. pneumoniae* within atherosclerotic tissues; For the satisfaction of Koch's 1st postulate, PCR is the most definitive in identifying *C. pneumoniae* within atherosclerotic tissue and elucidating the strong affinity of *C. pneumoniae* for atheromas. CABG; *Coronary artery bypass grafting.

| Primary Authors | Sample Size | Tissue Collected | Detection Methods | Results/Findings/ Association |
|---|---|---|---|---|
| **Sessa et al[41].** | 60 subjects with aortic atherosclerosis<br><br>30 (controls non-atherosclerotic tissue) | atherosclerotic tissue within coronary arteries | *Semi-nested PCR | *C. pneumoniae* DNA detected in 31% (of 60)<br><br>*No *C. pneumoniae* DNA detected within controls<br><br>**(+) Association between *C. pneumoniae* and atherosclerosis** |
| **Davidson et al[36].** | 60 (low risk for coronary artery disease) | Atherosclerotic tissue within coronary arteries | ICC *C. pneumoniae* specific monoclonal antibodies<br><br>*PCR | *C. pneumoniae* detected in raised atherosclerotic lesions-15 subjects<br><br>*C. pneumoniae* found within flat lesions-7 subjects<br><br>**(+) Association between *C. pneumoniae* and atherosclerosis** |
| **Cho-Chou Kuo[17].** | 36 subjects | Atherosclerotic tissue within coronary arteries | ICC<br><br>*PCR<br><br>Electron microscopy | *C. pneumoniae* detected by ICC in 15 of 36 subjects<br><br>*C. pneumoniae* detected in 13 of 30 by PCR<br><br>Elementary bodies of *C. pneumoniae* found in 6 of 21 harvested atheromatous plaques<br><br>**(+) Association between *C. pneumoniae* and atherosclerosis** |
| **Agmon et al [42].** | 385 subjects | Aortic plaque detection via transesophageal Echocardiography | Serology<br><br>Species-specific immunoglobulin titers | *C. pneumoniae* detected in 287 subjects<br><br>*C. pneumoniae* not associated with aortic plaques<br><br>**(-) Association between *C. pneumoniae* and atherosclerosis** |
| **Altman et al[43].** | 159 patients (severe arterial disease)<br><br>203 patients (heart valve prosthesis | Serum samples | Indirect immunofluorescence | *C. pneumoniae* detected in 107 patients in arterial group<br><br>Detected in 120 valvular group<br><br>Clinical events for *C. pneumoniae* (+) patients same as (-)<br><br>**(-) Association between *C. pneumoniae* and coronary artery disease** |

| Primary Authors | Sample Size | Tissue Collected | Detection Methods | Results/Findings/ Association |
|---|---|---|---|---|
| **Hagiwara et al[44].** | 50 samples | Carotid atherosclerotic plaques | *PCR<br><br>ICC<br><br>ELISA | *C. pneumoniae* specific IgA antibody detected in 24 samples, 29 for IgG<br><br>*C. pneumoniae* detected in 8 samples via ICC<br><br>No *C. pneumonia* DNA detected by PCR within plaques<br><br>**(-) Association between *C. pneumoniae* and atherosclerosis.** |
| **Podsiadly et al[40].** | 188 patients with atherosclerosis<br><br>115 patients without atherosclerosis | Serum | ELISA<br><br>*PCR | IgG and IgA *C. pneumoniae* specific antibodies detected in 63.8% and 49.0% atheroma patients,<br><br>46.9% and 54.8% patients without atherosclerosis<br><br>*C. pneumoniae* DNA detected via PCR in atheromatous plaques more than in vessels lacking atheromatous lesions<br><br>**(+) Association between *C. pneumoniae* and atherosclerosis** |
| **Asar et al[39].** | 85 patients with CAD | Atherosclerotic tissue within coronary arteries | *PCR | *C. pneumoniae* detected in 25 out of 85 atherosclerotic tissues via PCR and 5 out of 85 in control tissues.<br><br>**(+) Association between *C. pneumoniae* and atherosclerosis** |
| **Haider et al[38].** | 63 cardiovascular disease patients<br><br>40 healthy control patients | Serum | ELISA<br><br>*PCR | IgA antibodies for *C. pneumoniae* detected in 42 out of 63 cardiovascular disease patients and 12 out of 40 in controls.<br><br>*C. pneumoniae* DNA was detected via PCR was positive in 20 out of 63 cardiovascular disease patients and none in controls.<br><br>**(+) Association between *C. pneumoniae* and inflammation** |
| **Izadi et al[37].** | 105 patients undergoing *CABG<br><br>53 mamillary artery samples | Atherosclerotic tissue within coronary arteries | *PCR | *C. pneumoniae* DNA detected in 23 of 105 patients with coronary artery atherosclerosis and none found in mamillary artery controls<br><br>Significant portion of coronary atherosclerotic plaques are infected with *C. pneumoniae* while no such infection was found in controls.<br><br>**(+) Association between *C. pneumoniae* and atherosclerosis** |

### *Chlamydia pneumoniae, CVD, and Koch's 2nd postulate:*

Koch's 2nd postulate entails the isolation, in the form of pure culture, of the suspect organism from a diseased host. After intensive research within the literature of PubMed, we determined that the isolation of *C. pneumoniae* from atherosclerotic tissue has only been successfully accomplished twice. The low success rate is due in part from the fastidious nature of *C. pneumoniae* and the technical skills required for successful isolation. The findings/results of the two studies are summarized in the table below:

**Table 4:** Summary of results and findings from primary investigations able to isolate *C. pneumoniae* within atherosclerotic tissues.

| Primary Author | Sample Size | Tissue Collected | Isolation Method | Results/Findings/Associations |
|---|---|---|---|---|
| **Ramirez et al[47].** | 12 patients seeking heart transplantations | Atherosclerotic plaque from coronary artery | Characterization of isolates: PCR, immunohistochemistry, transmission electron microscopy<br><br>Culture in HEp-2 Cell monolayers. | Bacterial culture grown in Hep-2 cell monolayer-successful isolation from 1 of 12 patients/tissue samples<br><br>Isolate confirmed as *C. pneumoniae* via species-specific antibody reactivity, transmission electron microscopy/observable elementary bodies, and PCR.<br><br>**(+) Association of *C. pneumoniae* and atherosclerosis** |
| **Jackson et al[46].** | 25 patients undergoing Endarterectomy | Atherosclerotic tissue from carotid artery | Characterization of isolate: PCR, southern hybridization analysis, reactivity with species-specific monoclonal antibodies, electron microscopy.<br><br>Culture in HeLa cell monolayers | Successful characterization and a single isolation in 1 of 25 samples.<br><br>Isolate confirmed as *C. pneumoniae* via PCR, southern hybridization analysis, reactivity with species-specific monoclonal antibodies. Importantly, electron microscopy detected elementary bodies present within monolayer, consistent with *C. pneumoniae*.<br><br>**(+) Association of *C. pneumoniae* and atherosclerosis** |

### *Chlamydia pneumoniae, CVD, and Koch's 3rd postulate:*

Koch's 3rd postulate requires the inoculation of the suspect organism into an experimental host and the genesis of disease. In this investigation, multiple

studies demonstrated the ability of *C. pneumoniae* to induce atherosclerosis in experimental animal models. The findings/results of the analyzed investigations are summarized in the table below.

**Table 5:** Summary of research findings from studies analyzed aiming to demonstrate the atherogenic ability of C. pneumoniae within animal models.;*ApoE; apolipoprotein E deficient mice. *FVB mice; Friend leukemia virus B mouse. *C57BLK/6J; common inbred strain of laboratory mouse.

| Primary Authors | Sample Size | Animal Models | Tissue Collected | Detection Methods for *C. pneumonia*/ Atherosclerosis | Results/Findings/ Interpretations |
|---|---|---|---|---|---|
| **Campbell et al[51]** | *ApoE KO = 43<br><br>*C57BL/6J =18 | ApoE-KO<br><br>C57BL/6J | Aorta | C. pneumoniae: ICC and PCR<br><br>Atherosclerosis: Computer-assisted morphometric analysis of lesion area | *C. pneumoniae* has a tropism to developing lesions present in progressive atherosclerotic models[51].<br><br>**(+) *C. pneumoniae* can further aggravate atherosclerosis** |
| **Aalto-Setala et al[56].** | n/a | Apo-E KO<br><br>*FVB | Aortic root | *C. pneumoniae*: PCR<br><br>Atherosclerosis: histological interpretation | *C. pneumonia* infection did not influence lesion size in either mouse strain[56].<br><br>**(-) Association: *C. pneumoniae* not capable of inducing or aggravating atherosclerosis** |
| **Fong et al[53].** | 46 experimental animals 92 control animals | New Zealand White Rabbits | Aorta | *C. pneumoniae:* Immunohistochemistry, serological testing<br><br>Atherosclerosis: Light microscopy and lesion classification | *C. pneumoniae* found to induce atherosclerosis in rabbits[53]<br><br>**(+) Association: *C. pneumoniae* can be atherogenic in animal models** |
| **Fong et al[52].** | 12 experimental animals<br><br>5 controls | New Zealand White Rabbits | Lungs, liver spleen, aortic arch | *C. pneumoniae:* Serology and immunohistochemistry<br><br>Atherosclerosis: Histological interpretation | Rabbits within experimental groups demonstrated early, intermediate lesions not found in control animals[52]<br><br>**(+) Association: *C pneumoniae* can be atherogenic in animal models** |
| **Caligiuri et al[54].** | 43 experimental animals<br><br>8 controls | ApoE-KO<br><br>C57BLK6/J | Root of aorta and | *C. pneumoniae*: ELISA and PCR<br><br>Atherosclerosis: Histology interpretation | No statistical differences in aortic atherosclerotic lesions between C. |

| Primary Authors | Sample Size | Animal Models | Tissue Collected | Detection Methods for *C. pneumonia*/ Atherosclerosis | Results/Findings/ Interpretations |
|---|---|---|---|---|---|
| **Caligiuri et al[54]. continued** | | | | | pneumoniae infected animals and controls[54].<br><br>**(-) Association: *C. pneumoniae* not capable of inducing or aggravating atherosclerosis** |
| **Moazed et al[50].** | n/a | Apo-E KO | Aorta (curvature of aortic arch) | *C. pneumoniae:* n/a<br><br>Atherosclerosis: morphometric analysis | Results show that *C. pneumoniae* infection accelerates progression of atherosclerosis in Apo-E KO mouse models[50].<br><br>**(+) Association: *C pneumoniae* can be atherogenic in animal models** |
| **Moased et al[49].** | 68 ApoE-KO<br><br>74 C57Bl/6J | ApoE-KO<br><br>C57BLK6/J | Lung, Spleen, Ascending and abdominal aorta | C. pneumoniae: Serology and PCR<br><br>Atherosclerosis: Histological interpretation | *C. pneumoniae* were detected in the aorta 2 weeks after a single inoculation; suggestive of a tropism of C. pneumoniae to atherosclerotic lesions[49]<br><br>**(+) Association: *C. pneumoniae* may be atherogenic in animal models** |
| **Muhlsestein et al[48].** | n/a | Rabbits | Aorta | *C. pneumoniae*: Direct immunofluorescence<br><br>Atherosclerosis: Histological interpretation | *C. pneumoniae* infection is capable of aortic lesions where they do not otherwise occur[48].<br><br>**(+) Association: *C. pneumoniae* may be atherogenic in animal models** |

### *Chlamydia pneumoniae, CVD, and Koch's 4th postulate:*

Koch's 4th postulate entails the re-isolation, in pure culture, of the infectious organism from an experimentally infected animal. Despite extensive research within the literature in PubMed, no such experiment or process was found within the literature.

## Discussion

The goal of this paper was to use Koch's postulates as *guidelines* to determine if *C. pneumoniae* is a *risk factor* for cardiovascular disease. Exposure to *C. pneumonia* is common and the presence of *C. pneumoniae* antibodies within serum does not necessarily indicate infection. For this reason, many of the studies reviewed in this investigation utilized *C. pneumoniae* antibody detection in conjunction with other detection methods. Polymerase chain reaction is considered a reliable method for detection of *C. pneumoniae* within atherosclerotic tissues.

**Koch's 1st postulate: *Microorganisms should be found in heavy abundance in all disease hosts, and absent in healthy organisms*.** In alignment with Koch's 1st postulate, the results of studies analyzed in this investigation demonstrate a high prevalence of *C. pneumoniae within atherosclerotic tissue.* Kuo et al. found an association through detection of *C. pneumoniae* in atherosclerotic tissue using PCR, ICC and electron microscopy that revealed pear-shaped bodies within atheromatous tissues[17]; the finding of pear-shaped bodies is indicative of viable bacteria living within host tissue. The affinity of *C. pneumoniae* for atherosclerotic tissue was demonstrated by Izadi et al. using PCR to detect *C. pneumoniae* within atherosclerotic tissue harvested from coronary arteries. *Chlamydia pneumoniae* DNA was detected in 21.9% of samples tested and *was not found in control samples*[37]. The results reported by Izadi et al. were similar to those reported by Assar et al. who used PCR to detect

*C. pneumoniae* within atherosclerotic, and non-atherosclerotic tissues. *Chlamydia pneumoniae* was found in 25 of 85 atherosclerotic tissue samples compared to just 5 of 85 non-atherosclerotic tissue samples[39]. Sessa et al. also used PCR to detect *C. pneumoniae* within atherosclerotic tissues samples and found *C. pneumoniae* DNA present in 19 of 60 samples; *no such DNA was found in 30 non-atherosclerotic control samples*[41]. In total, seven of ten studies reviewed detected statistically significant greater numbers of *C. pneumoniae* within cardiovascular disease groups or tissues, compared to controls. Five of the seven above studies successfully detected *C. pneumoniae* directly within atherosclerotic tissues via PCR [17, 36, 37, 39, 41] . However, three of the studies reported no such detection compared to non-atherosclerotic controls[37, 38, 41] .

Further evidence of the association between *C. pneumoniae* and atherosclerosis was provided by serological data collected by Podsiadly et al. using ELISA for IgA and IgG antibodies to the bacteria[40]. Serum samples were collected from atherosclerotic patients and compared with that of non-atherosclerotic patients for the presence of species-specific antibodies. The prevalence of *C. pneumoniae* antibodies was reported as statistically greater within atherosclerotic patients when compared to non-atherosclerotic patients[40]. The results of Podsiadly et al. again show the association of *C. pneumoniae* with atherosclerosis.

In total, of the ten studies relevant to Koch's 1st postulate that were reviewed, *C. pneumoniae* DNA was detected within atherosclerotic tissue

samples in 195 of 641 tested samples, equivalent to 30.42%. Analysis of this review reveals, in total, *C. pneumoniae* was detected in atherosclerotic tissue by electron microscopy in 6 of 21 samples[17], by serology in 287 of 385 serum samples[42], by ICC in 38 of 146 samples[17, 36, 44], and by indirect immunofluorescence in 107 out of 159 samples[43]. Additionally, in total, IgA antibodies were detected via ELISA in 158 of 301 atherosclerotic samples. Similarly, analysis of all ten studies revealed total IgG antibodies detection via ELISA in 149 of 238 atherosclerotic samples[38, 40, 44]. Although the above values do not illustrate a causative relationship, it nonetheless should be noted that *C. pneumoniae* and atherosclerosis are frequently found together.

Three of ten studies analyzed relevant to Koch's 1st postulate asserted that *C. pneumoniae* was not associated with atherosclerotic tissues[42-44]. It is hypothesized that this lack of association is due to technical difficulties in harvesting samples, limited sample sizes, laboratory errors in detection, or factors unbeknownst to researchers.

The majority of aforementioned studies detected *C. pneumonia* within atherosclerotic tissues compared to nonatherosclerotic controls, and found a positive association between *C. pneumoniae* and atherosclerotic tissue. Because *C. pneumoniae* was not found within every occurrence of atherosclerosis, and was found in some healthy individuals, satisfying of Koch's 1st postulates is not possible. This is not unexpected, especially when considering the multifaceted process, and associated multitude of risk factors, involved in the development of

artherosclerotic plaques. However, the evidence provided, and frequent detection of *C. pneumoniae* within atherosclerotic tissue, clearly shows the affinity of *C. pneumoniae* for atherosclerotic tissue not to be a random occurrence, but an associative phenomenon, and suggests infection with *C. pneumoniae* is indeed a risk factor for CVD.

**Koch's 2nd postulate: *Microorganism must be isolated from diseased host, and grown in pure culture*.** As previously mentioned, *C. pneumoniae* is a very difficult organism to grow in the laboratory, and therefore it may be challenging to provide considerable evidence that supports Koch's 2nd postulate regarding *C. pneumoniae* and cardiovascular disease. However, a search of the literature clearly shows that although arduous, isolation for *C. pneumoniae* from a diseased organism is possible[46, 47]. One must also consider the overwhelming amount of *C. pneumoniae* detected via PCR, ELISA, immunohistochemistry, and serological methods as an indication that viable organisms are present within the diseased host tissues. The evidence within the literature demonstrates that isolation of *C. pneumoniae* from a diseased host is possible as outlined by Koch's 2nd postulate.

**Koch's 3rd postulate: *Microorganism must cause associated disease when introduced into healthy organism.*** Koch's 3rd postulate is perhaps the most significant in asserting that a suspect infectious agent is capable of causing disease. The postulate states that the inoculum must come from a pure culture isolated from an infected host, be inserted into a healthy experimental host, and

cause disease. However, given the limited number of studies that have been able to isolate *C. pneumoniae* and grow it in pure culture, a modified version of this postulate must be considered for this analysis; any viable culture of *C. pneumoniae* will suffice to serve this purpose. This investigation analyzed a total of eight studies that aimed to uncover the atherogenic capabilities of *C. pneumoniae* in animal models. Fong et al. inoculated a total of 58 New Zealand White rabbits with *C. pneumoniae* and compared these to 97 control animals; Fong et al, successfully demonstrated the atherogenic capability of *C. pneumoniae* in rabbit models [53, 57]. Additionally, a total 154 ApoE-deficient mice in three of the aforementioned studies were inoculated with *C. pneumoniae* under varying, but similar, conditions and compared to 100 control animals; all three studies found *C. pneumoniae* to be atherogenic when compared to control animals[49, 51, 54]. In total, six of the eight studies presented definitive evidence of *C. pneumoniae* as capable of inducing or aggravating atherogenesis in animal models when compared to controls[48-53].

Research conducted by Caligiuri et al. was unable to reproduce the atherogenic capabilities of *C. pneumoniae* within Apo-E deficient mice[54]; research conducted by Aalto-Setala et al. yielded similar results[56]. It is difficult to ascertain why *C. pneumoniae* was unable to produce or aggravate atherogenesis in experimental conditions so similar to the successful studies described above. One possibility could be differences in the metabolic activity of the bacterium at the time of inoculation. Bacteria in the exponential phase of growth are most

metabolically active and capable of causing infection. It is possible that at the time of inoculation, *C. pneumonia* was not at peak metabolic activity, and therefore unable to gain a foothold within the host. The expression of virulence factors also plays a significant role in the success of an infectious agent. It is possible that important virulence factors where not being expressed at the time of inoculation, unbeknownst to researchers. A future study that confirmed genetic expression profiles using microarrays before inoculation may be warranted to ensure successful infection; this could bring uniformity to the experimental results. Taken together, the experimental results within those studies mentioned above demonstrate the atherogenic ability of *C. pneumoniae* within animal models, and correspond with the criteria outlined by Koch's 3rd postulate.

**Koch's 4th postulate:** ***Microorganism must be re-isolated from diseased, experimental host and determined to be identical to original causative agent.*** As previously stated, despite an extensive search within the literature of Pubmed, no experiments were found that would correspond to Koch's 4th postulate. Such a task would entail inoculating a healthy host with *C. pneumoniae*, inducing measurable and statistically significant occurrences of atherosclerosis, and re-isolating the *C. pneumoniae* in pure culture. The challenges associated with isolating *C. pneumoniae* in pure culture make this task unlikely to succeed. However, establishing a causative relationship between *C. pneumoniae* and atherosclerosis/cardiovascular disease is not dependent on the starkly demarcated criteria of Koch's postulates, but upon the interpretation of

experimental results that use Koch's postulates as guidelines to establish a causative relationship.

Another important consideration when interpreting the results of each study, and the conclusions of this paper, is the possibility that initial infections by *C. pneumoniae* may initiate a cascade of immune responses within the infected host. The downstream of effects of such elicited immune responses could include, among other things, the genesis or aggravation of atherosclerosis. As with many infectious diseases, the immune system can respond in ways that are damaging to the body and are not always overtly obvious to researchers. With this consideration in mind, it is possible that atherosclerosis is a secondary consequence of infection by *C. pneumoniae* and not, a direct result of *C. pneumoniae* infection, and thus can occur after the C. pneumoniae is no longer present.

From around 1990 to 2005, *Chlamydia pneumoniae* was at center stage regarding biomedical research concerning the role it may play in cardiovascular disease. After 2005, there was stark drop-off in research conducted to establish a causative relationship between *C. pneumoniae* and CVD. It is possible that this drastic reduction was due to the fact there were hundreds of experiments that at a minimum, detected *C. pneumoniae* within atherosclerotic tissue and concluded that the two may therefore be associated with one another. It is possible, that from a clinical perspective, there has been enough research to support *C. pneumoniae* infection as a plausible risk factor for atherosclerosis and CVD, and

therefore no more research is warranted to further solidify the relationship between *C. pneumoniae* and CVD.

Within this investigation, *C. pneumoniae* was shown to be present in abundance in atherosclerotic tissues and patients with cardiovascular disease[17, 36-41]. Although in a limited capacity, *C. pneumoniae* has been isolated in pure culture from hosts who have atherosclerosis and cardiovascular disease. Additionally, *C. pneumoniae* demonstrated the ability to induce or aggravate atherosclerosis within animal models [46-53]. This investigation demonstrated a an association between *C. pneumoniae* and atherosclerosis/CVD through the use of Koch's postulates as guidelines. Future directions should include improving isolation techniques by assessing the efficacy of different mammalian cell lines in the passage of *C. pneumoniae*. Such an assessment may lead to improved passage techniques and congruence with Koch's 4$^{th}$ postulate. Future studies should also include antibiotic treatment of *C. pneumoniae* within animal models to determine if prophylactic treatment is warranted to attenuate the atherosclerotic process. Analysis and review of all experimental results summarized in this study suggest that *Chlamydiae pneumoniae should be considered a risk factor for atherosclerosis and cardiovascular disease*.

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