Comprehensive Summaries of Uppsala Dissertations
from the Faculty of Medicine 1168

Chlamydia pneumoniae in Aortic Valve Sclerosis and Thoracic Aortic Disease

Aspects of Pathogenesis and Therapy

BY

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ACTA UNIVERSITATIS UPSALIENSIS
UPPSALA 2002

Dissertation for the Degree of Doctor of Medical Science in Infectious Diseases presented at Uppsala University in 2002

ABSTRACT

Nyström-Rosander, C. 2002. *Chlamydia pneumoniae* in Aortic Valve Sclerosis and Thoracic Aortic Disease. Aspects of Pathogenesis and Therapy. Acta Universitatis Upsaliensis. *Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicin*e 1168. 65pp. Uppsala. ISBN 91-554-5356-2

The obligate intracellular bacterium *Chlamydia pneumoniae (Cp*), a common human pathogen, has been associated with atherosclerotic cardiovascular disease. The aetiology of non-rheumatic aortic valve sclerosis has, however, not been clarified. In two prospective studies of 42 and 46 patients undergoing surgical valve replacement because of aortic valve stenosis, the presence of *Cp* DNA could be demonstrated by polymerase chain reaction (PCR) in 49% and 35% of the sclerotic valves as compared to 9 % and 0%, respectively, of valves from forensic control cases with no heart valve disease. Some inflammatory and infectious diseases are associated with trace element changes. Eleven of 15 trace elements showed changed concentrations in sclerotic valve tissue compared to control valves in support of an active process in the sclerotic valves. Notable was an increased iron concentration in the patients´ valves suggesting a possible link to *Cp*. Furthermore, a disturbed trace element balance existed in the patients´ sera, the pattern of which was compatible with ongoing infection. In a prospective study of 38 patients operated on for thoracic aortic aneurysm or dissection, *Cp* DNA *w*as detected by PCR in 12 % of the aneurysms and the result was confirmed by electron microscopy (EM*)*. In none of the dissection patients could *Cp* be demonstrated in the removed tissues. The minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) values for doxycycline and azithromycin increased with longer *Cp* preincubation times when tested in vitro. EM was performed to visualise the inactivation at a cellular level. Thus, the results demonstrate *Cp* in the tissues in non-rheumatic aortic valve sclerosis and in thoracic aortic aneurysm but not in aortic dissection.

Keywords: Chlamydia pneumoniae, aortic valve sclerosis, trace elements, thoracic aorta, antibiotics, Polymerase Chain Reaction (PCR), electron microscopy (EM)

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ISSN 0282-7476
ISBN 91-554-5356-2

TABLE OF CONTENTS

ABBREVIATIONS

AAA	Abdominal Aortic Aneurysm
AI	Aortic Insufficiency
AS	Aortic Stenosis
CABG	Coronary Artery Bypass Grafting
CAD	Coronary Artery Disease
C. pneumoniae	*Chlamydia pneumoniae*
CRP	C-reactive protein
EB	Elementary Body
Ig G	Immunoglobulin G
IgA	Immunoglobulin A
IgM	Immunoglobulin M
MIC	Minimal Inhibitory Concentration
MBC	Minimal Bactericidal Concentration
MIF	Micro Immuno Fluorescence
MOMP	Major Outer Membrane Protein
LDL	Low Density Lipoprotein
LPS	Lipopolysaccharide
PCA	Principal Components Analysis
PCR	Polymerase Chain Reaction
RB	Reticulate Body
TAA	Thoracic Aortic Aneurysm
TWAR	Taiwan Acute Respiratory

INTRODUCTION

Chlamydia pneumoniae

History of *C. pneumoniae*

Chlamydia orginates in Greek khlamus-udos, cloak. Human diseases now known to be caused by *Chlamydia trachomatis* have been recognised since antiquity. Trachoma was recognised in China already in the 27th century B.C. and were described in the Egyptien papyruses. Lymphogranuloma venerum was probably described in the 18th century (1). *C. trachomatis* was first recognised in 1907 by Halberstaedter and Prowazek in conjunctival scrapings from orangutangs inoculated with trachomatous material (2). Similar inclusions were identified in human material from trachoma cases and in the conjunctival scrapings from infants with inclusion blennorrhoea (3). Repeated eye infections with *C. trachomatis* are responsible for scarring and it is the major cause of blindness in developing countries. The first isolate of *C. trachomatis* was made from the cervix of the mother of an infant with inclusion conjunctivitis (4). In women, *C. trachomatis* is responsible for genital infections, including cervicitis urethritis, pelvic inflammatory disease (PID) and perihepatitis; in men, *C. trachomatis* is responsible for urethritis and epididymitis (5)(6)(1)(7). In the 1930s, the *psittacosis* organism (formerly Bedsonia) was isolated by inoculation of embryonated eggs of hens (8)(9)(10). Human infections by *Chlamydia psittaci* are contracted from birds and farm animals. The most common clinical picture is consistent of one of pneumonia. Perimyocarditis, endocarditis, arthritis are common complications and abortion in women by contact with infected animals has been described (11)(12). *C. pecorum* is a newly recognised pathogen that is known to cause infections in animals (13).

C. pneumoniae "TWAR"

C. pneumoniae TWAR was first isolated as TW-183 from one eye specimen collected during a trachoma vaccine trial in Taiwan in 1965 and from another, IOL.27, from Iran. *C. pneumoniae* TWAR was also an isolate from a pharyngeal throat swab from a university student with pharyngitis as AR-39 (Acute Respiratory) in Seattle in 1983 (14). In 1986, it was first described

as a cause of acute respiratory tract infection (15)(14) and after DNA homology analysis and ultrastructural definition, it was declared as a new species in 1989 (16). Genus Chlamydia was recently divided into two genera, *Chlamydia* and *Chlamydophila*, and *Chlamydia pneumoniae* is currently classified as *Chlamydophila pneumoniae*. In this thesis the former name *Chlamydia pneumoniae* is used (17) (see Table 1).

Infection and disease epidemiology

Epidemiology of C. pneumoniae

C. pneumoniae is spread worldwide with cyclic variations over time (18)(19)(20).Human all ages can be infected but the prevalence under five years of age is low. Approximately 70% of elderly people have antibodies to *C. pneumoniae,* males having a higher sero-prevalence than females (21)(22). There is no lifelong immunity and reinfection is common. In addition, there is no vector or animal reservoir known. Transmission from person to person can occur through respiratory droplets during infectious outbreaks in families and institutions but infections from asymtomatic carriers probably predominate.(23)(22).

Clinical manifestations

C. pneumoniae causes upper and lower respiratory tract infections of various severity, from mild upper respiratory infections to severe pneumonia. *C. pneumoniae* has been diagnosed in patients with pharyngitis, otitis media, sinusitis, laryngitis , bronchitis and pneumonia (16)(24)(25). In some patients, long-lasting fatigue with or without respiratory symptoms have been noted in the aftermath of a *C. pneumoniae* infection (18)(26). Some earlier reports of *C. psittaci* have subsequently been diagnosed as *C. pneumoniae* infections (27)(28). Furthermore, subacute or chronic conditions have been associated with *C. pneumoniae*, including arthritis (29)(30)(31), bronchial asthma (32), erytema nodosum (15)(33), thyroiditis, myocarditis (34)(30)(35), endocarditis, (36)(37) sarcoidosis ((38)(39), Guillain-Barre´s syndrome (40), meningoencephalitis (41) and lung cancer. (42). Finally, an association between *C. pneumoniae* and atherosclerotic cardiovascular disease has been proposed (43)(44)(45)(46).

Taxonomy

Table 1. Phylogenetic analyses of 16 S and 23 S rRNA genes have addressed *C. pneumoniae* to belong to the order **Chlamydiales** including two genera (*Chlamydia* and *Chlamydophila*) and four families.

I Chlamydiaceae

Chlamydia	*C. muridarum* sp nov
	C. suis sp nov
	C. trachomatis
	Biovar Trachom
	Biovar LGV
Chlamydophila	***C. psittaci*** comb nov
	C. abortus sp nov
	caviae sp nov
	felis sp nov
	pecorum comb nov
	C. pneumoniae comb nov
	Biovar TWAR
	Biovar Koala
	Bivar Equine

II Simkaniaceae fam nov

Simkania negevensis sp nov

III Parachlamydiaceae fam nov

Parachlamydia acanthamoebae sp nov

IV Family (Unnamed)

The organism and its cell biology

C. trachomatis was identified by accumulation of glycogen in cell inclusions and sensitivity to sulfadiazine. This observation was in contrast to *C. psittaci* that did not accumulate glycogen and was resistant to sulfadiazine. New techniques during the 1980s with DNA based classifications provided new methods for differentiating chlamydial groups. Apart from the newly recognised species *C. pneumoniae* and *C. pecorum* a new *Chlamydia* was reported from swine isolates 1993 (47). Species within the Chlamydiaceae have 16S rRNA gene sequences that are >90% identical. (17).

Life cycle and cell structure

C. pneumoniae is an obligate intracellular bacterium that cannot synthesise adenosine triphosphate or guanosine triphosphate. Moreover, the bacterium is dependent on the energy production of the host cell. There is a unique, two-stage development cycle of replication. The infectious, metabolically inactive form, named elementary body (EB, approximately 100-300nm), can survive in the extracellular environment and infect the susceptible host cells. The mechanism to mediate the attachment and entry into the cell is not completely understood. After entering the cell the EB is transformed to a metabolically active, but non-infectious, larger reticulate body (RB, approximately 500-1000nm) and replicates by binary fission within an enlarged vacuole called inclusion body, using the host cells energy and nutrients. Finally, the RBs that cannot survive extracellularly transform into a metabolically inactive infectious form and are released through cell rupture or fusion of the inclusion and plasma membranes. However, sometimes the host cell is preserved and a persistent cryptic form of *C. pneumoniae* can persist intracellularly (48)(49). The EBs can be pear shaped and probably infect the host cell via a receptor-mediated process (51). The pear shape of *C. pneumoniae* EBs has been proposed as a possible criterion for distinguishing *C. pneumoniae* EBs from EBs of other chlamydial species. This particular morphology does not appear to be common to all strains of *C. pneumoniae*, however (50)(49). The *C. pneumoniae* is classified as gram-negative bacterium. The cell wall has an outer cell membrane that consists of the genus specific lipopolysaccharide (LPS) and an outer membrane protein (OMP), with the dominating major outer membrane protein (MOMP) and an inner

membrane. Elements in the membrane include highly reactive proteins that help adherence to host cells. The activity of endotoxin is lower than that of other common disease-causing gram-negative bacteria. This is why host immune defence is not triggered as much as with other bacteria and may also explain the persistence of RB. On the other hand, the MOMPs stimulate a neutralising antibody response (52).

Diagnostic methods

Microbiological culture

Traditionally, culture in embryonated egg has been the standard method for *C. psittaci* isolation and has been used for *C. pneumoniae* though not as a routine method today. *C. pneumoniae* is difficult to isolate compared with *C. trachomatis.* Yet, it is possible to isolate in acute and primary infection but rarely in chronic infections. Established cell lines of HEp-2 and HL cells are preferable to HeLa and McCoy cells. The organism is susceptible to freezing, which is why immediate transport to the laboratory is essential.

Serology

The Complement fixation test (CF) is based on the common lipopolysaccharide (LPS) antigen and can be used for detection of *C. psittaci.* Acute infections of *C. pneumoniae* may be diagnosed but the sensitivity is low, specially in reinfection. Furthermore, the test is non-specific. The enzyme-linked immunoassay (ELISA) test, based on different chlamydial antigens, is available but the microimmunofluorescence (MIF) test is the most widely used (53). The MIF test was first developed for diagnosis of *C. trachomatis* (54) but has been adapted for *C. pneumoniae* using *C. pneumoniae* TW183 elementary bodies (18). MIF is a sensitive and species-specific test that can differentiate IgM, IgG and IgA responses; it is considered the standard reference test to date. In acute infection the diagnostic criteria have been defined as a 4-fold rise in IgG titre, a single IgM titre of >16, or a single titre of IgG >512. Past or pre-existing infection has been defined as an IgG titre between 16 and 512. IgA antibodies have been demonstrated in acute infection and in association with chronic infections but there have been variable results in different studies. Moreover, there have been no recommendations to evaluate a specific titre as a diagnostic criterion for chronic infection. Acute infections are expected to generate an antibody response with titre changes; however, in

asymtomatic chronic persistent infection testing may not result in a significant antibody titre (55).

Polymerase chain reaction (PCR)

Nucleid acid amplification technique has been applied for *C. pneumoniae* and subsequent *C. pneumoniae* DNA detection by PCR has been found to be more sensitive than cell culture and by this procedure a single organism can be detected. There are severel different protocols published but no standardised PCRs and the in-house assays are using different extraction procedures, primers, reaction conditions and methods of detection (56)(57)(58).

Antibiotic susceptibility testing

Susceptibility testing of *C. pneumoniae* is complex. Determination of minimal inhibitory concentration (MIC) is performed with a microdilution plate technique using immunofluorescence staining. The MIC is taken as the lowest concentration that completely inhibits the normal inclusion. The minimal bactericidal concentration (MBC) is taken as the lowest concentration of an antimicrobial agent preventing demonstrable inclusions after several passages (59). Knowledge about antimicrobial resistance of *C. pneumoniae* is limited, with only a few clinical isolates from patients with clinical failure to antibiotic treatment, having been tested (60). The conditions of the methods currently used do not reflect those in natural *Chlamydia* infection where *Chlamydia* is exposed to the antibiotic long after an intracellular infection has been established. A persistent infection in continuous *C. pneumoniae* infected HEp-2 cells has been observed for up to one year where each of azithromycin and ofloxacin reduced but did not eliminate the infection (61). In vitro data have shown induction of resistance to quinolones in *C. trachomatis* after serial passing of the organism in subinhibitory concentrations of the drug (62).

Therapy

C. pneumoniae is susceptible in vitro to several groups of antimicrobial agents, including tetracyclines, macrolides, azalides and quinolones. Although these antibiotics are frequently used for treatment of respiratory

C. pneumonia infections, little is known about dosage and duration of therapy needed. Most treatment studies have been based on the serological results, which is the most common method used for the diagnosis of C. *pneumoniae.* This is the reason why evaluation of eradication of *C. pneumoniae* has not been possible. In a few studies nasopharyngeal culture showed eradication in approximately 70-80% in children and adults with pneumoniae but persistent infection could be suspected in some patients patients (63)(64). Several antibiotic treatment trials for prevention of secondary cardiovascular events are under evaluation. A few small randomised studies have been published and several larger studies are either ongoing or under evaluation (55).

Cardiovascular diseases

Aortic valve stenosis

Epidemiology

Aortic valve stenosis is the most common valvular disease among the adult population with an estimated prevalence of 3% (65)(66). Stenotic aortic valves are always anatomically abnormal with fibrous thickening, commissural fusion and calcium deposits (67). Studies indicate, that the incidence increases with age (68)(65). The natural history is characterised by a long asymptomatic period with gradually increasing valvular obstruction. The number of heart valve operations due to aortic stenosis has increased over the past decades and the mean age of patients undergoing surgery has gradually increased. The prognosis of aortic valve stenosis is good as long as the patient is asymtomatic. With modern surgical techniques, valvular replacement greatly enhances the prognosis of those who develop symptoms and require surgery. Aortic valve stenosis can be congenital or acquired. Both congenitally malformed and previously normal valves may calcify. The most common malformation is the bicuspid valve that is thought to calcify as a result of “wear and tear” of the valve (68)(69). Bicuspid valves are considered a predisposing factor for aortic valve stenosis but most malformed valves will not become stenotic. In aortic valve stenosis the bicuspid valve accounts for approximately one third of the cases. The acquired stenotic valve has been classified as being caused by degenerative

and post-inflammatory factors. Rheumatic fever was a previously dominant cause of post-inflammatory stenosis that is rarely diagnosed today.

Aetiology and pathogenesis of aortic valve stenosis

The mechanisms resulting in valve calcification are not fully understood. Mechanical factors may contribute to the development of degenerative aortic stenosis (68). Histopathology reveals scaring and focal calcification with inflammatory cells and interstitial fibrosis. Calcification is a result of complex processes, including deposition of bone matrix protein expressed by proliferating fibroblasts, macrophages, platelets and vascular cells (70). Furthermore, in non-rheumatic stenotic aortic valves activated T-lymphocytes and fibroblasts are expressing smooth muscle cell makers and HLA-DR that indicate chronic activation and suggest that immune reactions against antigen in aortic valves may be involved in the formation of aortic valve fibrosis and calcification (71). A comparison with the pathogenesis of atherosclerosis will show some similarities, including the accumulation of T-cells and HLA-DR expression occuring in smooth muscle cells in atherosclerosis and in fibroblasts in aortic stenosis (72)(73)(71). However, there are many patients without any classical risk factors for atherosclerosis and with no major atherosclerotic diseases who develop aortic stenosis, indicating alternative aetiopathogenesis.

Thoracic aortic aneurysm and dissection

”There is no disease more conductive to clinical humanity than aneurysm of the aorta.”Sir William Osler.The term aneurysm is derived from Greek *aneurusma*, from eurus, to “widen out”.

Thoracic aortic aneurysm

An aneurysm is a dilatation of all three wall layers in the aorta increasing the diameter with more than 50%. Indication for surgery is usually a diameter of 5.5 cm in the ascending aorta and 6.5 cm in the descending aorta. All mechanisms that weaken the aortic wall, and lamina media in particular, lead to higher wall stress, a condition that can induce aortic dilatation and aneurysm formation. Cystic medial necrosis is frequently seen in thoracic aortic aneurysms. The cause for this degeneration is unknown in most cases but in a minority (10%) it is caused by defined inherited syndromes. Among the inherited conditions is Marfan's syndrome, a connective tissue disorder

with involvement of many systems, particularly the skeletal system. A number of mutations have been identified for the fibrillin. Another disorder is Ehler-Danlos´ syndrome. This pattern of symptoms is a hereditary connective disorder with articular hypermobility and skin tissue fragility with defects in collagens. However, predisposing for aneurysm development. In annuloaortic ectasia that is considered as a separate condition, also associated with aneurysm, no abnormal type of collagen or fibrillin has been found. Aortitis secondary to giant cell artertitis in Takayasu's disease are syndromes in which arteritis is also seen (74)(75). Other non-infectious syndromes are associated with rheumatoid arthritis, ankylosing spondylitis, psoriasis arthritis and Reiter's syndrome. Syphilis was once the most common cause of infectious aortitis. The infection with the spirochetes produced inflammation in the media, adventitia and vasa vasorum and a fibrotic process caused replacement of the muscle fibres that weakened the arterial wall. The declining incidence of syphilis makes luetic aneurysm a rare condition today. Another infectious aortitis involves the mycotic aneurysm. Traumatic aneurysm can be caused by crushing or penetrating injuries (76).

Dissection

Aortic dissection is a splitting of the wall layers in the aorta. The condition has an acute onset, is very painful and carries a high risk of aortic rupture if the ascending aorta is involved. The condition is sometimes misdiagnosed. If ascending aortic dissection is present, immediate surgical therapy is indicated. Aortic dissection originates at the site of an intimal tear in the ascending aorta in the majority of the patients (77). This results in an exposure to pulsatile aortic flow, creating a "false" aortic lumen that then dissects in the outer layer of the aortic media. As in thoracic aneurysms cystic medial degeneration is commonly seen during microscopic examination. Risk factors include advanced age, systemic hypertension, congenital abnormalities and hereditary disorders of the connective tissue, such as Marfan's and Ehlers-Danlos' syndrome (78). Aortic dissection is most common in men. Iatrogenic aortic dissection can be induced through cardiac surgery and catheter procedures (79)(80). Furthermore, dissection has been described in giant cell aortitis, systemic lupus erythomatosus, Turner's syndrome, fibromuscular dysplasia, annuloaortic ectasia, polycystic

kidney disease, polyarteritis nodosa, cocaine use and trauma. In familial aortic dissection no abnormality of collagen or fibrillin has been found. (76).

Pathogenesis of aneurysm and dissection formation

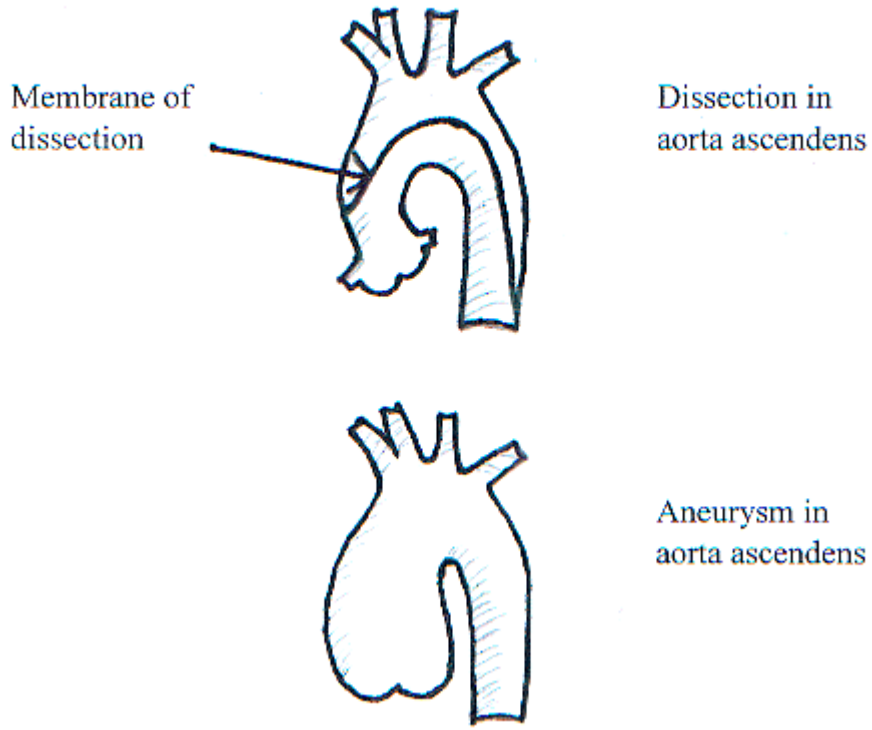
In atherosclerosis there is an intimal fibrosis and calcification that increases extracellular fatty acids. Extracellular matrix is degraded by histiocytic cells. Degenerative changes can be identified within the fibrous tissue. There is a reduced cellularity and collagen fibre hyalinisation resulting both in intimal thickening and increased fragility. This mechanism can lead to intimal rupture. The intimal thickening increases the distance between the endothelial layer and the media, comprising the nutrient and oxygen supply. Adventitial fibrosis may obstruct vessels feeding small intramural vasa vasorum. A reduced nutritional supply of the media results in medial thinning secondary to necrosis primary, which is due to necrosis of the smooth muscle cells. Another consequence is a fibrotic change in the elastic structures of the medial layer. All these changes contribute to increased vessel stiffness and to higher vulnerability to shear stress, a condition that ultimately leads to the formation of aneurysm and dissection. Known risk factors are hypertension, smoking and hypercholesterolaemia (81)(82)(83)(84)**.**

Atherosclerosis

Background

A potential correlation between infection, atherosclerosis and chronic heart disease has been emphasised in recent animal and human research studies. A proposed association between *C. pneumoniae* and cardiovascular diseases has been addressed. Such an association is biologically plausible but causality has yet to be proven (85)(55).

Aneurysm-Dissection



Differentiate between atherosclerotic aortic aneurysm and aortic dissection		
	Atherosclerosis	**Dissection**
Aortic diameter	++	+
Wall thickness	+(+)	-
Luminal surface	Rough	Smooth
Thrombos formation	Lumen	False lumen

Chlamydia pneumoniae and cardiovascular disease

Sero-epidemiological studies in Finland in 1988 were able to demonstrate an association between IgG antibodies to *C. pneumoniae* and myocardial infarction in a male study population (43)(86). In the larger Helsinki Heart Study where traditional risk factors for cardiovascular disease *C. pneumoniae* IgA antibodies and circulating immune complexes were analysed, a significant difference in the cardiovascular disease rate between *C. pneumoniae* positive individuals and individuals lacking *C. pneumoniae* makers was confirmed (43)(86). These studies were followed by several other sero-epidemiological investigations, including coronary angiographic trials. Most of the early prospective studies showed an association of a positive serological result of *C. pneumoniae* to cardiovascular disease, but some subsequent controlled studies could not confirm these data (87) In some cases *C. pneumoniae* has been detected in atherosclerotic tissue samples in individuals who have not developed a serological response (88).

Pathology

In 1992, Shor and co-workers detected *C. pneumoniae* in autopsy coronary arteries using the electron microscopy technique and immune histochemistry (45). Kuo and associates utilised the polymerase chain reaction (PCR) technique and found that *C. pneumoniae* DNA could be demonstrated in atheromatous areas of the arteries from autopsy cases but not in normal arteries (89). In several studies the organism has been found by PCR and/or immune histochemistry using specific *C. pneumoniae* antibodies in cardiovascular tissue materials from abdominal aortic aneurysms, carotid arteries and femoral and iliac arteries (90)(91)(46). In some studies in situ hybridisation and transmission electron microscopy have been used. In a few studies *C. pneumoniae* has been isolated from atheromatous plaque and carotid arteries (92)(91). In many, but not all, studies *C. pneumoniae* was detected more often in atherosclerotic arteries than in with normal arteries.(91). The causality of these findings has been intensively discussed and an innocent bystander hypothesis has been suggested. To address this hypothesis studies have focused on animal and human experimental, in vitro and therapeutic studies in order to elucidate this association. Circulating *C. pneumoniae* has been detected in monocytes from blood in patients with cardiovascular disease and studies on the association to CAD have been undertaken (93)(94).

Animal Studies

C. pneumoniae animal models were initially established to study respiratory infections. Intranasally inoculated mice developed multifocal interstitial pneumonia and a disseminated infection could be detected through lung macrophages and monocytes in blood (95). Studies on genetically modified mice on high cholesterol vs. normal diet, with and without induced *C. pneumoniae* infection, have been undertaken. At normal diet, minor atherosclerotic changes could be demonstrated in the aorta of infected animals in comparison with more advanced artherosclerotic and intimal changes in infected animals fed a high cholesterol diet (96). Furthermore, a small number of animal studies using high cholesterol diet in infection have shown that early installation of antibiotic treatment retarded the development of atherosclerotic changes (97).

In vitro studies

C. pneumoniae multiplies in endothelial cells, macrophages and smooth muscle cells known to be involved in the early stage of the atherosclerotic process in the arterial intimal *C. pneumoniae* is thought to invade the endothelial cells through the respiratory tract. Some T-cells isolated from atherosclerotic plaques have been shown to have an immune response with human heat shock protein 60 (HSP 60) and T-cells isolated from human atherosclerotic carotid plaques have been able to react with *C. pneumoniae* specific HSP 60 (98). Infected macrophages incubated with low density lipoprotein (LDL) were transformed to cells known to be involved in the early atherom process (99).

Inflammatory and atherosclerosis

It is conceivable that *C. pneumoniae* may interact with traditional cardiovascular risk factors. In systemic infection an acute phase response occurs that could cause injury. Pro-inflammatory cytokines and lipopolysaccharide (LPS**)** is released during acute events. It is conceivable that infectious agents can interact in plaque development and make rupture more likely (100)(101)(89). At endothelial dysfunction, there is a systemic and local inflammatory response as reflected in modesty elevated serum levels of C-reactive protein (CRP) (102)(103)(104)(105). It has been shown that *C. pneumoniae* HSP 60 may increase the synthesis of

metalloproteinases and cytokine secretion (100). The amount of inflammation is related to the severity of the coronary syndrome, and plaque complications are often associated with an inflammatory infiltrate (106).

Trace Elements

Several trace elements are important co-factors of numerous enzymes and are essential for immune cell production, activation and function. During most infections, an alteration in the trace element balance occurs, which is reflected in the serum concentrations. A well-documented and common response to infection is the reduction of zinc (Zn) and iron (Fe) levels in serum and a concomitant increase in the copper (Cu) level (107). Thus, an increased Cu/Zn ratio in serum is commonly encountered in active infections. These changes are paralleled by induction of synthesis of metal-binding acute phase proteins including Zn binding metallothionein and the Fe binding ferritin, as well as the plasma Cu transporter protein ceruloplasmin in the liver (108). Calcification in inflammatory heart lesions is known to be associated with a prognosis in animal and worsened human myocarditis (109)(110) and a recent study has shown the myocardial calcium (Ca) concentration to be increased already in the early phase of myocarditis (111). Tissue Ca accumulation in aortic valve disease is correlated to histopathological severity. Few studies have focused on trace elements in human cardiac valves. A recent study found differences in the amounts of Ca accumulation in mitral, tricuspid, aortic and pulmonary valves (112)(113).

Iron and *C. pneumonia*

Iron (Fe) is an essential metal involved in vital cell functions and deposition has been demonstrated in atherosclerotic lesions. Although mechanisms are not fully understood it has been proposed that the availability of iron would stimulate the atherosclerotic process by an interaction of macrophages and endothelial cells with Fe and low density lipoprotein (LDL). This “iron hypothesis has been suggested to explain the lower morbidity and mortality in atherosclerotic diseases in women than in man (114)(115). Evidence indicates that Fe is essential for the growth of *C. pneumoniae* that is inhibited by iron restriction (116)(117). In a recent study it was suggested that *C. pneumoniae* might use the iron transport pathways of the host by

attracting transferrin receptors to the phagosom (118). There is increasing evidence that microorganisms can damage endothelium involved in atherogenesis (104)(119). Cultured endothelial cells respond to infection and iron incubation with increased production of IL-6 and an inflammatory process, indicating that iron overload and interaction with *C. pneumoniae* infection may promote disease (120).

AIM OF STUDY

- To investigate the presence of *C. pneumoniae* in aortic valve in patients undergoing surgery due to aortic valve sclerosis.

- To demonstrate concentration of 15 trace elements in aortic valve tissue in patients undergoing surgery for aortic valve stenosis.

- To study the interaction of *C. pneumoniae* with four trace elements with known biologic activity in aortic valves.

- To investigate the presence and ultra structure of *C. pneumoniae* in thoracic aortic aneurysm and thoracic aortic dissection.

- To study *C. pneumoniae* in cell culture under the influence of two antibiotics with respect to antibacterial and intracellular effects

MATERIALS AND METHODS

Patients, clinical characteristics and procedures

Patients (Papers I-IV)

Paper I

During the period November 1992 - March 1993, patients who underwent surgery for aortic valve stenosis were consecutively included in the present study. The study group comprised 42 patients (21 males and 21 females). In 55% of the cases aortic valve replacement was combined with coronary bypass grafting. The mean age of the patients was 67.5 years (range 38-85 years) with no significant gender difference. Control valves were collected and selected from patients who had died of other causes than heart disease and who had no known history of heart disease. Their mean age was 50.8 years (range 42-74 years), with a male/female ratio of 8/3. In addition, serum samples collected in 1992 from 100 healthy blood donors (50 donors with an age range 20-40 years, male/female ratio 32/18, and 50 donors with an age range 41-60 years, male/female ratio 32:18) were tested for IgG and IgA *C. pneumoniae* antibodies. The mean ages in the two donor groups were 30.2 and 49.6 years, respectively.

Papers II and III

Between 1997 and 1999, aortic valve tissue and serum samples were taken from 46 consecutive patients (20 women and 26 men, aged 34-83 years, mean age 73 years) undergoing heart surgery for the replacement of stenotic aortic valves due to aortic sclerosis. The surgical procedures in the 46 patients were as follows: aortic valve replacement (AVR) only (26 patients), AVR combined with coronary by pass (3 patients) and AVR combined with mitral valve replacement (3 patients). Fifteen forensic autopsy controls (6 women and 9 men, aged 17-88 years, mean age 60 years) without known heart valve disease served as controls for the valve study; no serum samples were available from the autopsy controls. Aortic valve tissue in the controls was collected at the same time as for the patient group.

Paper IV

From 1997 to 2000, excised aortic tissue, serum and throat samples were taken from 32 (12 women and 20 men) consecutive patients (mean age total group 62.0 years, mean age of women 61.7 years, range 28-73 years; mean age of men 59.9 years, range 46–76 years) undergoing open-heart surgery for thoracic aortic aneurysm (TAA) and from 6 consecutive patients undergoing surgery for aortic dissection (2 women and 4 men: mean age 46.3 years, range 31-58 years). The surgical procedures in the 38 patients were as follows: graft replacement because of aortic dissection (6 patients), ascending aortic aneurysm only (4 patients), ascending aortic aneurysm with concomitant aortic valvular replacement/repair (24 patients), ascending aortic aneurysm with concomitant coronary artery bypass (CABG) or mitral valve repair (9 patients) and descending aortic aneurysm (4 patients).

Demographic data

Paper I

In the 42 patients the following features were observed: bicuspid valve (8 patients), history suggestive of rheumatic fever (2 patients), family history of coronary heart disease (17 patients), diabetes mellitus (5 patients), arterial hypertension (10 patients), chronic obstructive lung disease (3 patients) and (current or former) smoker (15 patients).

Papers II and III

Features of the patients were as follows: bicuspid valve (3 patients), history suggestive of rheumatic fever (1 patient), family history of coronary heart disease (13 patients), diabetes mellitus (4 patients), arterial hypertension (15 patients), chronic obstructive lung disease (2 patients) and (current or former) smoker (20 patients). With reference to these factors, the present group of patients is comparable to the one reported in Paper I.

Paper IV

Features of the patients in Paper IV were the following. In the 32 thoracic aortic aneurysm patients 1 patient had a history suggestive of rheumatic fever, 12 patients had a family history of coronary heart disease, 2 patients had a family history of ruptured aortic aneurysm, 1 patient had diabetes mellitus, 25 patients suffered from angina pectoris, 20 patients had arterial hypertension, 3 patients had hyperlipidaemia, 1 patient had chronic

obstructive lung disease and 23 patients were current or former smokers. None of the described features was applicable for the 6 patients with aortic dissection.

Tissue Sampling

Paper I

During the operation, the to-be-replaced aortic valve was excised in toto and placed in an empty sterile plastic tube. For 39 of the 42 patients, the tubes were immediately transferred to the laboratory and placed in a freezer (-70° C) pending PCR analysis; for the remaining 3 patients, the samples had to be discarded because of non-optimal handling. Nasopharyngeal and throat specimens were collected before surgery for culture and PCR of *C. pneumoniae*. Furthermore, a blood sample was obtained and the serum was kept at 4°C pending *C. pneumoniae* antibody analysis.

Papers II and III

During surgery, the to-be-replaced aortic valve was excised in toto and placed in an empty sterile plastic tube. Sterile surgical instruments of stainless steel were used. The tubes were immediately frozen at -150° C and subsequently transferred to the laboratory and placed in a freezer at -70°C. The tissue samples were excised from the soft parts of the valve tissue and stored for later analysis.

Paper IV

During the operation, in which sterile surgical instruments of stainless steel were used, aortic tissue was excised and placed in an empty sterile plastic tube. The tubes were immediately transferred to the laboratory and tissues were aseptically divided into four pieces: one piece was put in formaldehyde for pathology and light microscopy, one in buffered glutaraldehyde, one in glutaraldehyde/paraformaldehyde for electron microscopy and one was frozen at -150°C and subsequently stored at -70°C for later analysis by PCR.

Plasma

Paper III

Plasma samples collected in 1997-1998 from 46 healthy volunteers (20 women aged 22-77 years, mean age 47 years and 26 men aged 30-69 years, mean age 51 years) were used as controls in the Cu/Zn ratio study. This

control group, not currently undergoing any medical therapy or medication, was selected from 250 healthy volunteers, the majority of whom were employees of Uppsala University or the University Hospital, Uppsala. Volunteer plasma samples used in this study were sex- and age-matched to a feasible extent. Unfortunately, a good age-match could not be achieved because of the relatively high mean age of the patients in this study.

Nasopharyngeal and throat sampling

Paper I

CTA swabs (Biohospital AB, Sweden) swabs were used for nasopharyngeal and throat sampling. Specimens for culture were transported in 2-SP-medium while specimens for PCR were transported in Tris-buffer at pH 7.0. The culture specimens were kept at -70°C and the PCR specimens at 4°C pending analysis.

Throat sampling

Papers III and IV

The throat samples were obtained with CTA swabs and transported to the laboratory in Tris-buffer pH 7.0. The samples were immediately frozen at -70°C until tested.

Serum sampling

Papers I, III and IV

The blood specimens were immediately sent to the laboratory and centrifuged. The sera and plasma were frozen at -20°C and thawed only once.

DNA preparation and PCR for *C. pneumoniae* in valve tissue

Papers I, III and IV

An approximately 3 mm x 3 mm piece of frozen valve tissue was dissected aseptically and transferred to Eppendorf tubes with lysis buffer (50 M Tris, pH 8.5,1 mM EDTA, pH 8.0 and 1% Trition X-100). Proteinase-K was added to a final concentration of 200 μg/ml. The samples were mixed and incubated at 56°C for 1h followed by incubation for a 10min period at 95°C in order to inactivate the enzymes. An equal volume of phenol was added to the samples after vortexing and centrifugation for 7min. Soon afterward the

supernatant was discharged and the water phase was re-extracted with chloroform, added in equal proportions, and centrifuged for 5min. The supernatant was then purified by ultrafiltration with Microcon-100 (Amicon Bevely Md, USA) by centrifugation at 500 xg for 20min, washed with distilled water and centrifuged a second time at 500 xg for 20min. The final retentate was collected by centrifugation at 1,000 xg for 3min or precipitated overnight with a 1/10 volume of sodium acetate and 3 volumes of absolute alcohol. A nested PCR assay was performed. The first pair of primers (Cpn A and Cpn B) was adapted from published data from Gaydos (56). This first amplification was followed by an in-house PCR with primers Cpn 1 and Cpn 2 with the following sequence: Cpn 1;5´ CCG CAA GGA CAT ATA CAC AGG 3´. Cpn 2; 5´CCA GTT CGG ATT GTA GTC TGC 3´. The primers span from position 1036 to 1327 in 16 S rDNA. The PCR protocol for Cpn A and Cpn B was 94°C for 3min followed by 30 cycles of 94°C for 30s, 56°C for 30s , 72° C for 30s and finally a 5 minutes extension at 72C. The protocol for Cpn 1 and 2 consisted of 94° for 3 minutes followed by 30 cycles of 94°C for 30s, 64°C for 30 s, 72°C for 30 s followed by extension at 72°C for 5min.The reactions were carried out in a Perkin-Elmer Cetus thermocycler 9600. The PCR products were electrophoresed through a 1.4% agarose gel for 2 h at 70V. The DNA was detected on a UV transilluminator after staining with ethidium bromide. Due to the fact that the primers in this test were determined before the sequence of the actual gene had been published the sequence number mentioned in the published paper I differs, indicating that the primers Cpn1 starts at 1039 instead of 1036.

DNA sequencing

Paper I

In order to confirm that the amplified products were *C. pneumoniae,* the DNA products were sequenced using the method of DNA sequencing of Sanger (121)(122).

DNA sequencing

Paper IV

The amplified DNA products were sequenced using a Perkin Elmer ABI-PRISM 310 Genetic Analyser (Applied Biosystems) following the instructions of the manufacturer.

PCR in nasopharyngeal specimens

Paper I

The DNA preparation and PCR procedures for the respiratory tract specimens were similar to those employed for the valve specimens.

DNA preparation and PCR of the throat specimens

Papers III and IV

The DNA was extracted from the TRIS-buffer with Amplicor Respiratory Specimen kit (Roche, Diagnostic System, Basel, Switzerland). PCR was performed using two different methods; both with the nested PCR described above and with the method of Campbell et al using HL-HR primers (123).

Cell culture of *C. pneumonia*

Papers I, IV and V

Paper I

The cultures were performed in duplicate in 48-well microplates on HL-cells grown in RPMI 1640 (Gibco), supplemented with 10% fetal calf serum, Hepes, $NaHCO_3$, glutamine and gentamicin. The specimens were centrifuged onto the cells for 1h at 2,800 xg. After 3h of incubation at 35°C, the medium was changed to the same medium as above but supplemented with glucose and cycloheximide. After incubation for 3 days at 35°C, the cells were stained with FITC-conjugated monoclonal antibodies reactive with *Chlamydia* (Pathfinder, Kallestad, Diagnostics, Chaska, Minnesota, USA) and studied in a fluorescence microscope (Zeiss Axiovert).

Paper IV

C. pneumoniae was inoculated on monolayers of HEp-2 cells, strain G 954, and then incubated for 72h. The cell suspension was centrifuged and the pellet was fixed, embedded and sectioned as described for the processing for the TEM processing. Non-infected Hep-2 cells were prepared by the same procedure.

Paper V

HEp-2 cells (ATCC CCL 23) were grown in a medium consisting of RPMI 1640 (Gibco BRL, Life Technologies™, Paisley, Scotland), 10% fetal calf serum (FCS), 20 mM HEPES (N-2-hydroxyethylpiperazine-N-2-

ethanesulfonic acid), 2mM glutamine, 0.05% $NaHCO_3$ at 35°C and 5% CO_2 (D1-medium). The cells were cultured in monolayers in 48-well cell culture clusters (Costar®, Cambridge, MA, USA) before each experiment. A clinical strain of *C. pneumoniae*, G 954, derived from a patient at the University Hospital, Uppsala, Sweden with sinuitis was used in the experiments.

Microimmunofluorescence

Papers I, III and IV

Paper I

A microimmunofluorescence (MIF) test was employed to determine the presence of *C. pneumoniae* -specific IgG, IgA and IgM antibodies in patient and blood donor sera. The microscopic slides were obtained from I.O. International Ltd., London, U.K.The antigens prepared on the slides were yolk sac grown elementary bodies from *C. trachomatis*, serotypes B and D-K (pooled), *C. pneumoniae*, strain IOL 207, and *C. psittaci*. Normal yolk sac served as a negative control antigen. Before testing for IgM-antibodies, the sera were absorbed with Protein A sepharose CL4B (Pharmacia LKB Biotechnology AB, Uppsala, Sweden) to prevent possible interference with rheumatoid factor. The sera were allowed to bind to the antigens for 18h at 4°C. After washing in phosphate-buffered saline (PBS) at pH 7.2, each of the fluorescein-iso-thiocyanate conjugated antihuman IgG, IgA and IgM antibodies (Dakopatts AB, Stockholm, Sweden) were allowed to react for 30min at 37°C. The slides were rinsed with PBS containing 0.003% Evan's Blue and mounted with a glycerol buffer at pH 7.2. The slides were examined under a fluorescence microscope (Nikon) equipped with incident light. Only even fluorescence of all the elementary bodies in an antigen dot was considered as a positive result, except in dots containing pooled *C. trachomatis* antigens that were considered positive if only portions of elementary bodies fluoresced. An IgM and IgA titre of $\geq$16 was considered positive. An IgG titre of $\geq$64 was considered indicative of previous infection.

In papers III and IV the microscopic slides were obtained from Labsystems OY (Helsinki, Finland) and the sera were absorbed with RF Absorbent (Dade Behring Marburg, Germany).

Electron microscopy

Papers I, IV and V

Paper I

Frozen valve tissue was thawed and fixed for 1h at 4°C in cacodylate-buffered 3 % glutaraldehyde, pH 7.2, and postfixed in OsO_4. The tissue was then embedded in Epon. Ultrathin sections of valve tissue were stained with uranyl acetate and lead citrate and examined in a Philips TEM 420 transmission electron microscope at 60 kV.

Tissue and cell processing for transmission electron microscopy (TEM)

Paper IV

Morphological analysis: The samples were fixed in 2% glutaraldehyde in 0.1 mol/l cacodylate buffer, pH 7.2 (CAC buffer) for 6h and postfixed in 1% osmium tetroxide in the same buffer for 1.5h. The specimens were subsequently dehydrated in 50-100% ethanol, immersed in propylene oxide and infiltrated and embedded in the epoxy resin Agar 100 (Agar Scientific Ltd., Stansted, Essex, UK). Polymerisation was performed in 60^{o}C. Ultrathin sections were placed on Formvar-coated (Sigma Biochemicals, St Louis, MO) copper grids and contrasted with 4% uranyl acetate in water for 30min at 20^{o}C and Reynolds lead citrate for 5min at 20^{o}C before examination in a Phillips 201 transmission electron microscope. Low temperature processing for immunocytochemistry: The samples were fixed in 4% paraformaldehyde/0.5% glutaraldehyde in the cac-buffer for 4h at 4^{o}C. During the rapid dehydration in 50-95% ethanol, the temperature was lowered to -20^{o}C. Infiltration of the acrylic resin Lowicryl K4M (Agar Scientific Ltd.) and subsequent polymerisation in ultraviolet light was performed at -20^{o}C (56). Ultrathin sections were placed on Formvar-coated (Sigma Biochemicals) nickel grids. The cultured positive control cells were pelleted between each step in the embedding processes.

Preparation of cells for electron microscopy

Paper V

C. pneumoniae (G-954) was added to monolayers of HEp-2 cells. The cell plates were centrifuged as described previously and incubated for either 24 or 72h with D2-medium and 4 mg/l of either doxycycline or azithromycin.

Controls without antibiotic supplements were incubated for the same periods of time. The cells were rinsed with PBS and transferred to Eppendorf tubes; the cell suspensions were centrifuged at 12,000 rpm for 5min. The pellet was fixed for 1h at 4°C in cacodylate-buffered 3% glutaraldehyde (pH 7.2) and postfixed in OsO_4. The fixed cells were washed in buffer, dehydrated gradually in ethanol and embedded in Epon. Ultrathin sections were stained with uranyl acetate and lead citrate and examined with a Philips EM 420 transmission electron microscope.

Immunocytochemistry

Paper IV

The immunogold labelling technique has been described earlier (124). Shortly, ultrathin sections on nickel grids were blocked for unspecific binding with normal goat serum, type V (Sigma Biochemicals), incubated in the primary anti-*C. pneumoniae* antibodies over night and in the gold-conjugated secondary antibodies for 2h the day after. The sections were rinsed thoroughly and contrasted with uranyl acetate and lead citrate before examination in the electron microscope. TBS (0.05 mol/l), pH 7.2, with 0.1% BSA (Sigma Biochemicals) was used to dilute the primary antibodies. TBS, pH 8.2, with 1% BSA was used to dilute the secondary antibodies. For rinsing between incubation of the primary and secondary antibodies, TBS, pH 7.2, with 0.2 % BSA was used. Serial dilution tests determined the optimal dilution of the *C. pneumoniae* antibody. Four labelling experiments were performed in which the same result was evident each time. Replacing the primary antibody with TBS performed negative controls.

Antibodies

Monoclonal mouse anti-*C. pneumoniae* IgG, diluted 1:1, clone RR402 (Dakopatts AB, Älvsjö, Sweden) was used as primary antibody; as secondary antibody, goat-antimouse IgG conjugated to 10 and 15 nm colloidal gold (Amersham International, Amersham, UK) diluted 1:20 was used.

Detection of trace elements

Papers II and III

Heart valve tissue samples were decomposed using ultra-pure nitric acid (Scan Pure, Chem Scand AS Elverum, Norway) in steel bombs (Me Ana-Konsult, Uppsala, Sweden). Tissue samples of about 0.1 g were weighed and put in quartz tubes. One ml of 65% nitric acid per 0.1 g sample dry weight was added. Soon afterward, the tubes were sealed with a Teflon lid and put into the steel bombs, which were sealed with exactly the same momentum. The bombs were then heated in an oven to 180°C for 4h. After decomposition, an internal standard (indium) was added and the samples diluted in 10 ml of high purity water from an Elga Stat UHP (Elga Ltd., High Wycomb Buckshire, England). The water quality was maintained at more than 18MΩ cm. All handling of samples was done in a clean room. The trace element content of the sample was then measured in an ICP-MS (Perkin-Elmer SCIEX ELAN 6000, Perkin Elmer Corp, Norwalk, CONN, USA). Quality control was assessed with Certified Reference Materials (CRM). Every fifth sample was a CRM (BCR of bovine muscle, Community Bureau of Reference, Brussels, Belgium). The quality control procedures employed resulted in an overall precision of less than 5% and an overall accuracy of less than 8%. The trace elements aluminium (Al), arsenic (As), cadmium (Cd), calcium (Ca), cobalt (Co), copper (Cu), iron (Fe), lead (Pb), magnesium (Mg), manganese (Mn), mercury (Hg), selenium (Se), silver (Ag), vanadium (V) and zinc (Zn) were measured.

Histopathology

Papers II and IV

A normal aortic heart valve with no calcification or inflammatory lesions from a healthy forensic control case and a stenotic valve operatively excised from a patient having severe changes characteristic of sclerotic heart valve disease **(Paper II)** and tissue pieces from operated aortic aneurysm **(Paper IV)** were fixed in 10% formalin solution, embedded in paraffin, sectioned and stained with May Grünwald Giemsa and Grocotte stain.

Determination of antibiotic susceptibility

Paper V

Antimicrobial agents A stock solution of azithromycin (kindly provided by Pfizer Inc., New York, USA) was freshly prepared for each experiment by dissolving 10 mg of the drug in 1 ml methanol. Doxycycline (Sigma Chemical Co, St. Louis, MO, USA) was prepared in the same manner.

Experimental procedure *C. pneumoniae* was added to the monolayers of HEp-2 cells at concentrations of approximately 10^4 inclusion forming units/ml. The cell culture plates were centrifuged at 3000g for 1h at 30°C and preincubated (5% CO_2 and 35°C) for different periods of time (1, 12 and 24h), before the addition of the antibiotics. Two-fold serial dilutions (from 16 to 0.015 mg/l) of azithromycin and doxycycline were prepared in cell culture medium (D1), supplemented with 40 mM glucose and 0.0001% cycloheximide (D2 medium). Because of the pH sensitivity of this drug (Hulten 96), the pH in the medium was adjusted to 7.4 in the azithromycin experiments while the doxycycline experiments were performed at a standard cultivation pH of 7.2. Three sets of wells contained medium with different dilutions of the two antibiotics; in each set, one well without any antibiotic treatment was included for control purposes. After 72h of incubation in 5% CO_2 at 35°C, the cells in two of the wells exposed to each concentration of drug were fixed with methanol and stained with trisodium-citrate buffered propidium-iodide (Sigma Chemical Co., St. Louis, MO, USA) and fluorescein-conjugated murine monoclonal antibodies reactive with Chlamydia spp (Pathfinder®, Kallestad Diagnostics, Chaska, MN, USA). The results were assessed in an inverted Zeiss microscope equipped with incident UV illumination at x 100 and x 400. MIC was defined as the lowest concentration with a minimum of 95% reduction in inclusions compared with the controls. The remaining well at each antibiotic concentration was washed five times with PBS. The cells were subsequently harvested and transferred to new monolayers of HEp-2. The 48-well plates were centrifuged as described previously. After 2h of incubation, the medium was changed to a D2 medium without antibiotic supplementation, and the plates were incubated for a further 72h before staining and evaluation. MBC was defined as the lowest antibiotic concentration of which no inclusions were observed. In order to test the effect of exposure time of azithromycin and doxycycline on MIC and MBC after different

periods of incubation (24, 48 and 72h), cells grown in 48-well culture plates were infected with *C. pneumoniae*, centrifuged and preincubated for 1h. D2 medium with azithromycin or doxycycline was added at the concentrations mentioned above, to three sets of wells for each antibiotic and controls were included as previously. After 24 and 48h, the medium was changed to D2 medium without antibiotic supplementation in two of the sets. Growth was continued for 72h. Duplicate samples were stained for MIC evaluation and one sample of each concentration of a drug from the different sets was used to assess the MBC values.

Ethics

Papers I-IV

This study was approved by the Research Ethics Committee, Uppsala University Hospital, Uppsala (D.no. 97078). The investigation conforms to the principles outlined in the Declaration of Helsinki.

Statistical analysis

Papers I- III

Paper I

The Chi-Square test and ordinal logistic regression were used for the statistical analyses of the data.

Principal components analysis (Papers II and III) is designed to reduce the number of variables to a small number of indices (called the principal components) that are linear combinations of the original variables. These linear combinations are selected such that the first contains those of the original variables that correlate well and explains most of the variability in the data. The second linear combination is chosen in a similar way to the first one but it does not correlate with the first one, and so forth. Principal components analysis, therefore, provides an objective way of finding indices so that the variation in the data can be accounted for in a concise manner. It may turn out that two or three principal components provide a good summary of all the original variables. Consideration of the values of the principal components instead of the values of the original variables may then make it easier to interpret the data.

Discriminant analysis (Paper III) is a form of regression analysis concerned with the problem of determining whether it is possible to distinguish between groups on the basis of the available measurements. It is based firstly on a set of variables, often called training samples, in which the group identity is known a priori and subsequently a second set, refereed to as test samples, consists of observations for which group membership is unknown and which has to be assigned to one of the groups. The main objective is to determine functions of the measured variables that separate the groupings. The discriminant functions are calculated in an ordered sequence whereby the first function explains most of the group differences, the second some of the remaining group differences not explained by the first, and so on. The solution approach is comparable to that of principal components analysis. Discriminant functions represent linear combinations of the original variables. The value this function takes, based on the recorded values for the variables, is called the discriminant score. The values of the first discriminant function best separate out the samples into the groups and reflect most of the group differences. The second function captures as much as possible of the group differences not displayed in the first function.

Box-and-whisker plots (Paper II) are a type of graph in which boxes and lines convey a distribution's shape, central tendency and variability. The diagram gives a highly informative picture of the values of a single variable and is especially helpful for indicating whether a distribution is skewed and has outliers. The bottom of the box shows the lower quartile of the data, the middle line represents the median and the top line is the upper quartile. The lower whisker extends to the smallest observation of the set. If, however, some of the smallest observations are farther than 1.5 times the interquartile range from the lower quartile, they are plotted individually. The upper whisker is plotted in an analogous fashion.

Spearman's rank correlation coefficient (Paper III). This is a nonparametric correlation concerned largely with paired observations consisting of ranks. The ranks may be the primary data or they may be derived from continuous measurements.

Mann-Whitney U test. (Papers II and III). This is the nonparametric correspondence of the t-test with two independent samples (Sharma S). .In **Papers IV** and **V** statistical methods were not applicable.

RESULTS

C. pneumoniae detection using Polymerase chain reaction (PCR)

Paper I

C. pneumoniae was detected by PCR in the sclerotic aortic valves in 19/39 (49%) patients undergoing surgery (11 females and 8 males), whereas 1/11 (9%) forensic autopsy controls was PCR positive in the aortic valves (p=0.018). Seventeen patients were PCR negative in the valves; in three patients the valves were not studied for practical reasons. Only one patient was PCR positive in the nasopharynx (the same patient was negative in the valves). Thus, 20 patients were PCR positive for *C. pneumoniae in* either the valves or nasopharynx. None of the patients had a positive nasopharyngeal *C. pneumoniae* culture. From one valve that was PCR positive for *C. pneumoniae* when using specific primers, the amplified product was sequenced. The DNA sequence was identical to the corresponding sequence of *C. pneumoniae*. This procedure was carried out in order to rule out *C. psittaci.*

Paper III

C. pneumoniae -specific nucleotide sequences could be demonstrated using PCR in the aortic valves in 16/46 patients (34.8 %) (in 9 of the 20 females and in 7 of the 26 males). A gender difference was, however, not statistically significant. None of the forensic autopsy controls tested positive. Furthermore, no *C. pneumoniae* DNA could be detected in the throat specimens from any of the patients.

Paper IV

C. pneumoniae could be demonstrated by PCR in 4/32 (12%) patients with thoracic aortic aneurysm but in none of the six patients with aortic dissection. None of the 17 tested aneurysm patients was *C. pneumoniae* PCR positive in throat swabs. Throat swabs from the rest of the patients were not taken in most of the cases because of logistic reasons. In one of the

C. pneumoniae CPR positive aneurysm patients the infection was confirmed by specific nucleotide sequencing.

Serology

Paper I

Antibodies reactive with *C. pneumoniae* were detected in 69% of the patients, in 40% of the forensic autopsy controls and in 53% of the blood donors, indicating prior infection. In 17% of the patients, but in only 3% of the blood donors (p=0.01) and in none of the controls, IgG levels exceeded 1/512, a finding suggestive of recent or reactivated infection**.** IgG antibodies reactive with *C. psittaci and C. trachomatis* were found in a minority of cases, probably as a result of cross-reaction with *C. pneumoniae.* IgA antibodies reactive with *C. pneumoniae*, possibly suggestive of chronic infection, were found in 19% (8/42) of the patients, in none of the autopsy controls and in 18% of the blood donors. None of the patients or the forensic autopsy controls had IgM antibodies. There was no statistically significant difference in IgG antibody positivity to *C. pneumoniae* in those patients who had *C. pneumoniae* in their valves or nasopharynx and those who had not (64% in each group). Of the eight IgA-positive patients, five had *C. pneumoniae* in their valves or nasopharynx. In the only patient who was PCR positive to *C. pneumoniae* in the nasopharynx IgA antibodies were detected.

Paper III

C. pneumoniae IgG antibodies were found in 26/46 (56.5%) patients, indicating prior infection with that agent. Sera were not available in autopsy control patients. Low titres of IgG antibodies that were reactive with *C. psittaci* and *C. trachomatis* were found in a minority of cases, probably as a result of cross-reaction with *C. pneumoniae.* IgA antibodies reactive with *C. pneumoniae,* suggestive of chronic infection, were found in 12 (26.1%) patients; none of the patients had IgM antibodies indicative of acute infection. Twelve (75.0%) of the 16 PCR positive patients had either IgG or IgA antibodies to *C. pneumoniae,* or both. In total, 32 (69.6%) of the patients were positive in at least one *C. pneumoniae* marker. Furthermore, no statistically significant difference in IgG antibody positivity to *C. pneumoniae* was noted in those patients who had *C. pneumoniae* in their valves and those who had not: 11/16 (68.8%) and 15/30 (50%), respectively.

Of the 12 patients with positive IgA antibodies, 6 (50%) had *C. pneumoniae* in their valves.

Paper IV

The *C. pneumoniae* IgG antibodies were detected in 17/31 (55%) patients and the IgA antibodies in 15/31 (48%) patients with thoracic aortic aneurysm. Neither IgG nor IgA antibodies could be detected in the 5 tested patients with aortic dissection. One of the four patients with positive PCR had IgG antibodies in titre 1/64 but no IgA antibodies. The other three had neither IgG nor IgA antibodies.

Electronmicroscopy (EM)

Paper I

Electron microscopy was carried out in one PCR positive valve and pear-shaped elementary bodies typical of *C. pneumoniae* were detected. Such particles were not observed in the valve tissue of a PCR negative forensic autopsy control case, however.

Paper V

A difference in the effect on the cells by the two drugs was observed by electron microscopy. The azithromycin-treated cells demonstrated vacuolisation, whereas preserved elementary body inclusions were found in the cells treated with doxycycline. Small fluorescing particles that lacked inclusion morphology were seen after treatment with high doses of azithromycin. This could also be found in doxycycline-treated cultures to a lesser extent.

EM and Immunocytochemistry

Paper IV

Tissue samples from an excised aortic aneurysm were further studied with electron microscopy and *C. pneumoniae* bacteria and inclusions were detected. The cytoplasm of infected cells was mostly filled with inclusion bodies and very few intact organelles were seen. Because many cells had a broken plasma membrane, inclusions with bacteria and free bacteria were also noted extra cellularly. All differentiation stages of *C. pneumoniae* were demonstrated. The morphological findings in patient specimens were

identical to the findings in the *C. pneumoniae* infected Hep-2 cells. The immunoreactivity of the anti-*C. pneumoniae* antibodies on the low temperature processed material was very sparse. However, the gold markers were exclusively demonstrated on elementary bodies and in the membranes of reticulate bodies. The same labelling pattern and low immunoreaction were obvious for the positive control material as well (not shown).

Histopathology

Paper IV

Excised aortic tissue from 23/32 patients with thoracic aortic aneurysm was histopathologically classified into two groups: cystic medial necrosis was present in 7/23 patients and degenerative changes, including atherosclerotic and fibrosis, in 16/23 patients. In eight of the patients in these two subgroups inflammatory changes were observed. Of the six patients with aortic dissection, cystic medial necrosis was identified in the cell wall of four patients, whereas unspecific degenerative changes were seen in the other two. No signs of inflammatory changes were observed in these patients. Tissue samples stained with May Grünwald Giemsa and silver stain from one patient, from whom tissue samples were analysed with EM as well, showed no signs of cystic medial necrosis though changes of inflammation and perivascular cell infiltration were demonstrated. In the other three *C. pneumoniae* PCR positive aneurysm patients degenerative changes were detected in two patients and minor changes in the elastin structures were noted in the third patient.

Trace elements

Aortic valve tissue

Paper II

The concentrations of 15 trace elements in sclerotic aortic valve tissue from patients operated on for aortic stenosis were compared with the concentrations in the valves of forensic autopsy controls without known heart disease. For 11 of the 15 trace elements, concentrations were significantly different in patients and controls; for several of the elements, the differences were dramatic. Results from PCA indicated that certain trace elements (Ag, Cd, Co, Fe, As, Ca, Mg and Zn) formed a cluster in the patient, whereas no such cluster could be identified among the controls. Some trace elements revealed no significant differences in their

concentrations between patients and controls, including Al, Mn, Hg and Ag. For V, the mean V concentration in the patients' valves was 42% lower as compared with the controls. For a few trace elements, the concentration differences were moderate but significant, with an increase in Cd (52%, $p<0.05$) and a decrease in Cu (45%, $p<0.001$) and Se (14%, $p<0.05$) in the patients. Pronounced differences between patients and controls were observed for As, Ca, Co, Fe, Pb, Mg and Zn. The relationship and associated probabilities are given within brackets. Thus, in the patients there were increased concentrations of As (5-fold, $p<0.001$), Ca (70-fold, $p<0.001$), Co (10-fold, $p<0.001$), Fe (20-fold, $p<0.001$), Pb (8-fold, $p<0.001$), Mg (20-fold, $p<0.001$) and Zn (10-fold, $p<0.001$). In the patient group all of the trace elements exhibited a large inter-individual variation in their concentrations, whereas in the control group several of the elements (i.e. Fe, Co, Zn, As, Ag, Cd, Mg, Ca and Pb) varied within narrow limits.

Paper III

In paper II, the use of principal components analysis identified a cluster formation of eight trace elements in the patient group that was not present in the control group. From this cluster, four trace elements (Ca, Fe, Mg and Zn) with known biological functions were selected for further studies. Pronounced differences in valve concentrations between patients and controls were observed for Ca, Mg, Fe and Zn. In the patients there were increased concentrations of Ca (70-fold), Fe (20-fold), Mg (20-fold) and Zn (10-fold). The analyses revealed no significant differences between women and men. In general, the trace element concentrations showed greater inter-individual variation among the patients than among the forensic controls. Furthermore, significant ($p<0.001$) correlations between Ca and Fe, Ca and Mg and Ca and Zn were demonstrated in the patient group but not in the controls. These correlations persisted among the 16 PCR positive patients though at a somewhat lower level, which could be accounted for by the low number of cases. Although the valve concentration of Fe indicated a more marked increase in the *C. pneumoniae* PCR positive patients (mean Fe concentration 391.6 ± 153.9 μ/g SD) than in the *C. pneumoniae* PCR negative patients (mean Fe concentration 337.4 ± 205.3 μ/g SD), comparisons of the concentration of each of the four trace elements in the subgroups of patients displaying PCR positivity or seropositivity to

C. pneumoniae with the subgroups that lacked these markers did not reveal any significant differences.

Serum/plasma

Paper III

Trace element concentrations of Cu and Zn in patient sera were compared to those in healthy control plasma showing a significantly increased Cu concentration and a significantly decreased Zn concentration in the patients (mean ± SD: Cu, pat: 1261 ± 408ng/ml, Cu, ctr: 1111 ± 160 ng/ml; $p<0.05$; Zn, pat: 753 ± 214 ng/ml, Zn, ctr: 859 ± 180ng/ml $p<0.05$). Thus, the serum Cu/Zn ratio was markedly elevated in the patient sera (Cu/Zn, pat: 1.71 ± 0.45, Cu/Zn, ctr: 1.36 ± 0.42; $p<0.001$). It is known that zinc concentrations may be higher in serum than in plasma. However, this possibility did not influence the conclusion arrived at in this study.

Antibiotic susceptibility

Paper V

The influence of different chlamydial preincubation times prior to addition of azithromycin and doxycycline showed that MIC values increased 20-fold and the MBC values about 3-fold when the preincubation time was prolonged from 1 to 24h. The MBC values were substantially higher than the MIC values for both drugs. Antibiotic concentrations containing more than 16 mg/l of doxycycline and 32 mg/l of azithromycin could not be evaluated because of cell death. A comparison of the difference in the range of the MIC and MBC assays showed that the MIC experiments gave narrower range than the MBC experiments. The MIC values for both drugs decreased more than 10-fold when antibiotic exposure was extended from 24 to 72h.

DISCUSSION

Paper I

Evidence suggests that aortic sclerosis develops over a period of many years and that patients can be asymptomatic for comparatively long periods before symptoms gradually appear. With currently employed diagnostic and surgical techniques and perioperative procedures, including intensive care

when needed, the prognosis of this disease is favourable. This study demonstrates *C. pneumoniae* DNA in the aortic valves in approximately 50% of patients undergoing valve replacement surgery due to aortic sclerosis, and in only one of 11 forensic autopsy controls without evident heart disease (p=0.018). The organism was confirmed by electron microscopy in one patient and might also be involved in the pathogenesis of aortic valve sclerosis The immunopathology of non-rheumatic aortic sclerosis seems to have some features in common and others at variance with that of atherosclerosis. For example, HLA-Dr expression occurs in both conditions and, in aortic sclerosis, HLA-DR is expressed by fibroblast, whereas in atherosclerosis HLA-DR is expressed by smooth muscle cells. Furthermore, in aortic sclerosis T-lymphocytes predominate, often expressing interleukin-2 receptors (71), whereas in the atherosclerotic plaque numerous lipid-rich macrophages are believed to play a key role (106). Whether similar or different antigens are involved in the pathogenesis of these two conditions remains to be established. The increasing number of epidemiological and microbiological studies showing a suggestive correlation between *C. pneumoniae* and atherosclerosis has stimulated pathogenetic investigations. It has been shown that *C. pneumoniae* infection may be latent (26) and that it may multiply in macrophages (125). There was no obvious correlation to classical risk factors for atherosclerosis among the present cardiac patients. Earlier studies have shown that the common risk factors predictive of atherosclerosis are not generally predictive of the development of isolated aortic valve sclerosis In conclusion, we found *C. pneumoniae* by PCR in the sclerotic aortic valves in approximately 50% of consecutive patients undergoing valve replacement surgery but in only one case in a control group of 11 forensic autopsies without discernible heart disease. The organism was demonstrated by electron microscopy. There was no correlation between *C. pneumoniae* positivity in the sclerotic valves and serological markers of *C. pneumoniae* infection. A role for *C. pneumoniae* in the pathogenesis of aortic sclerosis is suggested, a role similar to that previously proposed for this organism in atherosclerosis.

Paper II

For 11 of the 15 trace elements determined in the present study, the concentrations in the heart valve tissue of the patients suffering from non-rheumatic aortic sclerosis with concomitant aortic stenosis were

significantly different from those of the heart valves of the forensic controls with no known heart disease. Of these 11 elements, Zn, Fe, Se and Cu are believed to be involved in inflammatory processes. In addition, Ca is a major component of sclerotic valve tissue that is frequently macroscopically evident. The pronounced mean increase, as well as the notable inter-individual differences around this increased mean level, in the concentrations of Zn and Fe in the patients but not in the controls may reflect the presence of inflammatory activity at various levels in the patients' valves. Additional to being essential for immune cell function and host defence (108)(126)(111), the trace elements Cu, Zn, Fe and Mn are important components of several anti-oxidative enzymes protective against the action of free radicals (127). More recent evidence indicates that free radical processes, as well as imbalances in several trace elements, are involved in atherogenesis (127) and thus potentially even implicated in the development of aortic valve sclerosis. Accumulation of Ca in the inflammatory heart is considered a poor prognostic factor (110) and Mg deficiency has been associated with cardiac arrhythmia and sudden death (128). A limited number of human studies of the heart have focused on the role of trace elements in health or disease. The Zn level in serum is known to be lower during various infectious diseases (108). Zn is intimately involved in the regulation of immune function and is regarded as crucial for optimal T-cell function (129). The 10-fold mean elevation of Zn in the sclerotic valves may be related to the presence of immune cells as previously found in valve tissue in this disease (71). Fe has been regarded as a promoter of atherosclerosis through inducing lipid oxidation, although the interactions between macrophages and endothelial cells and Fe have not been fully clarified (114). In the present study the Fe content was increased 20-fold in the sclerotic valves as compared with the control valves. Dietary Cu deficiency may produce cardiac lesions and hypertrophy in rats (130) and it may impair cardiovascular health by contributing to high blood pressure, enhancement of anaemia, reduced blood clotting and atherosclerosis (131). The Cu content in the atherosclerotic aortic artery tissue is low and has been suggested to be caused by a shift of Cu from aortic tissue into the blood (132). Similarly, the Cu concentration in the sclerotic valves in the present study was approximately half of that found in the control valves. Furthermore, peroxidation of low density lipoproteins is known to occur more readily during coexisting Cu deficiency because of a reduction of the

antioxidative enzyme Cu-Zn superoxide dismutase (127). This effect is enhanced in the presence of increased Fe and, in the present sclerotic valves, Fe concentration was in fact greatly increased (128)(133). A possible link of sclerotic heart valve disease to *C. pneumoniae* infection has even been suggested in paper I and in a Finnish study of autopsy cases (134). In the study of Tohno and co-workers the concentrations of Zn in the aortic valves were at the same level as in our study, but their Ca, Mg and Fe concentrations were two to three times lower. The differences may be explained because different populations were investigated (112). In conclusion, in the sclerotic heart valves the concentrations of 11 of the 15 presently studied trace elements were significantly different from the concentrations in the normal control valves, and for Ca, Cd, Cu, Fe, Mg and Zn the pattern of change was comparable to that previously observed in infectious conditions. Furthermore, among the patients after principal components analysis (PCA), 8 of the elements (Ag, As, Ca, Cd, Co, Fe, Mg and Zn) formed a cluster showing co-variation that could not be demonstrated in the controls. These elements would seem to be of particular interest in further studies of the pathogenesis of aortic valve sclerosis.

Paper III

Direct infections and damage of cells as well as low-grade inflammation may be the starting point for atherosclerotic lesions and diseases. In this process such essential nutrients as trace elements are needed for host defence reactions and healing of the inflammatory lesions, as well as for replication and surveillance of the infecting microorganism. In this study the concentrations of Ca, Fe, Mg and Zn were increased to a greater extent in the sclerotic heart valve tissue of the patients as compared with the forensic control cases with no known heart disease. No significant differences in trace element concentrations were found between those patients with or without serologic or molecular evidence of *C. pneumoniae* infection, suggesting that *C. pneumoniae* positive patients are pathogenically representative of aortic sclerosis patients in general. Furthermore, a larger increase in the Cu/Zn ratio in serum, as commonly encountered in active infections (108)(135), was observed in the patients as compared with a control group of healthy individuals. The frequency of women and men was similar in patients with aortic stenosis severe enough to require surgery (altogether 41 women and 47 men). Tissue Ca accumulation is correlated to the histopathological severity of this and other cardiovascular diseases

(136)(109)(110). Thus, Ca was used as an independent variable in regression studies of the other elements. Noteworthy was the substantial inter-individual variation observed for all the studied elements in the patients' valves, although all the patients had developed aortic stenosis severe enough to require surgery. Fe exhibited a strong correlation to Ca in the total patient group that persisted among *C. pneumoniae* PCR-positive patients when tested separately. Fe is essential for *C. pneumoniae* growth that is inhibited by iron restriction (116)(117). A recent study proposed that *Chlamydia* might use the iron transport pathways of the host by attracting transferrin receptors to the phagosom (118). Furthermore, Fe is an essential metal involved in vital cell functions and deposition has been demonstrated in atherosclerotic lesions. Fe augments the development of atherosclerosis in rabbits (137)(138). The iron hypothesis in atherosclerotic heart disease includes an interaction of macrophages and endothelial cells with Fe and LDL, although the underlying mechanisms are not fully understood. (114). In the patients' valves Zn and Mg were found to correlate to Ca as well. Zn is intimately involved in the regulation of immune function and is regarded as crucial for optimal T-cell functioning (129)(108). The elevation of Zn in the sclerotic valves may be related to the presence of immune cells as previously found in valve tissue in this disease (71). Zn and Fe are both components of antioxidative enzymes protective against the action of free radicals, radicals that have been shown to be involved in atherosclerosis (127). Mg has no yet defined role in immune function, and studies on Mg in inflammatory disorders are few. In the granulocytes of ankylosing spondylitis patients accumulation of Mg was demonstrated (139). Further, Mg deficiency may cause cardiac arrhythmia and sudden death (128). During active infection, redistribution occurs of certain trace elements between body compartments, resulting in an increase of serum Cu concentration and a decrease of serum Zn concentration (108)(135). Further, in experimental studies of various infectious diseases an increased Cu/Zn ratio has been commonly used as a marker of infection, even in the absence of clinical symptoms (140)(108). Thus, the increased Cu/Zn ratio in the present patients is compatible with the existence of an active infection. In the present study the frequency of *C. pneumoniae PCR*-positive valves was lower (34.8%) than in the study with similar patients (49%) discussed in paper I. Because methodologies were similar, this discrepancy might be ascribable to epidemiological variation. Notably, however, in atherosclerosis studies considerable inter-study variation in *C. pneumoniae* positivity rates exists, although reported methodologies are similar (85), suggesting that

consistency in methodologies is difficult. Noteworthy was the finding of a tendency of higher positivity rates among the female patients, a trend that was also noted in our earlier study. The percentage of cases showing *C. pneumoniae* IgA, a suggestive marker of chronic infection (85), was found comparable in the two studies. However, in both aortic valve studies not all of the *C. pneumoniae PCR*-positive patients were seropositive to this agent and seropositivity was often not associated with *C. pneumoniae* in the valve tissues. Similar discrepancies have been found in atherosclerosis studies (141). In conclusion, the trace element changes observed in the present study of aortic valve sclerosis are compatible with an immunologically active process. Although autoimmune events cannot be excluded, several features of the trace element changes in valves and serum in all our patients, as well as the finding of *C. pneumoniae* in the valves in a considerable proportion of our patients, are suggestive of an active infection, where the Fe results might represent a putative link to *C. pneumoniae.*

Paper IV

An inflammatory injury of the aorta and a complex interaction of different factors that influence the layers of the aortic wall have been proposed (82)(83). No pathogenic mechanism is common for all aneurysms. The most common histopathological picture described is a medial necrosis that is due to destruction of elastin fibrils. A reduction and degradation of these fibrils in abdominal aortic aneurysms (AAA) and in Marfan's syndrome have been reported (82)(83)(84). In several studies of AAA *C. pneumoniae* has been detected using a host of methods. However, other studies have failed to confirm these findings of a connection between *C. pneumoniae and* AAA (142)(143)**.** Recently, detection of viable *C. pneumoniae* has been reported in AAA (144). Furthermore, *C. pneumoniae* reactive T-lymphocytes have been demonstrated in the wall of AAA (145). In a recent in vitro study a relationship between the presence of *C. pneumoniae* and increased degradation of aortic elastin was demonstrated, suggesting *C. pneumoniae* as one possible aetiologic factor in AAA development (146). In vitro studies have demonstrated the persistence of *Chlamydia* in a metabolically active but culture negative cryptic form. The interaction of *C. pneumoniae* with its host cells and the ability to produce persistent intracellular infection have been suggested as a major factor in the pathogenesis of chronic Chlamydial infections (147). A proteolytic process and inflammation have been

associated with the development of AAA (148). Aortic dilatation appears to be stimulated by specific activation of macrophages and *C. pneumoniae* seems to influence that activation causing endothelial cell damage (149)(101)(100). The IgG antibody results (55% positivity) in the thoracic aortic aneurysm (TAA) patients in this study are in accordance with expected prevalence in the studied age group, whereas the IgA results of 48% positivity are higher than excepted. In one of our earlier studies antibodies to *C. pneumoniae* IgG and IgA in sera from blood donors were detected in 53% and 18%, respectively. Sero-epidemiological studies have demonstrated a relation to chronic infection and persistence of IgG antibodies (150)(151), but a poor correlation between MIF serology and PCR detection of vascular *C. pneumoniae* infection has also been reported. A possible association between AAA expansion and *C. pneumoniae* infection as measured by *C. pneumoniae* IgG and IgA antibodies detection was recently reported (142)**.** In addition, evidence suggests that some patients seem to be unable to produce an antibody response (18)**.** None of our four *C. pneumoniae* PCR-positive patients had IgA antibodies and 3 of the 4 patients indicated no IgG antibodies at all. This finding may indicate an increased susceptibility to this infection and impaired immune reactivity to this organism as measured by MIF tests. Persistent *C. pneumoniae* is known in some individuals to stimulate an immune response as measured by immunoblot but not by the MIF test (63)(152)(153). Although the present group of patients with dissection is small, the absence of detectable antibodies, as well as of PCR positivity to *C. pneumoniae,* in these patients is notable when considering that several of the aneurysm patients showed such positivity. In aortic dissection there is no primary inflammatory reaction (154). Accordingly, in this study the histopathological examination showed no signs of inflammation or atherosclerosis but changes described as cystic medial necrosis occurred in 4/6 of the patients. Thus, the pathogenesis of this condition differs from that described in TAA, where some of the patients are expected to have signs of inflammation. (154). In conclusion, the present study of TAAs and aortic dissection, in which samples were collected during surgery, demonstrated *C. pneumoniae* at the cellular level in some of the aneurysms, suggesting a potential role for the organism in the complex pathoaetiology of TAA. A conclusion implicating an association between *C. pneumoniae* and aortic aneurysm and dissection from serological results in individual patients seems inadvisable based on our present

knowledge. Further, controlled studies are necessary to prove tentative causality. If so future intervention studies on antibiotic and /or anti-inflammatory therapy seem warranted.

Paper V

In several studies on the in vitro activity of different antibiotics on *C. pneumoniae* the cells have either been pre-treated with antibiotics or the antibiotic has been added to the cells at the time of bacterial inoculation (155)(156). In the in vivo situation, *C. pneumoniae* infection is usually established as an intracellular infection before antibiotic treatment is initiated. In the present study the results show a significant difference in the MIC and MBC values for both azithromycin and doxycycline when using preincubated cells compared with experiments in which antibiotics were included from the start. The MIC and MBC values obtained in this study are higher than in several earlier studies (26)(157) even after 1h of preincubation. This is probably because a higher inoculum was used in this study. Furthermore, in other studies the number of experiments and the observed range are not always documented. We found a higher variability for the MBC than for the MIC tests, which may be partly because, according to our definition of MBC, every single inclusion has to be counted whereas 95% reduction is recorded for the MIC. Although the MIC values are within the range of achievable serum levels in vivo, none of the two antibiotics studied could attain extracellular concentrations after normal doses that equalled the MBC values obtained after 24h of preincubation. These results have to be evaluated with respect to the fact that the unique pharmacokinetic properties of azithromycin cause the latter to accumulate within cells and attain high intracellular concentrations, after which a slow release occurs. This process results in prolonged high intracellular concentrations, which may facilitate clearance and inactivation of intracellular bacteria (158)(159). The observed decrease in MIC values with prolonged antibiotic exposure time nicely illustrates the importance of sufficient antibiotic availability during the complete growth cycle of *C. pneumoniae* (a minimum of 72h). Immunofluorescence staining that compares inclusion bodies in the azithromycin- and doxycycline-treated cells produced varying results. In the azithromycin-treated cells small fluorescing particles were found more often at concentrations at which no inclusions could normally be found. This has been described by Wyrick et al. (1993), who made the observation after

treating *C. trachomatis* with azithromycin (160). Further studies revealed that these particles were empty envelope sheets of the Chlamydia cell wall. In the transmission electron microscopy photographs intracellular vacuoles were identified after 72h of treatment with high concentrations of azithromycin. This observation was not found in cells treated with doxycycline at the same concentration. Further studies are needed to evaluate the importance of the residual chlamydial components seen in the cell cultures after antibiotic treatment. These may be artefacts in a static assay or they may have a role in the persistence of chlamydial infections. There are not sufficient data today to establish reliable clinical guidelines for the treatment of severe *C. pneumoniae* infection.

SUMMARY OF PAPERS I-V

Paper I

A prospective study of aortic valves from patients undergoing aortic valve replacement was performed. Excised aortic valves were investigated for the presence of *C. pneumoniae* with PCR technique. Positive results were achieved in 19/39 (49%) patients compared with1/11 (9%) of forensic medicine cases without known heart valve disease. The results were confirmed by electron microscopy. This study was the first in which *C. pneumoniae* was detected in patients operated on for aortic valve stenosis.

Paper II

The aim of this study was to measure and compare differences in concentrations of trace elements in accordance with biologic activity. The study investigated the presence of 15 trace elements in aortic valves from 46 patients undergoing aortic valve replacement due to aortic stenosis. These 46 patients were compared with 15 forensic medicine control cases without known aortic valve disease. Analyses were performed using plasma masspectrometry. A high increase in concentrations of the trace elements calcium, iron, magnesium and zinc and a decreased concentration of copper were detected. Based on the present results, extended studies of the

connection to the intracellular processes, including the presence of *C. pneumoniae,* were planned (see Paper III).

Paper III

In this paper trace elements in aortic valves from patients with aortic stenosis and forensic medicine control cases were studied. Using statistic discriminant analyses, a cluster of trace elements was detected in the patients (see Paper II). Included among these tested elements were calcium, iron, magnesium and zinc with known biologic activity. Studies of correlation with reference to iron, magnesium and zinc to calcium showed significant differences. Testing of aortic valve for *C. pneumoniae* using PCR showed PCR positive cases to have similar trace element results. Furthermore, copper and zinc in serum from patients were tested, including sera from 46 patients and plasma from 46 healthy controls. In the patients a significantly elevated copper/zinc ratio as a marker of an ongoing infectious process was observed. In earlier in vitro studies a correlation between *C. pneumoniae* growth and iron has been reported. In the current paper an increased concentration of iron in the aortic valves was detected which possibly could have influenced the growth and persistence of *C. pneumoniae* intracellularly.

Paper IV

There are several possible causes to thoracic aortic disease. Syphilis was once a common aetiology to thoracic aortic aneurysm but is rare today. A correlation between *C. pneumoniae* and abdominal aortic aneurysm has been reported. In this study *C. pneumoniae* was detected by PCR in tissue from thoracic aortic aneurysm in 4/32 (12%) of patients and in none of 6 patients with thoracic aortic dissection. The detected *C. pneumoniae* PCR product was sequenced and *C. pneumoniae* specific inclusion bodies were observed using electron microscopy and immunogold labelling technique. Cell-cultured *C. pneumoniae* cells were used as control. At histopathology, cystic medial necrosis was shown in some of the cases, and inflammation in some others.

Paper V

The intracellular bacteria *C. pneumoniae* is difficult to eradicate and treat. Normal sensitivity/bactericidal tests are not fully applicable because of the special features of *C. pneumoniae*. An in vitro assay to measure and compare the efficacy of two potentially active antibiotics, doxycycline and azithromycin, was established. Both the minimal inhibitory concentrations (MIC) and the minimal bactericidal concentrations (MBC) were increased with extended bacterial preincubation times. The intracellular activity was investigated by electron microscopy and intracellular vacuoles were more often identified after treatment with high doses of azithromycin compared to doxycyclin.

CONCLUSION

- In approximately half of the patients operated on for aortic valve stenosis *C. pneumoniae* was detected in the sclerotic valves by PCR. There was no correlation to *C. pneumoniae* antibodies in serum as detected by microimmunofluorescence tests.
- Investigation of the concentration of 15 trace elements in sclerotic aortic valves demonstrated a statistically confirmed cluster of 11 elements that were changed including four elements with known biologic activity.
- A study of the four previously identified cluster forming trace elements in sclerotic aortic valve tissue showed, when associated with *C. pneumoniae*, a pattern of changes in accordance with an immunologically active process. Associated changes were reflected in serum and suggested a possible connection to infection, where iron and *C. pneumoniae* might represent a putative link in sclerotic valve disease.
- *C. pneumoniae* was detected by PCR and electron microscopy in some patients with thoracic aortic aneurysm but in none of the patients with thoracic aortic dissection, a condition with a different pathogenesis. These observations might indicate a role for *C. pneumoniae* in some cases of thoracic aortic aneurysm.
- Susceptibility of *C. pneumoniae* to doxycycline and azithromycin showed that the minimal inhibitory and bactericidal concentration values increased significantly with longer bacterial preincubation times.

ACKNOWLEDGEMENTS

I wish to express my sincere gratitude to all those who helped and supported me to make this work possible, with special attention

Göran Friman, my supervisor, for everlasting enthusiasm and patience, for sharing your vast knowledge and for invaluable scientific guidance over the years.
Stefan Thelin, my co-supervisor, for always letting me through with my calls and questions during long-lasting surgery procedures in the operating room and far away journeys.
Nils-Gunnar Ilbäck, my co-supervisor in the field of trace elements, for introducing me to a whole new world and for always showing genuine interest and sharing new ideas.
Eva Hjelm, my skilled co-worker at the lab over the years, for being willing to try new diagnostic approaches and for truly sharing my devotion to understand and fight "*Chlamydia pneumoniae*".
Ulf Lindh, for being my co-author, statistic advisor and for providing the most inspiring advice in the fields of trace elements.
Carl Påhlsson for innovative ideas in establishing the PCR technique and co-authorship.
Olle Lindquist, co-author, for sharing ideas and for providing professionally valuable help in collecting and analysing the control specimens and for your contribution to the pictures.
Lars Eriksson, co-author**,** for skilful evaluation of the histopathology.
Agneta Lukinius, co-author, for generously sharing her time for precious discussion and advice in analysing and presenting the EM results.
Otto Cars, former head of the Section of Infectious Diseases and co-author, for introducing me to the research field of antibiotics.
Lars Engstrand, visiting professor at the Department of Clinical Microbiology and co-author on the antibiotic paper.
Tina Hultén and **Ingegerd Gustavsson** for co-authorship on the antibiotic paper.

All the staff members at the Department of Medical Microbiology, with special thanks to **Christina Nylander**, **Ai-Jie Liu** and **Olga Svensson** for skilful technical assistance and for struggling with endless tissue samples.

Colleagues and staff members at the Departments of Thoracic Surgery and Anaesthesiology for excellent work in collecting all the patient samples. Special thanks to **Eva-Lena Annebäck**, **Gunnel Arvidsson Carlbring** and **Elisabeth Christensen** for perfect organisation of the specimens and registration of the questionnaires. **Margareta Roselius** for superior secretarial aid.

Stellan Bengtsson (late head), **Anna Hambraeus** (former head) and **Måns Ullberg** present head of the Section of Medical Microbiology for providing excellent working facilities at the laboratory
Britt-Marie Ericsson, present head and **Jan Sjölin** associate head of the Section of Infectious Disease, for maintaining the tradition of excellent leadership at our clinic.
Birgitta Sembrant, not only for your excellent secretarial help but also for always being ready to solve both minor and major problems with the same positive and professional attitude. It has been a pleasure to work with you, not least during my London years. Thank you!
Helena Hermelin for all the invaluable help with the histopathology specimens.

All my present and former colleagues at the Section of Infectious Diseases for your friendship and kind collaboration in the clinical field that made all the commuting miles worthwhile.

Cecilia Rydén for valuable skill and co-operation with respect to teaching during the past few months.
Lars Wesslén for sharing my interest for the intracellular ones with enthusiasm.
Marie Edvinsson, the experienced newcomer, for showing genuine interest in my research field.
Medical students, **Åsa Elfgren, Mattias Karlquist, Magnus Andersson** and **Jon-Eric Johnson**, for efficient data collection.
Carl Löfman and **Frank Bittowski** for excellent photographic production and for your immediate help in reproducing the 'lost' picture!
Henry Letocha and **Leslie Shaps** for excellent linguistic revision of all the manuscripts. Special thanks to Leslie for working off hours.

All the staff at the Section of Infectious Diseases for creating a positive atmosphere that encourages clinical and research endeavours in the field of infectious diseases and their treatment. It is a pleasure working with you.

Dan Marklund for patience in computer assistance.
Johan Bring for constructive statistical advice.

The staff at the Medical Library for excellent service over the years.
A special gratitude to all the patients participating in the various studies.

My **mother** and **father** in memory for your never failing support and love.

My dear **Tomas**, for your love and your unique sense of humour.

Adam and **Sebastian** for being the most wonderful sons now, as ever.

Financial support for this study was provided by The Swedish Heart and Lung Foundation, Mary, Åke and Hans Ländell Foundation, Swedish Society for Medical Research, The Medical Faculty of Uppsala University and Pharmacia and Upjohn, Sweden

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