Infection and Inflammation in progression of Atherosclerosis : the role of Chlamydiae pneumoniae infection in different mouse models

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Infection and Inflammation in progression of Atherosclerosis:

the role of *Chlamydiae pneumoniae* infection
in different mouse models

Infection and Inflammation in progression of Atherosclerosis:

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PROEFSCHRIFT

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aan de Universiteit Maastricht,
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CHAPTER 1

General Introduction

List of abbreviations

AHA	American Heart Association
ApoE-/-	Apolipoprotein knockout
bFGF	basic fibroblast growth factor
CMV	cytomegalovirus
COPD	chronic obstructive pulmonary disease
Cpn	Chlamydia pneumoniae
EB	elementary body
EC	endothelial cells
ECM	extracellular matrix
EIA	enzyme immuno-assay
eNOS	endothelial nitric oxide synthase
E-selectin	endothelial selectin
HB-EGF	heparin-binding epidermal-like growth factor
H. pylori	Helicobacter pylori
HDL	high density lipoprotein
Hep-2	human epithelial cell
ICAM-1	intercellular adhesion molecule
IFN γ	interferon gamma
Ig	immunoglobulin
IGFBP4	insulin-like growth factor binding protein 4
IL	interleukin
LDL	low density lipoprotein
LPS	lipopolysaccharide
MCMV	murine CMV
MCP-1	macrophage colony stimulating protein
MHSP65	microbial heat shock protein 65
MIF	macrophage inducible factor
MMP	matrix metalloproteinase
MOMP	major outer membrane protein
NF- B	nuclear factor
NO	nitric oxide
oxLDL	oxidised LDL
PAI-1	plasminogen activator inhibitor 1
PCR	polymerase chain reaction
PDGF	platelet derived growth factor
RB	reticulate body
ROS	intracellular reactive oxygen species
SMC	smooth muscle cell
TF	tissue factor
TNF α	tumor necrosis factor
VCAM-1	vascular cell adhesion molecule
VLA-4	very late activation antigen

Preface

Atherosclerotic disease is a complex condition with multiple contributing factors that may induce progression of clinical symptoms leading to unstable atherosclerotic plaques, ischemia and ultimately death. Yet, more than one third of the patients dying of coronary disease do not have any of the known risk factors.^{1,2} Therefore, studies concerning possible other risk factors for atherosclerosis are of extreme importance in the treatment of cardiovascular disease.

One of these factors potentially involved in atherosclerosis is infection. In particular, *Chlamydia pneumoniae* (Cpn), an obligate intracellular micro-organisms which causes (asymptomatic) upper airway infections, has drawn the attention of both clinical and basic scientists. Since the late 80's of the previous century, sero-epidemiological as well as experimental studies have supported a role for Cpn in the development and/or progression of atherosclerosis. The mechanisms by which Cpn contributes, if any, are still unknown. In this thesis, we tried to unravel some of the mechanisms by studying the influence of Cpn infection on atherosclerosis by using well established animal models.

Introduction

In the next sections we will briefly describe the etiology of atherosclerotic disease and the factors that are involved in the progression of atherosclerosis. First, the process of chronic inflammation, various mediators of the inflammatory process and the role of several pathogens causing infection of the vascular wall will be described. Then the epidemiology of Cpn and possible role of this micro-organism in the pathogenesis of the atherosclerotic process will be mentioned. Sero-epidemiological studies, clinical trials and experimental studies will be discussed to clarify several lines of evidence that link Cpn and atherogenesis.

Atherosclerosis

Cardiovascular disease continues to be the principal cause of death in Western countries.³ Complications of cardiovascular disease result in high morbidity and disability. Since this disease has a multi-organ character (myocardial infarction, cerebrovascular accident, peripheral arterial disease) diagnosis and treatment require a multidisciplinary treatment. Consequently the health care systems are confronted with high costs due to cardiovascular disease.

The main cause of cardiovascular disease is atherosclerosis, a systemic disease of the medium-sized and large arteries which ultimately affects all long living humans and may be present throughout one's lifetime. This process starts with early lesions in infants and children and progresses to an advanced atherosclerotic plaque after the

third decade of life. Finally, clinical symptoms occur later in life usually from the fifth decade.⁴

The localisation of the atherosclerotic lesion in the vascular bed determines its clinical manifestation. Atherosclerotic lesions in the coronary arteries may cause myocardial infarctions, while instable plaques in the carotid arteries may lead to transient ischemic attacks and stroke. In the renal arteries, plaques may cause renal ischemia and hypertension. Peripheral arterial vascular disease results from stenosis of arteries in the peripheral segment of the arterial tree (iliac, femoral, crural arteries) leading to lower limb ischemia and in the long run to critical limb ischemia (rest pain and/or gangrene). Occlusion/stenosis of the mesenteric arteries causes mesenteric ischemia ("angine abdominale") and bowel infarction.

In contrast to the familiarity with the clinical symptoms of atherosclerosis, its pathogenesis still needs to be elucidated. In the last decades several risk factors have been identified in the initiation and/or progression of atherosclerosis, like hypercholesterolemia, diabetes mellitus, hypertension, smoking, genetic alterations and obesity. Diagnosis and treatment of these risk factors is important to prevent development and progression of atherosclerotic disease.

Severity of atherosclerosis: lesion classification and composition

During the course of atherosclerotic plaque development various lesion types have been identified, ranging from small clinically silent lesions to large ruptured lesions with prominent intraplaque hemorrhage. Atherosclerotic lesions are classified according to histological observations, as described by the Committee on Vascular Lesions of the Council on Arteriosclerosis and approved by the American Heart Association (AHA).^{4,5} The classification divides the atherosclerotic process in six stages as shown in table 1. Type I-II are classified as early lesions that are clinically silent, while type IV and V plaques are designated as advanced lesions consisting of a fibrous cap surrounding a necrotic core. Stable advanced lesions can progress into unstable plaques which ultimately can lead to rupture of the fibrous cap and formation of a thrombus. This final stage is called a ruptured plaque or type VI lesion and is the underlying process in most acute clinical manifestations.

The initiation of atherosclerosis is characterised by adhesion of monocytes to the endothelium of the vascular wall, followed by diapedesis of monocytes into the subendothelial space and differentiation of monocytes into macrophages. When lipids accumulate in the macrophages foam cells appear, which are characteristic for a fatty streak or type I/II lesion according to the AHA classification. When lysis of foam cells occurs and more monocytes are recruited to the plaque, the fatty streak lesion progresses into a type III lesion containing extracellular lipids. Advanced lesions, type IV, contain a necrotic core, which consists of extracellular lipids and cholesterol-ester crystals, and a fibrous cap containing smooth muscle cells (SMC) cells, attracted from the media, and extracellular matrix (collagen). The fibrous cap overlies the necrotic core. Type V lesions can be subdivided in (I)Va, the fibroatheroma, consisting of

fibrous tissue and lipids, (II)type Vb, containing calcified segments and (III)type Vc lesions showing a fibrous component. The advanced lesions can be either stable, with a thick fibrous cap, rich in collagen and SMC or unstable, with a thin fibrous cap and a large lipid core. A thin fibrous cap is prone to rupture and leads to thrombus formation on the highly thrombogenic necrotic core. The resulting lesion is called a type VI lesion and is responsible for the clinical symptoms of atherosclerotic cardiovascular disease.

Table 1: Classification of lesion types

Lesion type	Nomenclature	Main histology	
Type I	Initial lesion	Isolated macrophage foam cells, adaptive thickening intima (smooth muscle cells)	Early lesions
Type II	Fatty streak	Intracellular lipid accumulation	
Type III	Intermediate lesion	Type II changes with small extracellular lipid pools	
Type IV	Atheroma	Type II changes with lipid core	Advanced lesions
Type V	Fibroatheroma	Fibrotic cap surrounding a lipid core with fibrous tissue and lipids (Va), calcifications(Vb), or fibrous component (Vc)	
Type VI	Complicated lesion	Cap disruption, hematoma –hemorrhage, thrombus	

Aetiology of atherosclerosis

In the pathogenesis of atherosclerosis several hypotheses have been postulated to elucidate the mechanism by which atherosclerotic lesions develop and progress. In the 70's Ross and co-workers described the response-to-endothelial-injury hypothesis.⁶ According to this hypothesis, local injury and activation of the endothelium caused by hyperlipidemia, mechanical injury or low shear stress activates the arterial SMC of the media to migrate to the intima and proliferate, also formation of connective-tissue matrix and deposition of lipids occur.⁷ Furthermore, lipid-laden macrophages and T-lymphocytes appear in the subendothelial space.⁸ With continued injury to the endothelium, SMC further proliferate and accumulation of connective tissue and lipid occurs. The response-to-injury hypothesis has been continually modified² describing endothelial dysfunction rather than endothelial injury. Elevated levels of modified low density lipoprotein (LDL), free radicals due to cigarette smoking are, among others, possible causes of endothelial dysfunction. Due to dysfunction of the endothelium, compensatory responses occur that lead to an increased adhesiveness and perme-

ability of the endothelium for leukocytes (mainly macrophages and T-cells). These cells are the most important contributors in the initiation and progression of atherosclerotic lesions. Endothelial dysfunction also induces upregulation of leukocyte adhesion molecules, like VCAM and ICAM-1. Then, growth factors like PDGF and bFGF, and cytokines (IFN γ and IL-1) induce differentiation of monocytes into macrophages and activation of macrophages and T-cells. When the dysfunction of the endothelium (due to e.g. hypercholesterolemia or hypertension) continues, cell influx and proliferation lead to the formation of more advanced lesions.^{8,9}

Besides the endothelial dysfunction hypothesis, the hypothesis of atherosclerosis as a chronic inflammatory disease has prevailed since the 90's.^{2,8,10} An inflammatory response in atherosclerotic disease is initiated by chemotaxis and adhesion to the endothelium. The endothelium is activated to express leukocyte adhesion molecules like E-selectin, ICAM-1 and VCAM-1¹¹ which recruit mononuclear leukocytes, i.e. monocytes and T-cells, to the vessel wall. Adhesion of leukocytes to the endothelium is initiated by rolling on the endothelial surface under the influence of selectins, followed by adhesion to the endothelium which is dependent on the interaction between VCAM-1, ICAM-1 and very late- activation antigen-4 (VLA-4). The latter is expressed by monocytes and lymphocytes.¹¹ Adhesion is then followed by migration of the leukocytes through the endothelium into the subendothelial space. Several chemokines (MCP-1),¹¹ cytokines (IL-2, IFN γ)¹² and fragments of complement proteins (C5a)¹¹ promote chemotactic migration of monocytes and T-cells into the tissue of the arterial wall.

Once activated monocytes and T-cells have arrived at the site of injury, they act as scavengers to remove the injured tissue and start the regenerating process. Macrophages secrete cytokines, oxygen radicals, proteases, complement factors and present antigens. After antigen presentation by macrophages, T-cells are activated and either secrete cytokines (T helper cells), or mediate cytotoxic activity against cells that express proteosomally processed peptides (cytotoxic T-cells). Also, activated T-cells upregulate transcription factor NF- κ B resulting in the release of IFN- γ and activation of macrophages. Oxidation of low-density lipoprotein (oxLDL), in the subendothelial space stimulates SMC proliferation¹³ and the immune complex of oxLDL and anti-oxLDL antibody are taken up by macrophages resulting in additional release of pro-inflammatory cytokines such as IL-1 β and TNF- α .¹⁴ After uptake of oxLDL by macrophages, foam cells are formed, which represents early lesion formation.

At this stage, macrophages secrete cytokines and growth factors that induce activation and proliferation of SMCs which migrate to the neointima and contribute to collagen synthesis. All these processes enhance the formation of an advanced atherosclerotic lesion. Also, activated macrophages produce and secrete matrix metalloproteinases (MMPs), that may degrade extracellular matrix (ECM) ultimately leading to a vulnerable plaque with a high risk of rupture.

Infection and atherosclerosis

At the beginning of the 20th century, Sir William Osler was the first to suggest a potential link between infections and progression of atherosclerosis¹⁵ His initial observation was confirmed by Frothingham¹⁶ and Klotz and colleagues.¹⁷ Since then, bacteria such as *Helicobacter pylori*, *Chlamydia pneumoniae* (Cpn) and viruses such as cytomegalovirus (CMV) and influenza have been suggested as mediators of the inflammatory response leading to progression of atherosclerosis.¹⁸⁻²⁰ Several sero-epidemiological studies, randomised controlled trials and experimental studies have been performed to elucidate the mechanism by which infections contribute to the progression of atherosclerotic disease.

Cytomegalovirus

More than 25 years ago, Benditt²¹ and Fabricant²² proposed a potential role for viral infections in atherosclerosis by demonstrating that chickens develop atherosclerotic-like lesions after infection with an avian herpesvirus, Marek disease virus. Since then CMV has been linked to restenosis,²³ transplantation-associated arteriosclerosis²⁴⁻²⁶ and atherosclerosis.^{27,28} Evidence regarding a role for CMV in atherosclerosis is based on sero-epidemiological studies and detection of CMV by culture, immunohistochemical staining, PCR or in situ hybridisation.^{10,29,30} Furthermore, Zhou et al. demonstrated that high IgG CMV antibodies in patients prior to coronary atherectomy was an independent risk factor for restenosis.³¹ In transplantation associated arteriosclerosis, several cross-sectional and prospective studies have linked CMV infection to graft rejection^{32,33} while experimental studies in rats using the aortic transplant model³⁴⁻³⁶ have demonstrated that influx of monocytes/macrophages and T-cells was seen in the adventitia of the allograft, thereby indicating that CMV interacts with the immune system in the transplanted vessel wall. Besides the role of CMV in restenosis and transplantation-associated arteriosclerosis, CMV is also linked to atherosclerosis.³⁷ Sero-epidemiological studies³⁸ and animal models³⁹ were used to corroborate a line of evidence that CMV infection contributes to progression of atherosclerosis.^{31,40,41}

Influenza

Death from myocardial infarction is more common in winter than in summer thereby suggesting a role for respiratory infections in cardiovascular mortality.^{42,43} This has been affirmed by a large case-control study of 9571 patients which demonstrated that an acute respiratory infection is a risk factor for acute myocardial infarction.⁴⁴ Also, influenza vaccination has been reported to protect against recurrent myocardial infarction, primary cardiac arrest,^{45,46} ischemic stroke⁴⁷ and to reduce the rate of hospitalisation and deaths of congestive heart failure.⁴⁵ Several mechanisms for the role of influenza in cardiovascular disease have been postulated. Firstly, influenza A has been demonstrated to affect plasma lipoproteins in mice. This is caused by loss of the

anti-inflammatory nature of HDL, an increase in thrombogenic proteins and enhanced susceptibility of LDL oxidation.⁴⁸ Secondly, an increase in cytokine production of IL-6, TNF- α and MCP-1 has been demonstrated in lung tissue and blood after influenza infection.⁴⁹ These cytokines are known to enhance the progression of atherosclerosis. Other mechanisms potentially involved are endothelial apoptosis,⁵⁰ endothelial dysfunction,⁵¹ and loss of thromboresistance.⁵⁰

Helicobacter pylori

Helicobacter pylori (*H.pylori*) is a gram negative pathogen that infects the mucus layer of the stomach and is associated with gastritis and peptic ulcer disease.⁵² Since the first report of *H.pylori* seropositivity in patients with coronary heart disease in 1994,⁵³ several studies have either confirmed⁵⁴⁻⁵⁷ or refuted^{58,59} the hypothesis that *H.pylori* influences atherogenesis through low-grade, persistent inflammatory stimulation. Furthermore, a recent report describes the correlation of *H.pylori* infection with antibodies to mHSP65 and elevated levels of coronary calcification, suggesting that pathogen-triggered autoimmunity plays a role in early atherosclerosis.⁶⁰

Periodontal infections

Periodontal disease is a disease of the oral cavity characterised by infection and inflammation of the supporting structures of the teeth including periodontal ligament, cementum and alveolar bone.⁶¹ Chronic periodontitis has been reported to cause a low-grade asymptomatic bacteraemia with activation of the coagulation system and an increase in serum fibrinogen level thereby enhancing the inflammatory response of atherosclerosis.^{61,62} Case-control studies and population-based studies⁶³⁻⁶⁵ have corroborated the association between periodontal infection and atherosclerosis. In vivo studies with ApoE $\pm$ mice have shown that long term systemic challenge with *Porphyromonas gingivalis*, an oral pathogen, can accelerate atherogenic plaque progression.⁶⁶ In this study the oral pathogen was administered intravenously, while Lalla and coworkers used the oral infection route for inoculation of ApoE $\pm$ mice and found that periodontal infection increases vascular inflammation (elevated levels of IL-6, increased aortic expression of VCAM-1 and tissue factor) and early atherosclerotic lesion formation.⁶⁷ In patients, tooth loss, a marker of past periodontal disease, is related to carotid artery plaque prevalence as shown by Desvarieux et al.⁶⁸ Proposed explanations for the role of periodontal infection in atherosclerotic vascular disease are transient bacteremia with systemic spread of infection from the oral cavity, endothelium injury by circulating oral microbial toxins and systemic inflammation triggered by oral micro-organisms.^{66,67,69}

Chlamydia pneumoniae

While the pathogens mentioned previously have all been associated to some degree with atherosclerosis, the strongest evidence for an association between pathogens and atherosclerotic disease comes from studies on *Chlamydia pneumoniae* (Cpn).^{18,70}

In the following paragraph, an overview of taxonomy, epidemiology, pathogenesis, and diagnosis of Cpn will be discussed. Several lines of evidence that link Cpn infection with atherogenesis will be presented.

Taxonomy

Cpn was first isolated in 1965 from the eye of a child during the trachoma vaccine study in Taiwan by inoculation of material from the child's conjunctiva in the yolk sac of an embryonating egg.⁷¹ The isolated strain was called TW-183. It was approximately 20 years later that Cpn was isolated from several patients with acute respiratory tract infections.^{72,73} The isolated strain was called AR-39. Since then, this Cpn strain has been displayed as TWAR (TaiWan Acute Respiratory disease), which is a combination of TW-183 and AR-39 strain.⁷³

Recently, Everett described the taxonomy of Chlamydia bacteria (Chlamydiales) by classifying them in four families based on genetic relationships: Chlamydiaceae, Parachlamydiaceae, Simkaniaceae and Waddliaceae.⁷⁴ By using analyses of ribosomal and coding genes on Chlamydiaceae, evidence was found for 2 new genera, Chlamydia and Chlamydophila and 9 different species. (table 2)

Table 2: Main characteristics of the Chlamydiaceae⁷⁴

Genus	Species	Route of entry	Host	Infected tissues
<i>Chlamydophila</i>	<i>pneumoniae</i>	pharynx, eye	human, koala, horse	artery, brain, joint, lung
	<i>psittaci</i>	pharynx, eye, genitalia	bird	brain, eye, genitalia, intestine, liver, lung, spleen
	<i>pecorum</i>	oral	cattle, sheep, pig, koala	bladder, brain, eye, intestine, lymph, joint, prostate
	<i>felis</i>	pharynx, eye, genitalia	house cat	eye, genitalia, joint, lung
	<i>caviae</i>	pharynx, eye, genitalia	guinea pig	bladder, eye, genitalia, lung
	<i>abortus</i>	oral, genital mammals	sheep	intestine, placenta, spleen, fetal liver
<i>Chlamydia</i>	<i>trachomatis</i>	pharynx, eye, genitalia, rectum	human	eye, genitalia, joint, prostate, neonatal lung
	<i>suis</i>	pharynx	swine	eye, intestine, lung
	<i>muridarum</i>	pharynx, genitalia	mouse, hamster	genitalia, intestine, liver, lung, kidney, spleen

Microbiology

Chlamydiae are obligatory intracellular bacteria with a unique biphasic life cycle characterised by elementary bodies (EBs), which are infectious, 0.2-0.6 μm in diameter and reside in extracellular form, and the reticulate bodies (RBs). In the beginning of the replication cycle after attachment to the host cell, EBs are ingested by endocytosis and transformed into RBs. These RBs are up to 1.5 μm in diameter, take up nutrients from the host cell and undergo several rounds of binary division within the endosome, leading to enlargement of the cell and formation of an inclusion (RBs are also displayed as inclusion bodies). Transition from RB back into infectious EBs occurs approximately 36 hours after host cell infection, with subsequent release at about 48 hours through exocytosis or rupture of the host cell. A new replication cycle is initiated when infectious EBs attach to new host cells.

The cell-wall of Chlamydiae consists of a Gram-negative envelope without peptidoglycan, although *Chlamydia trachomatis* and Cpn encode for proteins forming a pathway for synthesis of peptidoglycan and penicillin binding proteins.^{75,76} This is indicated by the term "Chlamydial peptidoglycan paradox". Chlamydiae also have a lipopolysaccharide (LPS) antigen and utilize host adenosine triphosphate for synthesis of Chlamydial protein.⁷⁷ In addition, all Chlamydiae encode a protein called the major outer membrane protein (MOMP), that is the major determinant for serological classification of chlamydial isolates⁷⁸ and is surface-exposed in *Chlamydia trachomatis* and *Chlamydia psittaci*.^{79,80}

An interesting phenomenon is the fact that Chlamydiae may remain in a persistent state instead of initiating a new replication cycle. This may occur after treatment with antibiotics, interferon- γ (IFN- γ) or restriction of certain nutrients,⁷⁷ During this persistent state, metabolic activity is reduced and subclinical infection is prolonged, which is one of the major characteristics of Chlamydiaceae (including Cpn).⁸¹ This phenomenon is important in achieving eradication of Cpn by antibiotic therapy.

Pathogenesis of Cpn infection

Recent studies have shown that not only acute respiratory infections are associated with Cpn infection: such as Alzheimer's disease,⁸² asthma,⁸³ atherosclerosis,⁸⁴ COPD,⁸⁵ multiple sclerosis,⁸⁶ and reactive arthritis.⁸⁷

Most respiratory infections caused by Cpn are subclinical with only a small percentage resulting in clinically apparent pneumonia. This pneumonia is named "atypical" pneumonia. Several other pathogens have also been shown to be involved in this so called "atypical" pneumonia such as *Legionella* and *Mycoplasma pneumoniae*.⁸⁸ The so called "typical" pneumonia is caused by the more "classical" bacteria such as *Streptococcus pneumoniae*, *Hemophilus influenzae*, *Pseudomonas aeruginosa* and *Staphylococcus aureus*. Approximately 10% of all pneumonia cases and 5% of all pharyngitis and sinusitis cases are caused by Cpn.⁸⁹ There is not a set of signs and symptoms unique to Cpn lower respiratory tract infections, although several signs and symptoms may support that Cpn plays a causal role. Early presentation of the disease with pharyngitis, dry cough, and headache can be observed. Cough and malaise can

persist for many weeks but most cases of Cpn induced pneumonia have a mild course not necessitating hospitalisation.^{89,90}

Epidemiology of Cpn infection

Sero-prevalence of Cpn varies from 50 to 70% among adults, which indicates that this pathogen is a common cause of infection. Cpn IgG antibody levels are very low or absent in children under the age of 5.⁹¹ They increase from the beginning of primary school age until adolescence, with an incidence of about 10% in those aged 5-10 years. By the age of 20, prevalence of specific IgG has already reached 50%, which may even increase with age.⁹²⁻⁹⁵ A low endemic level of Cpn infections is alternated with epidemics occurring every 2-4 years and have been reported to occur especially in closed communities such as the military.⁹⁶ The mode of transmission is probably through respiratory tract secretions. The duration of spread of infection is low, 5 to 7 months, compared to 2 weeks for influenza.⁹⁷ Incubation period for Cpn infection is estimated at 3 to 4 weeks.^{98,99}

Diagnostic tests for Cpn

Laboratory tests are used to diagnose acute respiratory tract disease. Although no standard laboratory protocol is available yet,^{88,100} the following tests are used: antibody detection (serology), culture of Cpn, detection of Cpn DNA/RNA by PCR and detection of Cpn antigens by immunohistochemistry.¹⁰¹

First, diagnostic tools for acute Cpn infection will be evaluated in this paragraph followed by diagnostic tests used to detect chronic Cpn infection, which will be described in the next section.

Serology

Patients with acute primary Cpn infection, develop IgM antibodies 2 to 3 weeks after onset of the illness. IgM antibodies can be detected until 2 to 6 months afterwards. In contrast, IgG antibody reaches a high titer after 6-8 weeks and can be detected for over 3 years after primary infection,¹⁰² while IgA response is delayed, weak or absent.¹⁰³ In the case of reinfection, IgM may not appear at all and IgG increases fast within 1 to 2 weeks after onset. Because interpretation of single high IgG titers is difficult, especially in elderly patients who have persistently elevated IgG levels due to multiple Cpn infections in the past, convalescent serum specimens are needed to show a 4 fold increase in titer. This often provides only a retrospective diagnosis of acute infection, making this diagnostic tool not optimal for patient management. However, it is useful in determination of prevalence of infection in epidemiological studies and in the case of an outbreak.

To analyse Cpn antibody titers in serum, the micro-immuno-fluorescence (MIF) test or enzyme immunoassays (EIA) are used.¹⁰⁴ Although MIF is considered the golden standard in chlamydia serology, it is difficult to standardise and different criteria are used for interpretation.¹⁰⁵ Therefore, several laboratories prefer the EIA for antibody detection using either recombinant LPS, other Cpn proteins, Cpn bacteria or extract-

ed antigens.⁸¹ These assays are either genus-specific, or are species-specific, respectively. In order to detect acute Cpn infection, paired serum samples, obtained 4-8 weeks apart, should be used. When IgM is detected at a titer of $\geq 1:16$ or IgG shows a 4-fold increase, acute Cpn infection can be considered, while IgG titers of $\geq 1:512$ are suggestive of an acute Cpn infection.¹⁰¹

Culture

For detection of viable Cpn, the cell culture technique can be used. Culture procedures are based on inoculation via centrifugation of a specimen (swabs of oropharynx, nasopharynx, sputum specimens, bronchoalveolar lavage specimens or biopsy specimens) onto human cells, mainly Hep-2 cells, grown in vitro. After incubation, cells are stained with a fluorescent-labelled antibody specific to Cpn to visualise the bacteria that are multiplying within these cells.

This test is labour intensive since multiple passages (at least 2) are necessary before a specimen can be considered negative. Special expertise is required for distinguishing true Cpn inclusions from artefacts, which is corroborated by the fact that only few laboratories are using this technique to detect Cpn in specimens of patients with infections. Therefore, culture is not recommended for large scale use in diagnostic laboratories.⁹⁰

Because of the low yield and technical complexity of culture, other tests, such as polymerase chain reaction (PCR), have been developed and are widely used for detection of Cpn.¹⁰¹

PCR

PCR is considered a validated test for detection of Cpn DNA during acute infections,¹⁰⁶ because it is reasonably fast and highly sensitive.¹⁰⁷ PCR enables detection of DNA and of RNA of pathogens that are present in small numbers, nonviable or growing slowly. Also, material not suitable for culture such as large tissue specimens, can be used in PCR analysis.¹⁰⁸ However, for routine use of Cpn PCR, several issues should be taken into consideration: choice of primers, optimisation of the assay, extraction of nucleic acids, detection of amplification products and prevention and identification of false-positive and false-negative results.¹⁰⁶ The most frequently used primers have been based on the *omp1* gene, the 16S rRNA gene, the 16S-23S spacer rRNA genes and a Cpn-specific cloned PstI fragment.¹⁰⁶ For detection of the amplification product, agarose gel electrophoresis, Southern blot, EIA and polyacrylamide gel electrophoresis can be used.⁸¹ To establish reproducibility of Cpn, detection of cDNA by reverse transcription-PCR of mRNA may be useful.¹⁰⁹ Lately, real-time quantitative PCR assays, allow highly reproducible, sensitive and specific quantification of Cpn in cultures and clinical samples.¹¹⁰

Immunohistochemistry

Finally, detection of Cpn antigens by immunocytochemistry, using genus-or species-specific antibodies labelled with a peroxidase or a fluorescent marker provide valuable

information concerning presence and localisation of Cpn to specific areas and cells in infected tissue.¹¹¹ Also, it offers the advantage of preserving tissue morphology, although no information can be obtained about the presence of viable bacteria. Detection of LPS can be achieved by using CF-2, a Chlamydia genus-specific monoclonal antibody, while RR402 and TT401 are Cpn-specific antibodies directed against elementary bodies of Cpn. Background staining¹¹² and nonspecific staining¹¹³ can make interpretation of results difficult and may decrease the specificity of this diagnostic tool.

Cpn diagnosis in atherosclerotic vascular disease

In chronic diseases that have been linked to Cpn infections standardised approaches for laboratory testing are lacking. In the following paragraph, the diagnostic tools available for detection of Cpn infection in atherosclerotic vascular disease will be described.

Serology

Diagnosis of a chronic Cpn infection by means of serology is still very challenging, since no serological marker has been validated. Although several studies have attempted to correlate single sample IgG or IgA antibody levels with disease status, no equivocal results found mainly because of the use of different cut-off points and methodologies.^{103,114} Also, it has been proposed that high IgA titers (half-life of 5 to 7 days) may be a better marker of chronic Cpn infection than the use of IgG antibody (half-life of weeks to months).^{101,103} The line between chronic and past infection is not completely clear since 1) past infection is also diagnosed by antibody detection and 2) detection of Cpn remnants without viable bacteria, a method suggested for diagnosis of past infection, is almost impossible to demonstrate and is therefore not in use. When the MIF and EIA technique were compared, the agreement between EIA and MIF in detection of IgG and IgA antibodies was adequate.¹¹⁵ Therefore, EIA may be a practical alternative to the MIF technique to diagnose (chronic) Cpn infection in patients with cardiovascular disease.

Culture

Culture of Cpn from blood vessels is technically challenging and has been successful in only very few cases when multiple serial passages were used.¹¹⁶⁻¹¹⁸ Nonetheless, culture remains important to document the viability of the organism and, to provide isolates of the organism for characterisation and for evaluation of antimicrobial susceptibility. Also, for assessment of microbiological efficacy in treatment trials culture remains an essential diagnostic tool.¹¹⁹ Dowell and colleagues described recommendations for culture in atherosclerotic vascular disease.¹⁰¹ One of the controversies regards the number of passages used before the results are determined.^{116,120,121} Most laboratories used more than 2 passages to maximise the recovery of Cpn isolates from respiratory specimens. But when increased passages are used it may result in concentration of cell debris and possible non-specific staining of the monolayer thus resulting in false positive data.

PCR

Although PCR is considered a validated method for detection of Cpn DNA in acute infections, for chronic infections no standardised protocol is available yet. In chronic infections, the bacterium is mostly present in very small quantities, which necessitates a highly sensitive PCR thereby increasing the risk of contamination.¹⁰⁶ Studies have shown that in these cases DNA extraction procedures and storage conditions of samples play a pivotal role.¹⁰⁶ Also, atherosclerotic tissue is known to contain inhibitors of PCR, which makes it difficult to estimate the prevalence of Cpn in vascular tissue material. Nowadays, with the diversity of PCR techniques, a rise in shortcomings have also been detected, which has stimulated several laboratories to develop their own in-house PCRs. Therefore, consensus of the best Cpn PCR has not been ascertained yet.¹²² This also explains the reported interlaboratory variation in the performance of PCR tests.

Immunohistochemistry

Detection of Cpn antigens in human vascular plaque material using immunostaining showed variable detection rates from 21 to 71% between different laboratories.^{123,124} Moreover, when in the same atherosclerotic lesion presence of Cpn was analysed using different methods: culture, PCR and/or immunohistochemistry, a poor correlation was observed.¹²³⁻¹²⁵ Interestingly, Cpn detection rates determined by immunohistochemistry are higher than those determined by PCR. This can be assigned to several factors: 1. DNA shows faster degradation than antigens, 2. extraction of DNA from plaques can be difficult, due to calcifications and 3. presence of PCR inhibitors may result in false negative PCR results.^{123,124,126} These observations show that the interpretation of the discrepant results from different studies is difficult in the absence of a gold standard. Recently, guidelines have been formulated¹⁰¹ which makes it possible to obtain optimal results from immunohistochemistry. These guidelines recommend to use 2 Chlamydia antibodies and 2 control antibodies for each tissue block. Also, staining should run 1 positive and 1 negative tissue control, each incubated with the 4 antibodies. When interpreting the intracytoplasmatic staining of cells a granular pattern may be considered positive, while a homogeneous staining pattern is controversial.¹⁰¹

Since different monoclonal antibodies vary in type and specificity, detection rates vary widely, which emphasises the importance of the use of the same monoclonal antibodies when comparing results of different studies.¹⁰⁸

Association Cpn and atherosclerosis

Sero-epidemiological studies have reported an association of Cpn antibodies with myocardial infarction, chronic coronary heart disease⁶⁴ and an increased relative risk for cardiovascular complications.¹²⁷ The presence of Cpn DNA¹²⁸, Cpn antigen^{118,128} and viable Cpn¹¹⁸ in the vascular wall has been associated with atherosclerosis. However, these studies did not demonstrate the mechanism by which infections could contribute to the progression of atherosclerotic disease, leaving the "innocent bystander" hypothesis (Cpn would be an innocent bystander instead of a moderator of

the atherosclerotic process) open for discussion. In vitro and in vivo studies¹²⁹⁻¹³¹ have attempted to unravel the role of Cpn infection in atherosclerosis. Also, by using antibiotics directed against Cpn infection, insight can be gained in the role of Cpn infection in the progression of atherosclerotic disease.

Role of Cpn infection in the atherosclerotic process: studies in animal models

Animals that have been used to study the effect of Cpn infection on progression of atherosclerosis are mainly normocholesterolemic and hypercholesterolemic rabbits (New Zealand White rabbits) and mice (C57 Black/6J, Apolipoprotein knockout, ApoE3-Leiden and low density lipoprotein-receptor knockout mice). In the following paragraph, the role of Cpn infection on the progression of atherosclerosis will be discussed using animal models and in vitro studies.

C57 Black/6J (C57BL/6J) mice on a normal diet do not develop atherosclerosis, even after intranasal infection of Cpn.¹³² However, when an atherogenic diet was supplemented to these mice, fatty streak lesions were observed in the aortic root, which represents early atherosclerosis lesion formation.¹³³ By using this mouse strain to evaluate the effect of Cpn infection on atherosclerotic lesion progression, Blessing and colleagues found that chronic Cpn infection accelerates lesion development in hypercholesterolemic C57BL/6J mice.¹³⁴ When the atherogenic diet was given following Cpn infection, no atherosclerotic lesion progression was observed,¹³⁵ suggesting that Cpn is a co-risk factor for cardiovascular disease. Another study has confirmed this observation: in low density lipoprotein-receptor knockout mice (LDL-receptor^{-/-}) the atherogenic effect of Cpn infection is dependent on the presence of hyperlipidemia.^{136,137} Also, Tiirila and colleagues showed that acute Cpn infection causes a transient increase in serum triglycerides and lipid binding protein, one of the key proteins involved in lipoprotein metabolism in mice.¹³⁸ Hyperlipidemia plays a pivotal role in oxidation of LDL in the vascular wall, leading to foam cell formation. In contrast to these observations, several studies have reported progression of atherosclerosis independent of hyperlipidemia.¹³⁹⁻¹⁴¹

Moazed et al.^{140,142} demonstrated that intranasal Cpn infection of Apolipoprotein knockout (ApoE^{-/-}) mice results in a significant increase in lesion area. Other groups using ApoE^{-/-} mice studied the effect of repeated Cpn infections on endothelial dysfunction and found that infection impaired arterial endothelial function, with a pivotal role for the nitric oxide (NO) pathway in endothelial signalling.^{129,143-145} This is supported by Chesebro et al.¹³⁹ who demonstrated an increase in lesion size in wild type mice, fed a high cholesterol diet, following Cpn infection. This increase was comparable to the increase in lesion size in endothelial NO synthase knockout mice. However, no additional effect of Cpn infection was observed in these knockout mice suggesting that Cpn infection results in endothelial dysfunction with subsequent atherosclerosis. Besides in the thoracic aorta of ApoE^{-/-} mice, endothelial dysfunction after Cpn infection has also been demonstrated in epicardial and resistance vessels of pigs.¹⁴⁶

In addition to the association between Cpn infection and endothelial dysfunction, the role of pro-inflammatory cytokines in the progression of atherosclerosis has been

described in different reports. In vitro infection by Cpn of human U-937 macrophages increases the production of IL-1 β , IFN- γ and TNF- α , while infection of human coronary artery endothelial cells induces IL-8 production indicating a chemokine response to infection that may play a role in recruitment of inflammatory cells to sites of infected vascular cells.¹⁴⁷ A recent in vivo study by Burián et al.¹⁴⁸ evaluated the role of inflammatory cytokines after Cpn infection of Balb/c mice. Increased levels of IL-3, -6, -4 and IFN- γ were detected in lungs tissue. The same research group evaluated the inflammatory process in the aortic vascular wall of normocholesterolemic Balb/c mice after co-infection with Cpn and CMV.¹⁴⁹ Although no atherosclerotic lesions were observed, the combined effect of MCMV and Cpn induced inflammatory foci in the aortic wall which were more pronounced in the co-infected mice than in the mice infected with MCMV alone.

Also in rabbits a few studies have evaluated the role of Cpn infection in atherosclerotic lesion progression.^{141,150,151} Using normocholesterolemic New Zealand White rabbits, atherosclerotic lesions in the aorta were observed following single intranasal Cpn infection^{150,151} and multiple infections,¹⁴¹ providing further evidence for a role for Cpn in atherosclerotic disease initiation and/or progression.

Role of Cpn infection in the atherosclerotic process: in vitro studies

Next to Cpn research in animal models, in vitro studies have been used to elucidate mechanisms by which Cpn may contribute to atherosclerosis.

Previous studies demonstrated that the organism is able to infect many cell types at cardiovascular sites, including circulating monocytes, arterial smooth muscle cells (SMC) and vascular endothelial cells (EC). Infected cells exhibit characteristics associated with the development of cardiovascular disease, such as achievement of pro-coagulant state and secretion of pro-inflammatory cytokines by infected EC and foam cell formation by infected macrophages.¹⁵²

In the initiation of atherogenesis, endothelial dysfunction and subsequent release of inflammatory mediators plays an essential role. It has been demonstrated that Cpn is able to infect circulating CD14⁺ mononuclear cells of humans¹⁵³ and Cpn was detected in blood of mice infected intranasally with Cpn.¹⁵⁴ Probably, Cpn uses the mononuclear cells as a vehicle for entry into the vessel wall.¹⁵⁵ Instead of using mononuclear cells for entry into the vessel wall, Cpn may also enter through adventitial layers of large blood vessels. Another mechanism by which Cpn may enter the vessel wall is by direct cell-cell transmission from mononuclear cells to EC.¹⁵⁶ Molestina and colleagues¹⁵⁷ have demonstrated that Cpn infection of EC induces IL-8 and MCP-1 thereby stimulating transendothelial migration of monocytes and neutrophils and differentiation of monocytes into macrophages. In addition, after infection of human vascular EC an upregulation of various adhesion molecules (VCAM-1, ICAM-1, P-selectin, E-selectin) and IL-6, IL-8, IL-1 β , TNF- α , and MCP-1 has been reported.¹⁵⁶⁻¹⁶⁰ Since adhesion molecules are important in leukocyte rolling and adhesion and subsequent extravasation of leukocytes into the intima, these in vitro data suggest a contributory role for Cpn in atherosclerotic lesion development.

It has been demonstrated that Cpn infects the main cell types of the atherosclerotic lesion, such as macrophages, SMC, EC and T-cells.¹⁶¹ Activation of these cells results in a continuous release of inflammatory mediators. Collectively, these data support that Cpn infection of the vascular wall is involved in the chronic inflammatory process.^{155,162} Activation of T-lymphocytes in the plaque can be established by antigens like ox-LDL.¹⁶³⁻¹⁶⁵ Cpn has also been shown to activate T-cells cultured from peripheral blood of patients with atherosclerosis as well as T-cells cultured from carotid endarterectomy tissue.¹⁶⁶ Recently, it has even been demonstrated that Cpn can infect lymphocytes of human peripheral blood and mouse spleens as well as a T-lymphocyte cell line.¹⁶⁷ T-cells upregulate the transcription factor NF- κ B resulting in release of IFN γ and activation of macrophages.¹⁵⁵ The extracellular release of chlamydial antigens including LPS and HSP-60 all contribute to the inflammatory response. cHSP-60 induces T-cells to produce TNF- α ,¹⁶⁸ a potent pro-inflammatory cytokine which contributes to oxidation of LDL¹⁶⁹ while antibodies to HSP-60 are cytotoxic for EC.¹⁷⁰ This could result in additional release of inflammatory mediators. LPS induces EC production of reactive oxygen species which contributes to the formation of oxLDL within the neointima.⁶² Subendothelial oxLDL stimulates SMC proliferation¹³ and immune complexes of oxLDL and anti-oxLDL antibody are taken up by macrophages resulting in additional release of pro-inflammatory cytokines IL-1 β and TNF- α .¹⁴

Evidence for involvement of Cpn in the formation of foam cells and fibrous caps has been established by several in vitro studies.¹⁵⁵ Kalayoglu and coworkers demonstrated that Cpn LPS induced oxidation of LDL, uptake of oxLDL by macrophages and subsequent foam cell formation.^{169,171} Atherosclerotic lesion progression is characterised by advanced plaques consisting of a fibrous cap surrounding a necrotic core. In the process of cap formation, activation of SMC and production of the ECM and MMPs play a pivotal role.² Kol et al.¹⁵⁹ have shown that cHSP-60 stimulates MMP-2 and -9 production by macrophages. This may contribute to breakdown of ECM and internal elastic lamina. Cpn infected EC release soluble factors that stimulate SMC proliferation¹³⁰ and induce upregulation of SMC growth factors and related proteins (PDGF-B, b-FGF, HB-EGF, IGFBP4) as demonstrated by DNA microarray studies.¹⁶⁰

In the final stage of atherogenesis, plaque erosion, rupture and thrombus formation have been linked to the presence of Cpn. Macrophages have been shown to produce MMPs after being stimulated with cHSP-60 or Cpn^{159,172} This may alter the ECM, thereby contributing to plaque destabilisation and rupture. After Cpn infection of SMC, an upregulation of TF and PAI-1 was observed, thereby enhancing a procoagulant condition.^{173,174} Furthermore, activated macrophages and SMC release reactive oxygen species. This contributes to cellular cytotoxicity, activation of CD8+ T-cells and formation of necrotic core.¹⁷⁵⁻¹⁷⁷ A recent study by Nazzari and co workers¹⁷⁸ observed an increase in necrotic cell death after infection of human EC with Cpn. This process was modulated by ox LDL and was correlated with an increase in intracellular reactive oxygen species (ROS) concentration, which is considered to be involved in the orientation of cells toward necrosis. Increase of necrotic cell death in the atherosclerotic lesion could further aggregate to rupture of the plaque.

Antibiotic therapy for prevention or stabilisation of the atherosclerotic process in animals

Since several lines of evidence have linked Cpn infection with progression of atherosclerotic vascular disease,¹⁷⁹ the question was posed whether antibiotic therapy would be an option for prevention or stabilisation of the atherosclerotic process.¹⁸⁰ To define the duration and quantity of antibiotic treatment several animal models were used.

Muhlestein and coworkers infected 30 New Zealand White rabbits on an atherogenic diet with 3 separate Cpn inoculations (3.105 inclusion forming units, IFU) or saline at 3 weeks intervals and treated them with azithromycin or no therapy for 7 weeks.¹⁸¹ Maximal intimal thickness as well as plaque area in the thoracic aorta was increased in the infected rabbits compared to both uninfected controls and infected rabbits receiving antibiotics.

Using clarithromycin therapy after 3 intranasal Cpn infections (2 weeks apart) in rabbits, Fong et al. found a reduction in the number of plaques and a reduction in the Cpn antigen content in the vascular tree.¹⁸² Treatment beginning early after inoculation, was more effective than delayed treatment (starting 2 weeks after inoculation and continuing for 6 weeks).

Earlier studies performed by the same group,^{183,184} reported similar results although azithromycin was used instead of clarithromycin with shorter treatment protocols.¹⁸³ Comparison of different antimicrobials with antichlamydial activity (azithromycin, clarithromycin, moxifloxacin, doxycycline) demonstrated that early treatment i.e., within 5 days after infection with all antibiotics, prevents aortic lesions (efficacy 75% for clarithromycin and 85% for azithromycin). Delayed treatment, 6 weeks after infection, with azithromycin was ineffective in reducing atherosclerotic lesions, while clarithromycin resulted in 62.5% reduction.¹⁸⁴

Using ApoE^{-/-} mice, Rothstein and colleagues explored the hypothesis that azithromycin therapy inhibits the atherogenic effects of Cpn infection.¹⁸⁵ Ten-week old mice were infected twice with 5.106 IFU Cpn or placebo and treated with azithromycin 2 weeks after the last infection. Although Cpn infection increased lesion size of the aortic lesion in infected mice, azithromycin therapy did not reduce plaque area. Therefore, the authors concluded that immediate therapy of acute infection may be necessary to prevent the proatherogenic effects of Cpn infection.

In line with these results, Blessing and coworkers¹⁸⁶ observed no effect of azithromycin therapy on reduction of atherosclerotic lesion progression in ApoE^{-/-} mice after infection with Cpn. Mice were inoculated three times once a week with 3.107 IFU Cpn and treated with azithromycin for 6 weeks starting 3 days after the last inoculation. This study shows that the start of antibiotic treatment immediately after the last inoculation¹⁸¹ is essential in these animal models to prevent acceleration of lesion progression. Furthermore, the inoculation protocol used in the present study¹⁸⁶ is much higher (weekly 3.107 IFU) than the dosage used by Muhlestein¹⁸¹ (3 separate inoculations, 3.105 IFU, 3 weeks apart).

Outline of this thesis

Atherosclerosis is a multifactorial disease. It is generally accepted that inflammation of the vascular wall plays an important role in its development. Cpn infection has been proposed as one of the multiple moderators of the inflammatory process.

In this thesis, several possible mechanisms by which Cpn may aggravate the atherosclerotic process have been evaluated. First we studied the systemic dissemination of Cpn after intratracheal and intraperitoneal infection in C57Bl6/J mice (**Chapter 2**). This mouse strain was used since it is the genetic background for breeding of most of the transgenic and knockout mice (ApoE3-Leiden, ApoE $-/-$, and LDL-receptor, ApoE double knockout mice) used in atherosclerotic studies. In **Chapter 3**, we used one of these atherosclerotic mouse strains (ApoE3-Leiden) to study the effect of Cpn infection on atherosclerosis progression (plaque area/type) and inflammatory processes in the atherosclerotic vascular wall. Mice were infected with Cpn and sacrificed at different time points post infection. As Cpn significantly accelerated the formation of complex lesions, we decided to study the effect of Cpn infection on plaque formation in normal C57Bl6J mice fed an atherogenic diet. These mice normally develop only early lesions and we hypothesised that additional, multiple Cpn infection may result in the formation of more complex lesions (**Chapter 4**). In a subsequent study we evaluated whether the accelerated formation of more complex lesions, as observed earlier in ApoE3-Leiden mice, may ultimately result in destabilisation of atherosclerotic plaques with an increased risk for plaque rupture. As ApoE3-Leiden mice develop complex atherosclerotic plaques only slowly we decided to use the ApoE, LDL-receptor double knockout strain (ApoE/LDLr $-/-$), which develops atherosclerosis much faster and studied plaque composition in detail (**Chapter 5**). Azithromycin has been shown in both human and animal studies to be an effective therapy for reduction of the effects of Cpn on atherosclerosis. By using ApoE/LDLr $-/-$ mice, we evaluated the effect of early treatment with this antibiotic on Cpn-induced alterations in plaque size as well as plaque composition (**Chapter 6**). Finally, in **Chapter 7** the observations as described in this thesis are discussed and related to the hypothesis that Cpn contributes to the progression of atherosclerosis.

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CHAPTER 2

Short communication:
systemic dissemination of *Chlamydia pneumoniae*
after intratracheal and intraperitoneal infection, a
study in C57 Black mice

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Abstract

Objective: Although infection with *Chlamydia pneumoniae* (Cpn), a respiratory pathogen, has been associated with several chronic inflammatory diseases, knowledge about the dissemination of the pathogen in its host is scarce. To investigate this, mice were infected with Cpn through different inoculation routes. Dissemination was determined by means of Cpn DNA presence in various organs.

Methods: C57Bl/6J mice were inoculated with Cpn intraperitoneally (ip, 5.107 IFU) or intratracheally (it, 105 IFU) and sacrificed at 2, 4, 6, 14, and 28 days post infection to study presence of Cpn DNA in various organs.

Results: Real time Cpn PCR demonstrated that both ip and it Cpn inoculation induces systemic dissemination to lung, bone marrow, spleen and vascular tree. After ip inoculation the vascular tree contains Cpn DNA up till 6 days after inoculation, while after it inoculation significantly lower copy numbers were observed in the vascular tree (ip: 1711 ± 1563 Cpn copy numbers vs. it: 264 ± 256 , $p < 0.05$).

Conclusion: This study demonstrates that Cpn has the ability to disseminate systemically after both it and ip inoculation. Furthermore, as ip inoculation resulted in a significant higher number of Cpn DNA copies in vascular tissue material, this inoculation route is preferred over it inoculation if you want to study the effect of Cpn on atherosclerosis.

Chlamydia pneumoniae (Cpn) is an intracellular replicating pathogen, which causes respiratory infections such as pneumonia, bronchitis and pharyngitis.¹ The pathogen has also been associated with extrapulmonary chronic inflammatory diseases as arthritis² and atherosclerosis.³ Serological evidence of anti-Cpn antibodies in patients with cardiovascular disease³ as well as the presence of Cpn DNA in atheromatous plaques⁴ contribute to the alleged role of Cpn in the process of atherosclerosis.

Nevertheless, although Cpn has been found frequently in vascular tissue, the exact mechanism by which this pathogen disseminates from the lungs to the vascular wall and as such contributes to atherosclerosis has not been clarified yet. It has been postulated that monocytes or macrophages may take up and transport Cpn to the vascular wall. The mechanism of systemic dissemination to lung, thymus, spleen and abdominal lymph nodes has been studied in C57 BL/6J mice after intranasal infection with Cpn.⁵ Although intranasal inoculation "mimics" the natural route of Cpn infection, it also has several disadvantages. Intranasal inoculation stimulates sneezing which may lead to less volume of Cpn in the lungs but it also has the risk of infection of the stomach instead of lungs. Intratracheal (it) inoculation does not have the disadvantages of intranasal inoculation. Therefore, intratracheal Cpn inoculation might be superior to intranasal infections to study the effect of Cpn on various pathologies as atherosclerosis. In our previous experiments,⁶⁻⁸ we used intraperitoneal inoculation. Alternatively, mice can be infected by intraperitoneal injections, which might be the most exact way with respect to amount of inclusion forming units (IFU) applied to the experimental model. However, no data concerning systemic dissemination for both routes have been published so far. Therefore, to determine which route is superior in dissemination in particular to the vascular wall, we evaluated both inoculation routes and studied the systemic dissemination of Cpn to target organs.

Chlamydia pneumoniae

Cpn strain TWAR 2043 (ATCC VR-1355) was cultured in Hep-2 cells as previously published.⁹ Bacterial titres were determined by titration in Hep-2 cells and stained with fluorescein-isothiocyanate-conjugated (FITC) monoclonal antibody RR 402 (Dako, Denmark) directed against elementary bodies of Cpn.

Dissemination study

To study the dissemination of Cpn, 34 10-week old C57BL/6J mice were either infected intratracheally (it, n=16) or intraperitoneally (ip, n=18) with Cpn. A pilot study was performed to evaluate the exact dose for it inoculation with Cpn. In this study, mice were inoculated with different doses of Cpn (105-106 IFU). Inoculation of mice with 105 IFU resulted in successful Cpn infection of the mice as shown by presence of Cpn DNA in lung tissue of all mice (data not shown). Higher doses of Cpn were lethal and were not used in our study. For ip inoculation 5.107 IFU Cpn was used as shown in previous work of our laboratory.⁶⁻⁸

Before inoculation, mice were sedated with a weight-adjusted dose of pentobarbital (Nembutal, Sanofi BV, Maassluis, The Netherlands). Then, a volume of 50 μ l sterile PBS containing 105 IFU Cpn was instilled intratracheally via a canule, followed by 0.1 ml of air. After intratracheal treatment, the mice were kept in an upright position for several minutes to allow sufficient spreading of the fluid throughout the lungs. Ip infection was performed by injection of 100 μ l sterile PBS containing 5.107 IFU Cpn into the abdominal cavity. At sacrifice, 2, 4, 6, 14, and 28 days post inoculation, mice were anesthetized with pentobarbital and blood was collected from the left ventricular apex of the heart. The arterial tree was perfused with phosphate-buffered saline through a catheter placed in the left ventricular apex. Lung, spleen, bone marrow cells, and vascular tree (aortic arch, thoracic and abdominal aorta) were dissected and stored at -70°C for Cpn DNA analysis.

Cpn Polymerase Chain Reaction(PCR)

DNA was extracted from vascular tissue, lung, spleen, and bone marrow with the Wizard Genomic DNA purification kit (Promega, Germany). For detection of Cpn DNA in target organs, the real-time Cpn PCR (ABI Prism 7000) was used. Primers and probes for the detection of Cpn were based on a variable domain of the ompA gene, VD4: forward primer 5' TCCGCATTGCTCAGCC3' reverse primer 5'AAACAATTTG-CATGAAGTCTGAGAA3' and probe 5'-FAM-TAAACTTAACTGCATGGAACCCTTCTT-TACTAGG3'-TAMRA. PCR was carried out in 96-well optical reaction plates (Applied Biosystems, Foster City, CA) with 25 μ l total volume, containing: 12.5 μ l TaqMan Universal Master Mix, (2 times concentrated, Applied Biosystems) 300nM of forward primer, 900nM reverse primer, 200nM probe, 3.5 μ l water, and 5 μ l of the sample (1 μ g of DNA). Also, to generate a standard curve, serial dilutions of Cpn strain were used. The concentrations of the dilutions were: 2.5.102, 2.5.103, 2.5.104, 2.5.105, 2.5.106 copies. A negative control, which consisted of reagents only was included. The ABI Prism 7000 SDS Software was used to calculate the Cpn DNA concentrations in the unknown samples using the data from the standard curve. Every sample was measured twice and the mean values were used for analysis.

The probe carried a 5' FAM reporter and a 3' TAMRA quencher group. Thermal cycling started with UNG activation for 2 minutes at 50°C, followed by HotStarTaq activation during 10 minutes at 95°C. Thereafter 42 cycles of amplification were run consisting of 15 seconds at 95°C and 1 minute at 60°C.

Statistical analysis

Differences in copy numbers of Cpn DNA in different target organs were determined by the Mann-Whitney U-test. Data are expressed as mean $\pm$ SEM. A P-value of < 0.05 was considered statistically significant.

Systemic dissemination of *Cpn*

The results of the dissemination profile of *Cpn* after ip and it infection, are expressed in figures 1 A, B, C, D.

Real-time PCR performed on vascular tree material revealed a significant number of *Cpn* DNA copies after ip inoculation, while in it-inoculated mice *Cpn* DNA was undetectable or only a limited number of copies were found (figure 1A).

Cpn DNA levels in spleen were high at 2, and 4 days after ip inoculation with the highest copy number found at 2 days post infection. Thereafter, copy number decreased rapidly. In it-inoculated mice low to undetectable *Cpn* DNA copy numbers were found in spleen tissue (figure 1B).

In bone marrow cells, *Cpn* DNA copy number was highest after ip inoculation at 2 days post inoculation. After it inoculation *Cpn* DNA was detected only at 2 days post inoculation, while copy number at other time points was negligible (figure 1C).

Real-time PCR performed on lung samples showed detectable copies of *Cpn* DNA at every time point post infection. It-infected mice showed, as expected, higher copy numbers with a peak at 4 days post infection. Copy number in ip-inoculated mice was highest at 4 days post inoculation (figure 1D).

Figure 1: A Copies of *Cpn* DNA detected in vascular tree(A), spleen(B), bone marrow cells (C), and lung (D), $n=3$ at each time point post inoculation, ip: intraperitoneal inoculation, it: intratracheal inoculation, nd: not detected. Data are expressed as mean $\pm$ SEM. * statistically significant difference between intraperitoneal and intratracheal infected mice at the specific days post inoculation ($p<0.05$)

In these experiments we clearly showed that although Cpn is a respiratory pathogen, systemic dissemination is obtained both after intratracheal and intraperitoneal inoculation. In our study we used a quantitative technique (real-time Cpn PCR), which enabled us to quantify Cpn DNA copy numbers while other studies^{5,10} used conventional Cpn PCR which only reports presence of Cpn DNA without information about quantity. In this study, we demonstrated that intraperitoneal Cpn inoculation is the preferred inoculation route to study the effects of Cpn infection on atherogenesis, since by using this inoculation route higher concentrations of Cpn in the vascular tree are found. Before reaching the vascular wall, Cpn must be transported from the infection site to target organs. Since Cpn elementary bodies have never been detected freely in the circulation, the pathogen must depend on a host cell, possibly (alveolar) monocytes or macrophages,¹¹⁻¹³ to reach a target organ. In our study, we did not analyse the role of alveolar macrophages after Cpn infection.

Thus, whether alveolar macrophages act as a permanent source of Cpn-infected monocytes or macrophages still remains unclear. Moreover, it is not known whether the same Cpn-infected alveolar macrophage is able to migrate from the lung into the bloodstream and reach the vascular wall. Also, lymphocytes have been reported as a possible vehicle for Cpn, since they are susceptible to Cpn infection *in vitro* and invade an atherosclerotic plaque.¹⁴⁻¹⁷ Besides monocytes, macrophages, and lymphocytes, Cpn has the capacity to multiply in other immune cells as well. Gieffers and coworkers¹⁰ reported that granulocytes may act as carriers of Cpn to reach target organs and potentially cause chronic and persistent infections.

In summary, results from this study demonstrate that both intraperitoneal and intratracheal Cpn infection induce systemic dissemination. Intraperitoneal inoculation of Cpn is the preferred inoculation route in mice to study the effect of Cpn infection on atherosclerotic lesion progression, since it is an easy inoculation route to perform and high concentrations of Cpn in the vascular wall are obtained by this inoculation route.

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CHAPTER 3

Chlamydia pneumoniae accelerates formation of complex atherosclerotic lesions in Apo E3-Leiden mice

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Abstract

Objective: Atherosclerosis is an inflammatory process and is characterised by presence of T-lymphocytes in the lesions. To study the role of *Chlamydophila pneumoniae* (*C. pneumoniae*) in this process and the effect of infection on T-cell influx, we infected Apo E3-Leiden mice with *C. pneumoniae* and investigated the effect on lesion development and T-cell influx in atherosclerotic lesions at different time points post infection (pi).

Methods: Nine week old mice, fed an atherogenic diet, were either mock-infected or infected with *C. pneumoniae* and sacrificed at 1, 6 and 9 months pi. Longitudinal sections of the aortic arches of the mice were stained with hematoxylin-eosin for atherosclerotic lesion type and lesion area analysis, or with rabbit-anti-CD3+ to detect the presence of T-cells in the atherosclerotic lesions. T-cell influx was expressed as number of T-lymphocytes/lesion area.

Results: At 1 month pi, type 1, 2 and 3 lesions were present. At other time points pi, more complex lesion types 4, 5a and 5b were present as well. Although infection did not influence the total lesion number or area, we observed an effect of *C. pneumoniae* infection on lesion type. Infection resulted in a significant shift in lesion formation from type 3 to type 4 ($p=0.022$) at 6 months pi, and from type 4 to type 5a ($p=0.002$) at 9 months pi. T-cells were observed at every time point pi. At 1 month pi a significant increase in T-cell influx in the *C. pneumoniae*-infected atherosclerotic lesions was observed ($p=0.0005$).

Conclusion: This study shows that *C. pneumoniae* infection enhances the inflammatory process by the increase of T-lymphocytes in the plaque and accelerates the formation of complex lesions.

Cardiovascular complications due to atherosclerosis are the major causes of morbidity and mortality in the Western world. Besides several well known risk factors, such as smoking and hypercholesterolemia,¹ infections have been suggested to play an important role in the development of atherosclerosis over the last decades.²⁻⁴ Infectious agents like Cytomegalovirus, *Chlamydophila pneumoniae* (*C. pneumoniae*), *Helicobacter pylori*, and bacteria causing dental infections all have been linked to atherosclerosis.^{1,5-7} For *C. pneumoniae* the association has been demonstrated in sero-epidemiological, experimental, and clinical studies.^{4,8-15} However, the mechanism by which *C. pneumoniae* contributes to atherosclerosis has not been clarified yet. To understand this mechanism, various animal models, mostly rabbits and mice, have been used.^{11-14,16} Progression of atherosclerosis is reported in New Zealand White rabbits,¹⁵ as well as in low-density lipoprotein receptor (LDLR) knockout mice¹¹ inoculated with *C. pneumoniae*. In all of these studies, the effect of *C. pneumoniae* infection was only documented on lesion size. However, little is known on the effect of infection on the development and/or progression of lesion types. Since lesion type is an important indicator of severity of atherosclerosis, we studied the effect of *C. pneumoniae* infection on the type of atherosclerotic lesions by grading the lesions according to the guidelines of the American Heart Association.^{17,18}

In understanding the atherogenicity of *C. pneumoniae*, insight should be gained concerning the ability of this micro-organism to cause local infection and inflammation of the vascular wall as characterised by influx of leukocytes, consisting mainly of monocytes and T-lymphocytes. When T-lymphocytes are activated they can act as a powerful source of pro-atherogenic cytokines thereby contributing to the progression of atherosclerotic lesion formation from fatty streaks into complex atherosclerotic plaques.¹⁹ Immunohistochemical studies have demonstrated the presence of T-lymphocytes in human^{20,21} and animal²² atherosclerotic plaques. Activation of T-lymphocytes in the plaque can be established by antigens like ox-LDL.²³⁻²⁵ However, *C. pneumoniae* has also proven to be a good candidate for activating T-cells cultured from peripheral blood of patients with atherosclerosis as well as T-cells cultured from carotid endarterectomy tissue.²⁰ Recently it has even been demonstrated that *C. pneumoniae* can infect lymphocytes of human peripheral blood and mouse spleens as well as a T-lymphocyte cell line.²⁶

In this study, the role of *C. pneumoniae* in atherosclerotic lesion progression was studied using Apo E3-Leiden mice. Mice were infected with *C. pneumoniae* and sacrificed at different time points post infection (pi) to analyse lesion progression in the aortic arch and main branchpoints. To understand the possible mechanism by which *C. pneumoniae* accelerates atherosclerotic lesion progression, we studied inflammation of the vascular wall by determining the influx of T-lymphocytes in the atherosclerotic lesion.

Materials and methods

Mice

Sixty-four transgenic Apo E3-Leiden mice on a C57Bl/6J background were bred at the Central Animal Facility at the Maastricht University.²⁷ For the study, both males and

females were used and randomly divided over all groups. All mice were specific-pathogen-free (SPF) and were housed under standard conditions. This study was approved by the institutional committee for the welfare of laboratory animals of the Maastricht University.

C. pneumoniae

C. pneumoniae strain TWAR 2043 (ATCC VR-1355) was cultured in HEp-2 cells as previously described.²⁸ Bacterial titers were determined by titration in HEp-2 cells and staining with monoclonal antibody RR 402 (Dako, Glostrup, Denmark) followed by fluorescein-isothiocyanate (FITC) conjugated rabbit-anti-mouse (Dako, Glostrup, Denmark). Titers were expressed as inclusion forming units per milliliter (IFU/ml).

Experimental design

At the age of 5 weeks, the mice received a high-fat cholesterol (HFC) diet containing 15% cacao butter, 0.5% cholate, 1% cholesterol, 40.5% sucrose, 10% corn starch, 1% corn oil, and 4.7% cellulose (Hopefarms BV, Woerden, The Netherlands). Since it has been demonstrated that multiple infections, the age of the animals at the time of inoculation and the interval between inoculations are all critical factors in mice for enabling *C. pneumoniae* to promote atherosclerosis,^{11,14,29,30} we decided to inoculate our mice twice with *C. pneumoniae* using different time intervals between inoculations. By infecting the mice for the second time at different time points after the primary infection we also aimed to study the effect of *C. pneumoniae* either on early lesions or more advanced lesions. All mice received the first infection by intraperitoneal injection of *C. pneumoniae* 6.10⁷ IFU at the age of 9 weeks (figure 1). Control mice were injected with sterile phosphate-buffered saline (PBS, mock infection). Since no differences in dissemination of *C. pneumoniae* after intraperitoneal or intranasal inoculation have been reported,³¹ we selected intraperitoneal infection in our mice. In group 1, mice received a second inoculation at the age of 11 weeks and were sacrificed at the age of 13 weeks, 1 month after the first infection (figure 1).

Figure 1: Design of animal experiment, showing the different mice groups analysed in this study, the inoculation time points and time points of sacrifice.

At this time point type 2 lesions are the predominant phenotype in the aortic arch¹⁸. In group 2, mice were inoculated for the second time at the age of 29 weeks and were sacrificed at the age of 33 weeks, 6 months after the first infection when type 4 and 5a lesions are present in the aortic arch¹⁸. Finally, mice in group 3 received a second inoculation at the age of 41 weeks and were sacrificed at the age of 45 weeks, 9 months after the first infection with calcified type 5b lesions most prominent¹⁸.

Tissue handling

At sacrifice, the mice were anesthetized with a weight-adjusted dose of pentobarbital³² (Nembutal, Sanofi Sante BV, Maassluis, The Netherlands) and blood was collected from the left ventricular apex for assessment of lipid profiles and anti-*C. pneumoniae* antibody titers. The arterial tree was perfused with PBS followed by 1.85% PBS-buffered formaldehyde, both containing 0.1 mg/ml sodium-nitroprusside (Merck, Darmstadt, Germany), through a catheter placed in the left ventricular apex. The arterial tree was removed, fixed overnight in 3.7% PBS-buffered formaldehyde and embedded in paraffin. Four μ m thick longitudinal sections were consecutively cut, stained and analysed.¹⁸

Evaluation of atherosclerotic lesions

Since all lesion types were found in the aortic arch,¹⁸ lesion analysis was restricted to this segment of the arterial tree. Only aortic arches with an intact morphology (brachiocephalic trunk, left and right common carotid artery and left subclavian artery) were used.

Of the entire arch, longitudinal, coronal, consecutive sections were made. Every fifth section was hematoxylin-eosin (HE) stained to evaluate lesion type, size and number. Since atherosclerotic lesion development progresses over time thereby increasing the size of the aortic arch, more sections were collected from the aortic arches of group 2 (33 weeks of age) and 3 (45 weeks of age) than from arches of group 1 (13 weeks of age). Five HE-stained sections from each aortic arch were analysed in group 1, while in group 2 and 3, 11-15 sections per aortic arch were analysed. The type of atherosclerotic lesions was determined according to the guidelines of the American Heart Association (table 1).¹⁷ The size of the lesions was determined using a microscope coupled to a computer-assisted morphometry system (Quantimet 570, Leica, The Netherlands). For every lesion, the mean area was calculated and total lesion area was calculated as the sum of all mean areas. All sections were analysed by impartial investigators (interobserver variability <10%), who were blinded with respect to the presence or absence of *C. pneumoniae* infection as well as to the time point of sacrifice.

Detection and evaluation of T-lymphocytes in atherosclerotic lesion

In order to detect T-lymphocytes in the atherosclerotic lesions, a rabbit-anti-CD3⁺ polyclonal antibody was used (Dako, Glostrup, Denmark). After a blocking step with Bovine Serum Albumin (BSA)/PBS 2% for aspecific binding, slides were incubated with the CD3⁺ polyclonal antibody for 60 minutes at a dilution of 1:200, followed by a second-

step incubation with a biotinylated swine-anti-rabbit IgG (1:1000, Dako, Glostrup, Denmark) for 30 minutes. Sections were then labelled with an alkaline phosphatase coupled ABC reagent (Dako, Denmark) for 30 minutes. Alkaline phosphatase activity was visualised using Fast-red (Sigma Chemical Co, St. Louis, United States). Rabbit-anti-rat IgG (Dako, Glostrup, Denmark) was used as a negative control. For positive controls, tissue sections from mice spleens were used. T-lymphocytes in the atherosclerotic lesion were counted and expressed as the number of T-lymphocytes in the plaque divided by the total plaque area. The investigator was blinded for the presence or absence of *C. pneumoniae* infection as well as to the time point of sacrifice.

Table 1: Classification of atherosclerotic lesion types according to the Stary classification defined in 1995

Lesion type	Nomenclature	Main histology	
Type I	Initial lesion	Isolated macrophage foam cells, adaptive thickening intima (smooth muscle cells)	Early lesions
Type II	Fatty streak	Intracellular lipid accumulation	
Type III	Intermediate lesion	Type II changes with small extracellular lipid pools	
Type IV	Atheroma	Type II changes with lipid core	Advanced lesions
Type V	Fibroatheroma	Fibrotic cap surrounding a lipid core with fibrous tissue and lipids (Va), calcifications(Vb), or fibrous component (Vc)	
Type VI	Complicated lesion	Cap disruption, hematoma –hemorrhage, thrombus	

Detection of C. pneumoniae antigens and anti-C. pneumoniae antibodies

In order to detect *C. pneumoniae* antigens, a Chlamydia genus-specific mouse monoclonal antibody, CF-2 (Washington Research Foundation, Seattle, Washington, USA) was used.³³ This monoclonal is directed against chlamydial lipopolysaccharide. For each mouse, a representative section with the highest lesion number and the most advanced lesion type, as analysed by the HE staining, was used for *C. pneumoniae* detection. The primary antibody was probed with goat-anti-mouse IgG conjugated with peroxidase (M.O.M.T Kit, Vector, Brunswick, Amsterdam, The Netherlands). To visualise the antibody binding, 3,3'-diaminobenzodine (DAB, Sigma Chemical Co, St. Louis, United States) was used. Normal mouse ascitic fluid (Sigma-Aldrich Chemie BV, Zwijndrecht, The Netherlands) was used as negative control. Tissue sections from mock-infected mice were used as controls. For positive controls, sections of *C. pneumoniae*-infected HEp-2 cells were used which were embedded in agarose, fixed overnight in 3.7% PBS-buffered formaldehyde and embedded in paraffin.

Anti-*C. pneumoniae* antibodies were measured with the indirect micro-immunofluorescence (MIF) technique using mouse plasma sample dilutions 1:10 and 1:100 on antigen-coated slides (Labsystems, Helsinki, Finland). Goat-anti-mouse IgG conjugated to FITC (Dako, Glostrup, Denmark) was used as the secondary antibody. The presence of anti-*C. pneumoniae* antibodies was determined by 2 independent observers.

Assessment of lipid profile

Total plasma cholesterol and triglyceride concentrations were determined by a standard cholesterol oxidase method performed on Beckman Synchron CX Systems.¹²

Statistical analysis

The difference in lesion type between the *C. pneumoniae* and mock group was compared and analysed with the Chi-square test. Differences in lesion number and size were analysed with the Mann-Whitney U test. To determine differences in T-cell influx in the lesions the Mann-Whitney U test was used. Plasma lipids of *C. pneumoniae*- and mock-infected groups were compared with the Student's 2-tailed t test. A P-value of < 0.05 was considered statistically significant. Data are expressed as mean $\pm$ SEM.

Results

To investigate the effect of *C. pneumoniae* infection on atherosclerotic lesion type, number and total lesion size, sixty-four Apo E3-Leiden mice were infected, sacrificed and analysed. Seven mice died before reaching point of sacrifice, leaving fifty-seven mice for analysis. The cause of death was unknown.

C. pneumoniae infection leads to more severe atherosclerotic lesions

In the *C. pneumoniae*-infected group as well as in the mock-infected group atherosclerotic lesions were found. The type of lesion was analysed according to the histology criteria expressed in table 1, which are based on the Stary classification defined in 1995.¹⁷

At 1 month pi, type 1, 2 and 3 lesions were found. The more advanced lesion types 4, 5a and 5b, were present at 6 and 9 months pi and consisted of a fibrous cap surrounding a necrotic central core. The presence of these lesion types at these time points is in agreement with previous report.¹⁸ Type 6 lesions were never observed in any of the mice at any given time point pi.

The effect of *C. pneumoniae* infection on atherosclerotic lesion types is presented in table 2. The complexity of the atherosclerotic lesion types increases in time when lesion types of 1 month pi were compared to lesion types of 6 and 9 months pi. At 6 months pi, all lesion types, except type 1 lesions, were present, while at 9 months pi, type 1 and 2 lesions were absent. When the distribution of the lesion types in the *C. pneumoniae*-infected and mock-infected group was analysed, a significant shift in lesion type was observed from type 3 to type 4 at 6 months pi ($p < 0.05$, figure 2), while at 9 months pi, a significant shift was seen from type 4 to type 5a ($p < 0.01$, figure 3).

Table 2: Atherosclerotic lesion types in *C. pneumoniae*- and mock-infected mice. a Presence of mean number of lesions of each type is expressed as the mean frequency $\pm$ SEM. pi post infection, * statistical significant shift from type 3 to type 4 ($p=0.022$), ** statistical significant shift from type 4 to type 5a ($p=0.002$), -- lesion type is not present. Total lesion area (right) in *C. pneumoniae*- and mock-infected group at different time points pi.

Inoculation (number of mice)	Lesion type ^a						Lesion area (mm ²)
	1	2	3	4	5a	5b	
<i>C. pneumoniae</i> (8)	0.38 $\pm$ 0.18	2.13 $\pm$ 0.79	2.00 $\pm$ 0.57	--	--	--	122 $\pm$ 9
mock (8)	0.25 $\pm$ 0.17	0.75 $\pm$ 0.34	1.50 $\pm$ 0.45	--	--	--	81 $\pm$ 6
<i>C. pneumoniae</i> (7)	--	0.14 $\pm$ 0.15	1.71 $\pm$ 0.56	2.86 $\pm$ 0.98*	1.57 $\pm$ 0.40	1.86 $\pm$ 0.28	969 $\pm$ 29
mock (7)	--	0.43 $\pm$ 0.32	3.14 $\pm$ 0.68*	1.29 $\pm$ 0.39	1.29 $\pm$ 0.20	1.43 $\pm$ 0.22	933 $\pm$ 34
<i>C. pneumoniae</i> (6)	--	--	0.83 $\pm$ 0.44	0.83 $\pm$ 0.18	3.08 $\pm$ 0.92**	2.00 $\pm$ 0.69	1802 $\pm$ 85
mock (8)	--	--	0.63 $\pm$ 0.40	2.63 $\pm$ 0.53**	1.88 $\pm$ 0.32	2.13 $\pm$ 0.24	1860 $\pm$ 105

Figure 2: Lesion types in the *C. pneumoniae* and mock group 6 months post infection. The values are presented as the mean frequency $\pm$ SEM at which that type of lesion was present. * $p=0.022$

Figure 3: Lesion types in the *C. pneumoniae* and mock group 9 months post infection. The values are presented as the mean frequency $\pm$ SEM at which that type of lesion was present. * $p=0.002$

C. pneumoniae infection has no effect on lesion size and number of lesions

The HE-stained sections used for classification of atherosclerotic lesion types were also used for determination of lesion area and number of lesions. Data on the total lesion area are presented in table 2. No statistically significant differences in lesion area could be detected when the *C. pneumoniae*-infected and mock-infected group were compared at the different time points.

The number of atherosclerotic lesions was analysed in all HE-stained sections. The number of lesions in the *C. pneumoniae*-infected and mock-infected group, at the different time points pi, was calculated. Data on lesion number are presented in table 3. There were no significant differences in total lesion number between *C. pneumoniae*-infected and mock-infected mice at any time point pi.

Table 3: Total lesion number in *C. pneumoniae*- and mock-infected group at different time points pi. Data are expressed as mean $\pm$ SEM.

Time pi	Inoculation (number of mice)	lesion number
1 month	<i>C. pneumoniae</i> (8)	4.38 $\pm$ 0.88
	mock (8)	2.75 $\pm$ 0.75
6 months	<i>C. pneumoniae</i> (7)	8.57 $\pm$ 0.85
	mock (7)	8.00 $\pm$ 0.88
9 months	<i>C. pneumoniae</i> (6)	6.67 $\pm$ 1.01
	mock (6)	7.25 $\pm$ 0.75

Table 4: Cholesterol and triglyceride levels in *C. pneumoniae*- and mock-infected group at different time points pi. Data are expressed as mean $\pm$ SEM.

Time pi	Inoculation (number of mice)	Cholesterol mmol/l	Triglycerides mmol/l
1 month	<i>C. pneumoniae</i> (8)	2.47 $\pm$ 0.73	4.85 $\pm$ 1.65
	mock (8)	2.93 $\pm$ 0.63	5.15 $\pm$ 1.41
6 months	<i>C. pneumoniae</i> (7)	3.69 $\pm$ 1.00	4.83 $\pm$ 0.97
	mock (7)	6.87 $\pm$ 3.30	2.90 $\pm$ 0.80
9 months	<i>C. pneumoniae</i> (6)	4.58 $\pm$ 1.17	6.76 $\pm$ 1.90
	mock (6)	6.83 $\pm$ 2.73	4.92 $\pm$ 1.44

Figure 4: Number of T-cells in atherosclerotic lesions at 1 month pi. Data are presented as number of T-cells divided by total plaque area. * $p=0.0005$

Figure 5: (A,B) T-lymphocyte staining showing positive cells (arrows) in atherosclerotic lesion of *C. pneumoniae*-infected mouse, 1 month pi. Magnification 100x (A) and 400x (B) (C) *Chlamydia pneumoniae* staining. *Chlamydia pneumoniae* was detected in a type 3 lesion of an infected mouse, 1 month post infection. Staining is concentrated in the subendothelial layer of the atherosclerotic lesion (see arrows). Magnification 200x.

C. pneumoniae infection results in an increased T-cell influx in the atherosclerotic lesions

The effect of *C. pneumoniae* infection on the influx of T-cells was determined at every time point pi in atherosclerotic lesions of the *C. pneumoniae*-infected mice and the mock-infected mice (figure 4). More specifically, at 1 month pi a significant increase in T-cell influx in the *C. pneumoniae*-infected atherosclerotic lesions was observed. Infection resulted in a significant increase in T-cells from 0.29 cells/m² in the mock group to 2.63 cells/m² in the *C. pneumoniae* group ($p=0.0005$, figure 5). At 6 and 9 months pi no effect of *C. pneumoniae* infection on the influx of T-cells was observed.

Lipid profiles

At 1, 6 and 9 months pi blood was collected from *C. pneumoniae*- and mock-infected mice for determination of plasma cholesterol and triglyceride levels. When the *C. pneumoniae* and the mock groups, within one time point pi, were compared, no statistically significant difference was found in cholesterol and triglyceride plasma levels, suggesting that *C. pneumoniae* infection does not affect plasma lipid levels in the long run (data not shown).

C. pneumoniae infection

All *C. pneumoniae*-infected mice seroconverted after inoculations with *C. pneumoniae*, indicating that all our inoculated mice had indeed been successfully infected. In all these mice anti-*C. pneumoniae* antibodies were found at plasma dilutions of 1:10 and 1:100. No anti-*C. pneumoniae* antibodies were detected in plasma of mock-infected mice.

C. pneumoniae antigen was detected by immunohistochemistry staining in about 30% of the aortic arches of the infected animals at the time points 1, 6 and 9 months pi. *C. pneumoniae* antigen was mainly found in the subendothelial layer of the atherosclerotic lesions of these mice as shown in figure 5c. Since the percentage of positive *C. pneumoniae* antigen was equally divided over all lesion types, no preference of *C. pneumoniae* antigen for a specific atherosclerotic lesion type was observed (data not shown). As expected, mock-infected mice were all negative.

Discussion

Infection with *C. pneumoniae* has been associated with the process of atherosclerosis,^{4,9} but whether infection influences the initiation or progression of atherosclerosis, or whether it plays a role in plaque (in)stability, has not been clarified yet. Most previously published experimental studies on *C. pneumoniae* infections have reported on the effect of infection on atherosclerotic lesion size.^{11,14,15,29} Nonetheless, no reports on the effect of *C. pneumoniae* infection on lesion type have been published yet. Since the type of atherosclerotic lesions may be a more important indicator for morbidity and mortality than lesion size or number, we evaluated the incidence of different

atherosclerotic lesion types. In this study we demonstrate that *C. pneumoniae* infection leads to an accelerated formation of complex atherosclerotic lesions. At 6 and 9 months pi, an acceleration in lesion type development was seen, whereas at 1 month pi, no effect of *C. pneumoniae* infection on early lesion formation was observed. This is an intriguing finding as it contributes to the suggestion that *C. pneumoniae* infection may promote progression^{11,14-16,29} of the atherosclerotic process rather than its initiation.³³ The effect of *C. pneumoniae* on the lesion formation seems to be the result of a long standing process since its effect is only found on later time points, 6 and 9 months pi. This suggests that an antigen-specific immune activation early after infection may contribute to the progression of atherosclerosis.^{1,6,9} Since *C. pneumoniae* has been shown to infect macrophages and use them as a transport vehicle for dissemination³¹ it may be speculated that *C. pneumoniae* enters the vascular wall in this way. Once entered the vascular wall *C. pneumoniae*-infected macrophages may act as antigen-presenting cells thereby attracting T-cells. Indeed, in the present study we demonstrated a marked enhanced T-cell influx shortly after the first infection. Since activated T-cells are a powerful source of pro-atherogenic and pro-inflammatory cytokines, such as IFN- γ , IL-2 and TNF- α ,³⁴ this may explain the observed acceleration of plaque development in the long run. On the other hand, T-cells themselves may also function as a transport vehicle for *C. pneumoniae*. Recently it has been shown that *C. pneumoniae* can infect and multiply in T-cells.²⁶ Furthermore, a significant higher proportion of *C. pneumoniae* positive T-cells were observed in patients with coronary artery disease compared to healthy blood donors.³⁵ Future co-localisation studies may shed some light on where *C. pneumoniae* is exactly located in the vascular wall of our infected mice.

Besides atherosclerotic lesion type, we also determined the effect of *C. pneumoniae* infection on lesion size and number and found no difference between the *C. pneumoniae*- and mock-infected mice. This corresponds to findings in previous studies, who also did not find any effect of *C. pneumoniae* infection on lesion size.^{36,37} In contrast, other studies reporting an increase in lesion size after infection with *C. pneumoniae* have been published as well.^{11,14} The difference between other studies and ours may result from differences in *C. pneumoniae* strain, inoculation schedule or type of animal model used. LDLR knockout¹¹ and Apo E knockout mice^{14,36,37} are the mice strains used by other groups. In the present study Apo E3-Leiden mice were used.¹⁸ It is known that different mouse strains vary in susceptibility to *C. pneumoniae* infection and the formation of atherosclerotic lesions.³⁸ Of the latter studies, only one reports of elevated lipid levels after *C. pneumoniae* infection in LDLR knockout mice.¹¹ However, in the present study and in previous reports^{14,36,37} no differences between infected and non-infected groups were observed in cholesterol or triglyceride levels, excluding this as a predominant factor.

In humans, the first association between *C. pneumoniae* infection, myocardial infarction and chronic heart disease was shown in patients with anti-*C. pneumoniae* antibodies by Saikku et al.⁴ Later on several other studies have supported this finding suggesting a correlation between *C. pneumoniae* infection and cardiovascular dis-

ease.^{8,9,39,40} However, the correlation between presence of anti-*C. pneumoniae* antibodies and *C. pneumoniae* antigen in atherosclerotic lesions is questionable.^{12,15,33,39,41} A similar finding is observed in our study as well, since all infected mice were positive for anti-*C. pneumoniae* antibodies while *C. pneumoniae* antigen was detected in only one third of the aortic arches. This discrepancy may be explained by the notion that immunohistochemical staining has a limited sensitivity detecting *C. pneumoniae* in vascular tissue. A second explanation could be that anti-*C. pneumoniae* antibody in plasma and *C. pneumoniae* antigen in aortic tissue do not necessarily have to be present at the same time.³⁶

In summary, this study demonstrates that *C. pneumoniae* infections result in an acceleration of the formation of complex atherosclerotic lesions in Apo E3-Leiden mice either by increasing the influx of activated T-cells to the vascular wall by infected and antigen presenting macrophages or by direct activation of T-cells.

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CHAPTER 4

Chlamydia pneumoniae infections augment atherosclerotic lesion formation: a role for serum amyloid P

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Abstract

Objectives: Multiple reports have demonstrated an association between *Chlamydia pneumoniae* (Cpn) and cardiovascular disease. In this study, we evaluated the effect of Cpn infections on early lesion progression in C57BL/6J mice. Since plaque formation in these mice does not develop past the initial stage, we thought these mice may be a better model for unravelling the effect of Cpn infection on early lesion type progression.

Methods: C57BL/6J mice were fed an atherogenic diet and injected 10 times with 5 107 IFU Cpn or mock. At sacrifice, lesion number, size and type were analysed. To study the role of Cpn on inflammation, serum amyloid P (SAP) in plasma was determined as well as T-cells, macrophages and SAP in the lesions.

Results: In the aortic sinus of both groups type 2 lesions were found. Cpn infection resulted in a 2.2-fold increase in total lesion size (Cpn: $10\,821 \pm 2429\text{ mm}^2$ vs. mock: $5022 \pm 1348\text{ mm}^2$; $p=0.04$). No difference in lesion number was observed. Also, Cpn infection increased SAP in the lesions from $1 \cdot 10^{-4} \pm 0.1 \cdot 10^{-4}$ SAP positive cells/lesion area to $10 \cdot 10^{-4} \pm 1 \cdot 10^{-4}$ SAP positive cells/lesion area ($p=0.05$). The influx of T-lymphocytes and macrophages in the lesions as well as SAP plasma levels were not different between groups.

Conclusion: Multiple Cpn infections resulted in a significant increase in total lesion size of C57BL/6J mice. Increase in total SAP positive area in infected mice suggests a role for this acute phase protein in lesion enlargement.

Introduction

Chlamydia pneumoniae (Cpn) infections are probably involved in human atherosclerosis. Since the original observation by Saikku and coworkers,¹ over 30 epidemiological studies have confirmed the association between Cpn infection and atherosclerosis.² The majority of these studies is based on serology or the presence of Cpn DNA or antigens in the vascular wall. While many in vitro studies have likewise demonstrated that Cpn is able to infect various components of the atherosclerotic vascular wall and results in production of multiple pro-inflammatory and pro-atherogenic cytokines and chemokines, the exact mechanisms by which Cpn contributes to plaque formation in vivo remains to be established. To unravel these mechanisms, genetically modified mice have been used. In low density lipoprotein-receptor knockout (LDLr^{-/-}) mice, Cpn infection has been shown to result in an enlargement of the total atherosclerotic plaque area in the aortic sinus.³ Along the same line, it has been demonstrated that Cpn infection accelerates the atherosclerotic process in ApoE^{-/-} mice.⁴⁻⁶ Furthermore, we recently demonstrated that Cpn infection accelerates the formation of advanced atherosclerotic lesions in ApoE3-Leiden mice,⁷ although the observed changes were subtle. In this study we evaluated the formation of atherosclerotic plaques in C57BL/6J mice following a prolonged period of high fat/high cholesterol diet with multiple Cpn infections. C57BL/6J mice, which are the parental background strain for most atherosclerotic mouse models, only develop initial lesions when fed an atherogenic diet.⁸⁻¹⁰ We hypothesised that concomitant Cpn infections result in the progression of early lesions into advanced lesion types.

Since the late 90's atherosclerosis has been described as an inflammatory disease.¹¹ Pathological and intervention studies have implicated organisms like Cpn in initiating or maintaining inflammation.^{2, 12, 13} Also, increased levels of acute phase proteins as C-reactive protein (CRP)¹⁴ and, in the mouse, serum amyloid P (SAP)¹⁵ have been linked to progression of atherosclerotic vascular disease. SAP has been demonstrated in amyloid deposits of tangles and plaques in Alzheimer's disease patients,¹⁶ but also in atherosclerotic intima of human abdominal aortas.¹⁵ In this study, the role of Cpn infection on inflammation was studied through assessment of SAP in plasma and in atherosclerotic lesions of the aortic sinus of C57BL/6J mice. In addition, we compared the presence of inflammatory cells in atherosclerotic lesions, total lesion area and lesion number in mice, which were fed an atherogenic diet for 10 months with or without 10 monthly inoculations of Cpn.

Material and Methods

Mice

Twenty male specific pathogen free C57BL/6J mice were bred and housed under standard conditions at the Central Animal Facility at the Maastricht University. During the study mice were fed a high fat/high cholesterol diet containing 15% cacao butter, 0.5%

cholate, 1% cholesterol, 40.5% sucrose, 10% cornstarch, 1% corn oil and 4.7% cellulose (Hope farms BV, Woerden, The Netherlands) and water ad libitum. This animal experiment was approved by the institutional committee for the welfare of laboratory animals of the Maastricht University.

C. pneumoniae

Cpn strain TWAR 2043 (ATCC VR-1355) was cultured in Hep-2 cells as previously published.¹⁷ Bacterial titres were determined by titration in Hep-2 cells and stained with fluorescein-isothiocyanate-conjugated (FITC) monoclonal antibody RR 402 (Dako, Glostrup, Denmark) directed against elementary bodies of Cpn.

Experimental design

At the age of seventeen weeks the high fat/high cholesterol diet was started and half of the mice were injected intraperitoneally with 5×10^7 IFU Cpn, dissolved in 200 μ l of sterile phosphate-buffered saline (PBS). In humans, multiple Cpn infections occur throughout one's lifetime. Therefore, mice received 9 consecutive monthly inoculations after the first injection (Cpn). Control mice (Mock) were injected with PBS only.

Tissue handling

At sacrifice, 25 days after the last inoculation, mice were anaesthetized with a weight-adjusted dose of pentobarbital (Nembutal®, Sanofi BV, Maassluis, The Netherlands) and blood was collected from the left ventricular apex of the heart for analysis of Cpn antibodies and serum amyloid P (SAP). The arterial tree was perfused with PBS through a catheter placed in the left ventricular apex. The aortic sinus was removed, fixed overnight in 3.7% PBS buffered formaldehyde and embedded in paraffin. Serial longitudinal 4 mm sections of the aortic sinus were cut, stained and analyzed. Thoracic and abdominal aorta as well as spleen and liver were dissected and stored at -70 °C for Cpn DNA analysis.

Atherosclerotic lesion analysis

Since atherosclerotic lesion development initiates in the aortic sinus, 4 mm sections of the aortic root were used from the level where valves were visible until the valves had disappeared. The middle section was used for an Elastica van Gieson (EvG) staining for analysis of atherosclerotic lesion type, size and number. This middle section was used as a "reference section" to select surrounding sections, 4 mm apart, for analysis of T-lymphocytes and macrophages. Atherosclerotic lesion type was determined according to the guidelines of the American Heart Association.^{7, 18, 19} For area analysis a microscope coupled to a computer-assisted morphometry system (Analysis Soft Imaging System, GmbH, Münster, Germany) was used.

Analysis of T-lymphocytes and macrophages in the atherosclerotic lesions

In order to detect T-lymphocytes in the atherosclerotic lesions, a rabbit-anti-CD3⁺ polyclonal antibody was used (Dako, Glostrup, Denmark). After a blocking step with

Bovine Serum Albumin(BSA)/PBS 2% for aspecific binding, slides were incubated with the CD3⁺ polyclonal antibody for 60 minutes at a dilution of 1:200, followed by a second-step incubation with a biotinylated swine-anti-rabbit IgG at a dilution of 1:1000 (Dako, Glostrup, Denmark) for 30 minutes. Sections were then labelled with an alkaline phosphatase coupled ABC reagent (Dako, Glostrup, Denmark) for 30 minutes. Alkaline phosphatase activity was visualized using Fast-red (Sigma Chemical Co, St. Louis, United States). Rabbit-anti-rat IgG (Dako, Glostrup, Denmark) was used as a negative control. For positive controls, tissue sections from mice spleens were used. T-lymphocytes in the atherosclerotic lesion were counted and expressed as the ratio between the number of T-lymphocytes and total plaque area.

Macrophages were detected in the lesion using a rat anti-mouse Mac-3 monoclonal antibody (Pharmingen, Heidelberg, Germany).²⁰ After a heating step with a mixture of AgR buffer (Dako, Glostrup, Denmark), citric acid (Merck, Darmstadt, Germany) and sodium citrate (Merck, Darmstadt, Germany) for 10 minutes, blocking steps were performed for aspecific binding. Slides were incubated with a mixture of methanol and H₂O₂ for 15 minutes followed by rabbit serum for 30 minutes. Then the slides were incubated with the Mac-3 monoclonal antibody at a 1:30 dilution for 24 hours, followed by a second step with biotinylated rabbit-anti-rat IgG (Dako, Glostrup, Denmark) at a dilution of 1:300 for 30 minutes. Finally, sections were labelled with an alkaline phosphatase coupled ABC reagent (Dako, Glostrup, Denmark) for 30 minutes. Alkaline phosphatase activity was visualized using Alkaline Phosphatase Substrate Kit I (Vector Laboratories, Burlingame, USA). For positive controls, tissue sections of mice spleens were used. The presence of macrophages was expressed as the ratio between the area positive for Mac-3 and total plaque area using a microscope coupled to a computer-assisted morphometry system (Analysis Soft Imaging System, GmbH, Münster, Germany).

Detection of SAP in serum and atherosclerotic lesions

SAP levels in the serum of Cpn infected and mock injected mice were measured using the enzyme linked immunosorbent assay (ELISA) technique previously used by Daemen et al.²¹

For detection of SAP in atherosclerotic lesions of the aortic sinus, a rabbit-anti-SAP polyclonal antibody was used (Calbiochem San Diego, United States). After blocking steps for peroxidase using 3% H₂O₂ and BSA/PBS 2% for aspecific binding, slides were incubated with rabbit-anti-SAP (Calbiochem, San Diego, United States) for 60 minutes at a dilution of 1:250, followed by a second-step incubation with a biotinylated swine-anti-rabbit IgG at a dilution of 1:1000 (Dako, Glostrup, Denmark) for 30 minutes. Finally, sections were labelled with an alkaline phosphatase coupled ABC reagent (Dako, Glostrup, Denmark) for 30 minutes. Alkaline phosphatase activity was visualised using Fast-red (Sigma Chemical Co, St. Louis, United States) For positive controls, tissue sections of murine brains with Alzheimer disease and liver sections were used. Rabbit-anti-rat IgG (Dako, Glostrup, Denmark) was used as a negative control. The presence of SAP was expressed as the ratio between the area positive

for SAP and total plaque area using a microscope coupled to a computer-assisted morphometry system.

Detection of Cpn antibodies and Cpn DNA

Cpn antibodies were measured with the indirect micro-immunofluorescence (MIF) technique using mouse plasma sample dilutions 1:10 and 1:100 on antigen-coated slides (Labsystems, Helsinki, Finland). Goat-anti-mouse IgG conjugated to FITC (Dako, Glostrup, Denmark) was used as the secondary antibody. The slides were analysed by 2 independent observers.

To detect the presence of Cpn DNA in the organs, a sensitive in house developed polymerase chain reaction (PCR) was used (detection ability of 1 copy of DNA). The following sequences were used as primers: sense 5'-TTA TTA ATT GAT GGT ACA ATA-3'; antisense 5'-ATC TAC GGC AGT AGT ATA GTT-3', accession number AF131889.²²

PCR was performed in a total volume of 50ml, containing 1mg DNA. DNA was extracted from vascular tissue with the Wizard Genomic DNA purification kit (Promega, Germany). Amplification was done by thermal cycling starting at 37°C for ten minutes and 95°C for fifteen minutes. Then 20 cycles of "Touchdown PCR" were performed from 60°C to 50°C, followed by 40 cycles of 1 minute at 94°C, 50°C, and 72°C.

The PCR products were resolved in a 2% agarose gel, stained with ethidium bromide, and photographed.

Lipid profiles

Total plasma cholesterol and triglyceride concentrations were measured with a standard cholesterol oxidase method performed on Beckman Synchron CX Systems.²³

Statistical analysis

Differences in lipid profile, atherosclerotic lesion size and type, influx of T-lymphocytes and macrophages and SAP levels in serum and plaque between the Cpn and mock group were compared with the Mann-Whitney test. Only mice with clear signs of atherosclerosis in the aortic sinus were included for analysis. A P-value of < 0.05 was considered statistically significant. All data are presented as mean $\pm$ SEM.

Results

To investigate the effect of Cpn infection on early lesion type progression, 20 male C57BL/6J mice were used. Mice in the present study showed no clinical signs following Cpn inoculations. No mortality was observed. This is in line with results of Blessing et al.,²⁴ that did not observe mortality in C57BL/6J mice inoculated with Cpn.

Detection of Cpn DNA and anti-Cpn antibodies

In all Cpn-infected mice antibodies against Cpn were detected in plasma, indicating that all mice were successfully infected. No Cpn antibodies were detected in the plasma of the mock-infected group. Furthermore, Cpn specific DNA was detected in 5/10 vascular tissue specimens of Cpn-infected group by PCR, which is comparable with earlier published Cpn PCR results in aortic tissue of mice.²⁵ In aortic tissue samples of mock-injected mice, no Cpn DNA was detected.

Lipid profiles

Blood was collected from the Cpn-infected and the mock-infected mice for analysis of total plasma cholesterol and triglyceride concentrations. Data are presented in table 1. No difference was observed in cholesterol and triglyceride plasma levels between the Cpn-infected and mock-infected group, suggesting that Cpn infection has no effect on plasma lipid levels.

Table 1. Lipid profiles, lesion number/aortic arch and number inflammatory cells/lesion area in mock injected or Cpn infected C57BL/6J mice, n: number of mice analysed.

	n	Mock-Infected	Cpn-Infected	p
Lipid profile (mmol/L)				
Cholesterol	10/10	7.4 ± 0.4	8.0 ± 0.2	p= 0.11
Triglycerides	10/10	0.051 ± 0.022	0.069 ± 0.017	p= 0.49
Lesion number/aortic sinus				
	9/9	1.67 ± 0.35	2.33 ± 0.53	p= 0.16
Inflammatory cells/lesion area				
T-cells (x 10 ⁻⁴)	8/8	2.96 ± 1.69	1.30 ± 0.66	p= 0.23
Macrophages	5/7	0.11 ± 0.04	0.17 ± 0.05	p= 0.17

Evaluation of atherosclerotic lesions

In the Cpn-infected as well as in the mock-infected group all atherosclerotic lesions found could be classified as type 2 lesions ("fatty streaks") according to the histological classification of AHA. The numbers of atherosclerotic lesions are presented in table 1. No significant difference in lesion number was observed between groups.

When atherosclerotic lesion area in the aortic sinus was measured a statistically significant increase was observed in Cpn infected C57BL/6J mice compared to mock-infected mice (Mock: 5022 ± 1348 µm² vs. Cpn: 10821 ± 2429 µm², p=0.04, figure 1).

Figure 1. Quantitative comparisons of mean aortic lesion areas between *Cpn* infected and mock injected 14 months old C56BL/6J mice, fed a high fat/high cholesterol diet for 10 months. Data are presented as mean $\pm$ SEM.

Figure 2. Results of SAP staining in atherosclerotic lesions of *Cpn* infected and mock injected C56BL/6J mice. Data are expressed as the number of SAP positive cells divided by total plaque area and presented as mean $\pm$ SEM.

Inflammatory cells in the atherosclerotic lesions

The effect of *Cpn* infection on influx of T-lymphocytes and macrophages was determined in the atherosclerotic lesions of the aortic sinus by immunohistochemical staining. Data are presented in table 1. There was no difference in T-cell or macrophage influx between *Cpn*-infected and mock-injected mice.

Figure 3. SAP staining showing SAP antigen in the subendothelial layer of the lesion of Cpn-infected mouse. Magnification 200x and 400x.

SAP in atherosclerotic lesions and serum

When SAP in the atherosclerotic lesions was determined, a 10 fold increase was observed in SAP positive area in the lesions of the aortic sinus from $1.10^{-4} \pm 0.1.10^{-4}$ SAP positive cells per lesion area in control mice to $10.10^{-4} \pm 1.10^{-4}$ SAP positive cells per lesion area in Cpn-infected mice ($p=0.05$, figure 2). SAP staining was mainly observed in the subendothelial layer of the plaque (figure 3).

The effect of Cpn infection on serum SAP levels was measured by the ELISA technique which demonstrated similar levels in SAP (13.69 mg/ml vs. 14.15 mg/ml, $p=0.4$) in Cpn and control group, respectively.

These data suggest that Cpn infection affects atherosclerotic lesion size through increasing SAP antigen in these lesions without changing SAP levels systemically.

Discussion

Many research groups have focused on the influence of Cpn infection in cardiovascular diseases.²⁶ In terms of atherosclerosis, the presence of Cpn in atherosclerotic lesions has been demonstrated by PCR, immunohistochemistry and electronmicroscopy.²⁷⁻³⁰ On a few occasions, Cpn has been cultured from lesions.³¹⁻³³ Furthermore, numerous in vitro studies have demonstrated that Cpn infection induces pro-atherogenic changes in various cellular components of the atherosclerotic vessel wall, such as foam cell formation,³⁴ endothelial expression of adhesion molecules and chemokines,³⁵ stimulation of transendothelial leukocyte migration,³⁶ smooth muscle cell proliferation,³⁷ endothelial production of tissue factor³⁸ and plasminogen activator

inhibitor 1³⁹ and macrophage production of matrix metalloproteinase 9.⁴⁰ All these findings suggest that Cpn may play a causal role in the development of atherosclerosis.

The effect of Cpn on atherogenesis has been studied in mice models. For these studies mostly genetically modified mice such as ApoE and LDLr knockout mice have been used. These mice develop atherosclerotic lesions in their vascular tree when fed a high fat/high cholesterol diet.⁴¹ Concomitant Cpn infection has been shown to stimulate plaque formation in these mice.⁴⁻⁶ Furthermore, we recently demonstrated that Cpn infection accelerates the formation of more advanced atherosclerotic lesions in ApoE3-Leiden mice.⁷ In C57BL/6J mice, the parental, normocholesterolemic background strain of the genetically modified mice (including the ApoE3-Leiden), plaque formation in the aortic sinus does not develop past the initial stage even after extended periods of a high fat/high cholesterol diet.⁸ As C57BL/6J mice on a high fat/high cholesterol diet may thus be a better model for unravelling the effect of Cpn infection on early lesion type progression, we hypothesized that Cpn infections induce more complicated lesion types in the aortic sinus of these mice. For this purpose, mice were fed a high fat/high cholesterol diet starting at the age of 17 weeks and sacrificed at approximately 14 months of age. As expected, only type 2 lesions were found in the aortic sinus of these uninfected control mice. The aortic sinus of mice which additionally received ten monthly inoculations with 5x10⁷ IFU Cpn also demonstrated no advanced lesion formation suggesting that Cpn infections do not lead to the formation of more complicated lesions. This finding is in contrast with our previous finding in ApoE3-Leiden mice,⁷ where Cpn infections accelerated formation of complex (advanced) atherosclerotic lesions without increasing lesion size. Some differences between high fat/high cholesterol fed C57BL/6J and ApoE3-Leiden mice may account for this discrepancy. Although our C57BL/6J mice show moderately elevated levels of serum cholesterol and triglycerides, levels are markedly higher in ApoE3-Leiden following prolonged periods of high fat/high cholesterol.⁴² As hypercholesterolemia may be a prerequisite for an effect of Cpn on plaque progression,³ this may explain the lack of effect of Cpn infection on plaque progression in C57BL/6J mice. Furthermore, genetic modulation of various components of the lipid metabolism pathway has been shown to alter the susceptibility of mice to various infections. ApoE^{-/-} mice display an increased susceptibility to *Klebsiella pneumoniae*⁴³ or *Listeria monocytogenes*.⁴⁴ LDLr^{-/-} mice, on the other hand, are protected from infections with *Klebsiella pneumoniae*⁴⁵ but show an increased mortality following systemic candidiasis.⁴⁶ Until now it has not been established whether the lack of ApoE or hypercholesterolemia affect the susceptibility of mice to Cpn infection. As Cpn belongs to the large family of Gram-negative bacteria, as *Klebsiella pneumoniae* also does, an increased susceptibility to Cpn infection may be anticipated in ApoE3-Leiden mice, which may eventually explain the observed differences in advanced lesion formation. Future studies using high sensitive techniques such as Cpn real time (RT)-PCR will be needed to elucidate this aspect.

Apart from lesion type, we also measured total lesion area in both groups and found a marked increase in total lesion area in the aortic root of Cpn infected mice. Blessing

and colleagues observed a similar increase in total lesion area in the aortic root of Cpn infected, high fat/high cholesterol fed C57BL/6J mice.⁹ In contrast to our study, they infected their mice less frequently than we did (3 vs. 10 times) and sacrificed them at a much younger age (7 months vs. 14 months). Despite these differences, total lesion sizes did not differ between their and our study.

Recently, we demonstrated that there is a significant increase in the number of T-cells, present in the aortic arch of ApoE3-Leiden mice early (1 month) after the first injection.⁷ We hypothesized that this increase in T cell influx may contribute to the observed acceleration of advanced plaque formation at later time points, i.e. 6 and 9 months p.i. However, at these later time points no differences are observed in T-cell number or inflammatory cells per lesion area. Nonetheless, in the present study T cell number at 1 month post infection was not evaluated.

Increasing evidence suggests a role for acute phase proteins in cardiovascular disorders. Elevated C-reactive protein (CRP) levels have been shown to be a strong independent risk factor for future cardiovascular complications in patients.^{14, 47-50} In addition, multiple pro-atherogenic effects, such as the upregulation of adhesion molecule expression by endothelial cells and production of tissue factor,⁵¹ TNF α and IL-1(52) by macrophages following exposure to CRP, have been demonstrated in vitro.

And although generally accepted that CRP is mainly produced by hepatocytes, we and other recently demonstrated the presence and production of CRP in the vascular wall.^{53, 54} Also SAP, the other member of the pentraxin family, has been suggested to contribute to atherosclerosis. Li et al. demonstrated the presence of SAP in human atherosclerotic lesions and suggested that the proatherogenic effects may be related to its interactions with various lipoproteins.¹⁵ In mice, SAP is the most important acute phase protein and SAP plasma levels are rapidly increased following LPS exposure as well as infection. Therefore, we investigated whether SAP is increased in our Cpn-infected mice. As Cpn could be demonstrated in vascular tissue, it may be speculated that this results in the formation of such cellular debris thereby explaining the presence of SAP at these sites. SAP has been shown to play a role in the binding and clearance of host- and pathogen-derived cellular debris at sites of acute inflammation as part of the innate immune response. Whether SAP enters the subendothelium from the circulation or is produced locally, as recently demonstrated for CRP,^{53, 54} remains to be established. Also, the mechanisms by which SAP may promote plaque formation are unclear until now. Following binding to host- and pathogen-derived cellular debris, SAP may activate the complement cascade which may represent an important initiating event in early atherogenesis.^{55, 56} In addition, pentraxins have been shown to bind to macrophages and to enhance the uptake and degradation of LDL via the macrophages scavenger receptor.^{57, 58} Intriguingly, Gates and Reese⁵⁹ demonstrated that activation of sphingomyelinase, an enzyme associated with the pathogenesis of atherosclerosis, requires the presence of SAP. This is supported by findings from Wong and colleagues,⁵⁹ who demonstrated that acute systemic inflammation up-regulates sphingomyelinase in vivo. This activated sphingomyelinase may then convert lipoproteins into potent inducers of macrophage foam cell formation in hypercholes-

terolemic mice,⁶⁰ which may results in increased formation of early lesions. In summary, various lines of evidence suggest a role for SAP in atherogenesis, in particular following infection, but further research is awaited to elucidate the exact mechanisms underlying its effects on lesion formation. In contrast to increased SAP in plaques, no differences in circulating SAP levels were observed between infected and uninfected mice. It may be speculated that the late time point after infection (25 days after the last inoculation) at which SAP levels were determined could explain the similar plasma SAP levels.

In summary, our results demonstrate that multiple infections with Cpn result in a marked enlargement of the atherosclerotic lesions in the aortic sinus of C57BL/6J mice. This was accompanied by an enhanced SAP immunoreactivity in lesions of Cpn-infected mice thereby suggesting a potential role for this acute phase protein in Cpn mediated aggravation of atherosclerosis. This finding provides further evidence for a role of Cpn in atherogenesis.

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CHAPTER 5

Unstable atherosclerotic lesions and premature death in LDL-receptor, ApoE double knockout mice following *Chlamydia pneumoniae* infection

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Abstract

Objective: Infections have been linked with progression of atherosclerosis. To study whether *Chlamydia pneumoniae* (Cpn) affects atherosclerosis, we infected atherogenic mice and studied plaque characteristics.

Methods: Mice were infected with Cpn or mock and sacrificed at 20 or 40 weeks of age or observed until natural death. Aortic arch sections were analyzed for lesion area, type, fibrous cap area, necrotic core content, collagen content, matrix metalloproteinases (MMP)-2 and -9, and signs of plaque rupture.

Results: All infected mice seroconverted and Cpn DNA was detected in the aorta of all infected mice. All mice developed atherosclerotic lesions from type 2 to 5b. Although infection did not affect lesion area or type, it significantly reduced the fibrous cap, increased necrotic core, and increased the presence of MMP-2 and -9 in Cpn-infected mice sacrificed at 20 and 40 weeks of age. MMP activity and mRNA expression did not differ between these groups. Furthermore, Cpn infection resulted in a decrease in survival when mice were followed until nature death occurred. Both groups showed equal numbers of animals with cardiovascular complications.

Conclusion: We demonstrate that Cpn infection has no effect on lesion area or type in the aortic arch of ApoE^{-/-}LDLr^{-/-}-mice. However, Cpn infection resulted in reduced fibrous cap thickness, increased necrotic core content, and a reduction in survival probably due to cardiovascular complications. These findings suggest that Cpn infection alters the plaque phenotype from stable to vulnerable and may provide an explanation for the previously observed correlation between myocardial infarction and Cpn.

Introduction

Key steps in the initiation of atherogenesis involve activation of the endothelium (e.g. by hypercholesterolemia), influx of leucocytes in the sub-endothelial layer and maintenance of the inflammatory state by production of pro-inflammatory cytokines by these leucocytes. Intriguingly, infections may initiate and/or stimulate most of these key events thereby adding themselves to the long list of potential risk factors. In particular *Chlamydia pneumoniae* (Cpn), an obligate intracellular gram negative bacterium, has frequently been associated with cardiovascular diseases. Sero-epidemiological^{1,2} as well as experimental in vivo^{3,4} and in vitro^{5,6} studies have demonstrated an association between Cpn and atherosclerosis. Furthermore, we recently demonstrated that Cpn infection stimulates the inflammatory activity in atherosclerotic plaques of ApoE3*Leiden mice.⁷ This invigorating effect on plaque inflammation may in the long run result in plaque destabilization and rupture. Earlier data demonstrated that the production of matrix metalloproteinases (MMPs) by both macrophages and smooth muscle cells is upregulated by Cpn, suggesting that these bacteria actively participate in the destruction of the extracellular matrix.^{8,9} Furthermore, increased immunoreactivity and gelatinolytic activity of MMP-2 and -9 were observed in Cpn positive human atheroma.^{10,11}

Based on these earlier observations, we hypothesized that Cpn infection increases vascular inflammation as well as MMP expression and activity, resulting in a modification of the plaque phenotype towards an unstable one, eventually plaque rupture with subsequent myocardial infarction and finally death. To explore this hypothesis, Apolipoprotein E, low density lipoprotein receptor double knockout (ApoE-/LDLr-/-) mice were infected with Cpn and the effect on plaque characteristics was studied. Furthermore, MMP immunoreactivity, expression, and activity were determined in the aortic arch following Cpn infection. Also survival characteristics following multiple Cpn infections were determined.

Methods

Mice

Eighty-six male specific pathogen free (SPF) ApoE-/LDLr-/- mice on a C57Bl/6J background were used. Mice were housed under standard conditions at the Central Animal Facility of the Maastricht University and fed a normal chow diet (Hopefarms BV, Woerden, The Netherlands) and water ad libitum. All experiments were performed according to the institutional guidelines of the committee for the welfare of laboratory animals of the Maastricht University.

Chlamydia pneumoniae (Cpn)

Cpn strain TWAR 2043 (ATCC VR-1355) was cultured in Hep-2 cells as described previously.¹² Bacterial titers were determined by titration in Hep-2 cells and stained with

fluorescein-isothiocyanate-conjugated (FITC) monoclonal antibody RR 402 (Dako, Glostrup, Denmark) directed against elementary bodies of Cpn. Titers were expressed as number of inclusion forming units per milliliter (IFU/ml).⁷

Study design

In humans, multiple Cpn infections occur throughout one's lifetime. Therefore, we infected mice 2 or 6 times starting at the age of 16 weeks by an intraperitoneal injection with Cpn (5.107 IFU) containing sucrose-phosphate-glucose (SPG) solution, while control mice (mock) received SPG only. The intraperitoneal inoculation route was chosen over intranasal injection since previous studies from our group had shown that systemic dissemination, analysed by real time PCR analysis, following intraperitoneal Cpn infection compared with intratracheal infection was not different (manuscript in preparation). Mice in group A received a second inoculation, 5.106 IFU Cpn or mock, (n=8 Cpn/n=8 mock) at the age of 18 weeks and were sacrificed at the age of 20 weeks. Mice in group B, (n=7/7) received 6 monthly infections (first infection with 5.107 IFU Cpn and following infections with 5.106 IFU Cpn) or mock injections and were sacrificed 1 month after the last injection (at the age of 40 weeks). Mice in group C (n=7/7) also received 6 successive monthly infections or mock injections, starting at the age of 16 weeks, and were observed until natural death had occurred. Post mortem examination was performed by an experienced pathologist specialized in animal pathology. Additionally 40 mice were included for analysis of MMP-2 and -9 expression (mRNA, n=10/10) and activity (zymography, n=10/10). These mice received the same inoculation protocol as mice in group A.

Tissue handling

Before sacrifice, mice were anaesthetized with a weight-adjusted dose of pentobarbital (Nembutal, Sanofi BV, Maassluis, The Netherlands) and blood was collected from the left ventricular apex of the heart for analysis of anti-Cpn antibodies.⁷ Then the arterial tree was perfused with phosphate-buffered saline (PBS) through a catheter placed in the left ventricular apex. From the mice in group A and B, the aortic arch with main branch points was removed, fixed overnight in 3.7% PBS buffered formaldehyde and embedded in paraffin. Serial longitudinal 4 μ m sections of the aortic arch were cut, stained and analyzed. The remainder of the aorta (thoracic and abdominal), lung, spleen, heart and brain were dissected and stored at -70°C for Cpn DNA analysis. For analysis of MMP-2 and -9 mRNA expression and gelatinolytic activity, the aortic arch with its main branch points was removed, frozen in liquid nitrogen and stored at -70°C.

Detection of anti-Cpn antibodies and Cpn DNA

To check whether the inoculation protocols were successful, the presence of anti-Cpn antibodies was determined in mouse plasma with the indirect micro-immunofluorescence (MIF) technique as described previously.¹³ Mouse plasma dilutions 1:10 and 1:100 were applied on antigen-coated slides (LabSystems, Helsinki, Finland). Sections were then incubated with goat-anti-mouse IgG conjugated to FITC (Dako, Glostrup,

Denmark) and analyzed by 2 independent observers. Furthermore, a sensitive (detection limit 1 copy of DNA) in house developed polymerase chain reaction (PCR) test was used to detect Cpn DNA in various organs. The following sequences were used as primers: sense CPC, 5'-TTA TTA ATT GAT GGT ACA ATA-3'; antisense CPD, 5'-ATC TAC GGC AGT AGT ATA GTT-3'.¹⁴ These primers specify MOMP of Cpn. PCR was performed in a total volume of 50 µl, containing 1 µg DNA. DNA was extracted from vascular tissue with the Wizard Genomic DNA purification kit (Promega, Germany). Amplification was done by thermal cycling starting at 37°C for ten minutes and 95°C for fifteen minutes. Then 20 cycles of "Touchdown PCR" were performed from 60°C to 50°C, followed by 40 cycles of 1 minute at 94°C, 50°C, and 72°C.

The PCR products were resolved in a 2% agarose gel, stained with ethidium bromide, and photographed

Atherosclerotic lesion analysis

The whole aortic arch from every mouse was cut in longitudinal, coronal, consecutive sections and used for analysis of atherosclerotic lesion type and lesion area. Only arches with an intact morphology (brachiocephalic trunk, left and right common carotid artery and left subclavian artery) were used in the study. The number of sections obtained from each aortic arch was calculated and the middle section was haematoxylin-eosin (HE) stained for analysis of atherosclerotic lesion types according to the guidelines of the American Heart Association.^{7,15-17} The total number of all lesions was measured and the contribution of each lesion type to the total number of lesions was expressed as a percentage of the total lesion number. For determination of total lesion area the same HE-stained section was used. Lesion area was measured using a microscope coupled to a computer-assisted morphometry system (Analysis Soft Imaging System, GmbH, Münster, Germany).⁷

Immunohistochemistry

For detection of smooth muscle cells (SMC) and to analyze the presence of matrix metalloproteinase (MMP) -2 and -9 in the atherosclerotic lesion, -smooth muscle cell actin (Sigma, St. Louis, USA) and MMP-2 and -9 (Oncogene Research Products, Cambridge, UK) mouse monoclonal antibody were used. The primary antibody was probed with biotin labeled goat-anti-mouse IgG. Sections were incubated with an alkaline phosphatase coupled ABC reagent (Dako, Glostrup, Denmark) and alkaline phosphatase activity was visualized using Fast-red (Sigma, St. Louis, USA). For negative control, normal mouse ascitic fluid (Sigma-Aldrich Chemie BV, Zwijndrecht, The Netherlands) was used. Since the media of the vascular wall mainly consists of SMC, this part of the slide was used as positive control for - actin staining. Since cardiomyocytes are known to contain MMP-2 and -9, mouse heart sections were used as positive controls for the MMP immunolabeling.

For quantification of fibrous cap area in the atherosclerotic lesion, non-media SMC positive area was analyzed using a microscope coupled to a computer assisted morphometry system (Analysis Soft Imaging System, GmbH, Münster, Germany). The

results were expressed as area SMC/total lesion area and are designated as cap area. MMP-2 and -9 immunoreactivity was analyzed by semi-quantitative measurements using 0 when no staining was visible, 1 when positive staining was observed and 2 when extensive positive staining was seen. Mean score of all aortic arch sections was calculated and results of Cpn-infected and control mice were compared.

Collagen content of the plaque

To evaluate the collagen content of the lesion, Sirius red staining was used.^{18,19} Sections were first incubated for 60 minutes with Susa fixative²⁰⁻²² followed by incubation with 0.1% Sirius red solution (Klinipath, Duiven, The Netherlands) in saturated picric acid for 30 minutes. After incubation with 0.01N HCl for 2 minutes, sections were stained with hematoxylin staining for 6 minutes. Liver mouse sections were used as positive control. For quantification of collagen content of the plaque, Sirius red positive area in the atherosclerotic lesion was analysed using a microscope coupled to a computer assisted morphometry system (Analysis Soft Imaging System, GmbH, Münster, Germany) and Scion Image Release Beta 3b (Scion Corporation, Frederick, Maryland, USA). The results were expressed as area collagen/total lesion area and are designated as percentage collagen of total lesion.

Lipid core evaluation of the atherosclerotic lesions

The lipid core area was measured in the atherosclerotic lesions that contained lipid cores using a microscope coupled to a computer assisted morphometry system (Analysis Soft Imaging System, GmbH, Münster, Germany) and expressed as percentage lipid core of the total lesion.

MMP-2 and -9 mRNA expression in the atherosclerotic aortic arch

To study the presence of MMP-2 and -9 mRNA in the aortic arch lesions of ApoE-/- LDLr-/- mice, real-time RT-PCR was used. Vascular tissue was homogenized and total RNA was isolated using the TRIzol® Reagent from Life Technologies, Paisley, United Kingdom for samples with high protein content. DNase treatment with DNase I (0.362U, Amersham Pharmacia Biotech) was performed and equal amounts of total RNA were reverse transcribed into cDNA using oligo dT (RACE-1, 1 pmol, Amersham Pharmacia Biotech) and Superscript II RNaseH- (25U, Invitrogen). For every total RNA isolate also a RT-PCR reaction was performed in the absence of the reverse transcriptase, to demonstrate the specific amplification of mRNA instead of genomic DNA. Real-time PCR reactions on cDNA were performed by the ABI Prism 7000 in a final volume of 25 l. Primers and probes for the detection of MMP-2 were: forward primer 5'-CTATGTCCACTGTGGGTGGAAAT-3'(300nM), reverse primer 5'-CTTGTTGCCAGGAAAGTGAA-3' (900Nm) and probe 5'-CAGAAGGTGCCCCATGTGTCTTCCC-3' (200nM). The probe carried a 5' FAM reporter and a 3' TAMRA quencher group. Thermal cycling started with UNG activation for 2 minutes at 50°C, followed by HotStarTaq activation during 10 minutes at 95°C. Thereafter 42 cycles of amplification were run consisting of 15 seconds at 95°C and 1 minute at 60°C. To

amplify GAPDH the Taqman® Rodent GAPDH Control Reagents kit (Applied Biosystems, Foster City, United States) was used. For amplification of MMP-9 the primers 5'-GACCCGAAGCGGACATTG-3' (300nM, forward primer) and 5'-TGC-CGTCTGAAGGGATACC-3' (900nM, reverse primer) and the probe 5'-ATCCAGTTTG-GTGTCTGCGGAGCA-3' (200 nM, VIC and TAMRA labelled) were used. Relative differences in the expression of MMP-2 and MMP-9 mRNA levels in the two groups were determined by dividing for every sample the mean Ct ("threshold cycle") value of GAPDH by the mean Ct value of MMP-2 or -9. Every sample was measured in duplo. This method was used to compensate for possible fluctuations in total RNA input in the real-time RT-PCR.

MMP-2 and -9 gelatinolytic activity

To study MMP-2 and -9 activity in atherosclerotic lesions, the zymography technique, the "golden standard" for analysis of MMP-2 and -9 activity, was used.²³ The aortic arch was weighed, cut into small pieces and incubated in extraction buffer (10mM cacodylic acid (pH 5.0), 0.15mM NaCl, 1 M ZnCl₂, 20mM CaCl₂, 1.5mM NaN₃ and 0.01% Triton X-100) at a concentration of 10 l/mg wet weight at 4°C. Then, samples were agitated continuously for 48 hours, extraction buffer was collected and 1M Tris (pH 8.0) was added to adjust the pH to 7.5. Protein concentration of the supernatant was determined using Bio-Rad protein assay (Bio-Rad Laboratories GmbH, München, Germany). Determination of MMP-2 and -9 activity was visualised using zymographic analysis on a gelatin-containing gel. Of every vascular tissue extract 30 µg of protein was loaded onto electrophoretic gels (SDS-PAGE) containing 1 mg/ml gelatin as described by Silence et al.²⁴ MMP-2 and -9 activity was quantified by measuring integrated optical densities of the lytic bands (Gel-Pro Analyzer v2.0).

Post mortem analysis of mice

Mice, included in group C (n=7/7), were inoculated with 6 monthly injections of Cpn or mock and observed until natural death had occurred. Post mortem examination was performed by an experienced pathologist specialized in animal pathology (MG). Consecutive sections of heart, lung, brain, liver, aortic arch and spleen were cut, stained with hematoxylin-eosin or Martius Scarlet Blue and screened for signs of plaque rupture, myocardial infarcts or other possible causes of death.

Statistical analysis

Difference in lesion type, size, fibrous cap, collagen, lipid core, MMP-2 and -9 expression, activity, and immunoreactivity between the Cpn-infected and control mice were analyzed with the Mann-Whitney-U test.

Differences in survival between Cpn-infected and control mice of group C were analyzed by Kaplan-Meier and log rank test. A p-value of <0.05 was considered statistically significant. Results are expressed as mean ± SEM. Analysis of sections was done by an observer who was blinded with respect to the presence or absence of Cpn as well as to the time point of sacrifice.

Results

Of the 86 male ApoE^{-/-}LDLr^{-/-} mice used in the study, 2 mice in group B (one Cpn-infected and one control mouse) died before reaching time point of sacrifice. These mice were excluded from the study, the cause of death remained unknown.

Presence of anti-Cpn antibodies and Cpn DNA

All Cpn-infected mice developed anti-Cpn antibodies at plasma dilutions of 1:10 and 1:100, indicating that mice had indeed been successfully infected. No anti-Cpn antibodies were detected in plasma of control mice. These results were confirmed by data obtained from Cpn PCR. Cpn DNA was detected in all aortic samples of group A showing that intra-peritoneal infection resulted in dissemination of Cpn to the vessel wall in all cases. Cpn DNA could also be detected in various other organs (spleen 100%, lung 86%, heart 57% and brain tissue 29% of the samples positive for Cpn DNA). In group B (sacrificed 1 month after the last infection), the percentage of Cpn positive aortic samples was slightly lower compared to group A (43% vs 100%) indicating that the immune system of the mouse is able to eradicate Cpn in the long run. In a similar way lower percentages of Cpn DNA positive organs were observed in group B (data not shown). No positive results were obtained in the mock infected group.

Cpn infection has no effect on atherosclerotic lesion type or lesion area

The aortic arches of mice in group A and B all contained atherosclerotic lesions ranging from type 2 (foam cell rich lesions) to type 5b (advanced calcified) lesions. In group B (sacrifice at 40 weeks of age) more advanced lesion types, especially type 5b, were found when compared to group A (sacrifice at 20 weeks of age) where type 4 and 5a lesions were predominant. This finding confirms earlier observations that the complexity of the atherosclerotic lesion types increases with time.^{7,15} Nevertheless, no statistically significant difference was found in the distribution of different lesion types between Cpn-infected and control mice in both group A and B. Similarly, no effect of Cpn infection on lesion area could be detected, either in group A or B. (Group A, mock: 0.22 ± 0.03 mm² vs. Cpn: 0.24 ± 0.03 mm², NS. Group B, mock: 0.75 ± 0.09 mm² vs. Cpn: 0.66 ± 0.08 mm², NS). Like lesion type, lesion area increases with time when control mice in group A and group B, and Cpn-infected mice of group A and B were compared.

These data indicate that Cpn infection does not affect atherosclerotic lesion type or lesion area in ApoE^{-/-}LDLr^{-/-} mice when these mice are infected at a stage when advanced atherosclerotic lesions are already present.

Cpn infection induces an unstable plaque phenotype

Fibrous cap and lipid core area

The fibrous cap and lipid core area, as well as the collagen content, were expressed as a percentage of the total lesion area. Positive staining for α -smooth muscle cell

actin was restricted to the fibrous cap of the atherosclerotic lesion with very few diffuse staining in the rest of the plaque.¹⁶ When the percentage of fibrous cap areas in Cpn-infected and control mice of group A were compared, a reduction was observed in the Cpn infected group (mock: 26% $\pm$ 3% vs. Cpn: 9% $\pm$ 3%, $p < 0.001$, figure 1A). Likewise, a reduction was seen following multiple infections (group B: mock: 10% $\pm$ 1% vs. Cpn 5% $\pm$ 2%, $p < 0.01$, figure 1A). When percentages of control mice in group A and group B were compared, a reduction from 26% $\pm$ 3% in group A to 10% $\pm$ 1% in group B ($p < 0.001$) was observed. Furthermore, when the percentage of lipid core area was studied an increase in the percentage of the lipid core was seen in Cpn-infected mice from groups (group A: 12% $\pm$ 2% (mock) vs. 24% $\pm$ 3% (Cpn), $p < 0.001$; Group B: 6% $\pm$ 1% (mock) vs. 9% $\pm$ 1% (Cpn), $p < 0.01$, figure 1B).

To determine the percentage of collagen, aortic arch sections were stained with Sirius red. All atherosclerotic lesions contained collagen as shown by a positive Sirius red staining. Nevertheless, no differences in percentage of collagen in the plaque was observed after either 2 or 6 Cpn infections (table 1).

In summary, Cpn reduced the percentage of fibrous cap and increased the lipid core. These results suggest that Cpn infection reduces the stability of the atherosclerotic plaque in these mice, despite the absence of changes in collagen percentage.

Table 1: Collagen percentage, MMP-2 and -9 antigen and mRNA in the plaque. Collagen is expressed as collagen percentage of total lesion area MMP-2 and -9 mRNA expression is designated as GAPDH/MMP-2 or GAPDH/MMP-9. # $p < 0.005$ vs. control.

	Group A (n=8/8)		Group B (n=7/7)	
	Control	Cpn	Control	Cpn
Collagen content	59% $\pm$ 2%	59% $\pm$ 1%	64% $\pm$ 1%	62% $\pm$ 1%
MMP-2 antigen	0.83 $\pm$ 0.18	#1.50 $\pm$ 0.24	1.00 $\pm$ 0.00	#1.43 $\pm$ 0.22
MMP-9 antigen	0.25 $\pm$ 0.17	#0.75 $\pm$ 0.17	0.57 $\pm$ 0.22	0.86 $\pm$ 0.15
MMP-2 mRNA	0.86 $\pm$ 0.01	0.86 $\pm$ 0.01	ND	ND
MMP-9 mRNA	0.63 $\pm$ 0.00	0.62 $\pm$ 0.01	ND	ND

Role of matrix metalloproteinase-2 and -9 in Cpn-induced plaque destabilization

Since previous reports have suggested a role for MMPs in plaque destabilization, possibly by extracellular matrix degradation, we studied the presence, expression and activity of two important candidates (MMP-2 and -9) in this respect.

Figure 1. **A:** Fibrous cap percentage in atherosclerotic lesions of Cpn-infected and control mice of groups A and B. The values are presented as percentage SMC area of total plaque area. # $p < 0.001$ vs. control in group A, * $p < 0.01$ vs. control in group B.

B: Necrotic core percentage in atherosclerotic lesions of Cpn-infected and control mice of groups A and B. Necrotic core is expressed as percentage necrotic core of the total plaque area. # $p < 0.001$ vs. control in group A, * $p < 0.01$ vs. control in group B.

MMP-2 and -9 antigens in the plaque

Presence of MMP antigens was shown by immunohistochemical stainings. Positive staining was mainly concentrated in the endothelial layer, lipid core and shoulder region of the atherosclerotic lesion. When the presence of MMP-2 and -9 antigen in Cpn-infected and control mice of group A and B was compared in a semi-quantitative way, an increase in MMP-2 and MMP-9 immunoreactivity was observed in Cpn-infected mice. MMP-2 positive staining was detected more frequently (1.5 ± 0.24) in Cpn-

infected mice from group A than in control mice (0.83 ± 0.18 , $p < 0.05$). Similar results were obtained in mice of group B. In this group MMP-2 positive staining was more frequently observed in Cpn-infected mice (1.43 ± 0.22) than in control mice (1.00 ± 0.05 , $p < 0.05$). Furthermore, in group A, MMP-9 positive staining increased from 0.25 ± 0.17 in control mice to 0.75 ± 0.17 in Cpn-infected mice ($p < 0.05$, table 1), while in group B MMP-9 positive staining was 0.57 ± 0.22 in control mice and 0.86 ± 0.15 in Cpn-infected mice (NS).

MMP-2 and -9 mRNA expression

Since the increase in MMP antigens was more pronounced at the age of 20 weeks (group A) as compared to group B (sacrificed at the age of 40 weeks), we determined the expression of MMP-2 and -9 mRNA in the aortic arch of ApoE^{-/-}LDLr^{-/-} mice at this time point either following Cpn infection or mock injection. All aortic arch samples showed presence of MMP-2 and -9 mRNA. Nonetheless, no significant difference in MMP-2 and -9 mRNA expression was observed between Cpn infected and control mice (NS, table 1).

Gelatinolytic activity

To study the effect of Cpn infection on MMP-2 and -9 activity, aortic arches of 20 ApoE^{-/-}LDLr^{-/-} mice were used for gelatinolytic analysis (figure 2). Quantification of the intensity of the bands demonstrated no differences in the intensities between both groups. Also, pro MMP-2 and -9 (latent form of MMP-2 and -9) activity did not differ between Cpn-infected and control mice (data not shown).

Figure 2. Gelatin zymography, demonstrating MMP-2 and -9 activity in aortic arches of Cpn-infected (lane 1-4) and control (lane 5-6) mice. The top bands represent pro-mmp-9 (94 kDa) and mmp-9 (83 kDa) while the bands with molecular weight ~ 66 kDa and ~60 kDa represent pro-mmp-2 and mmp-2 respectively. Lane 7 shows the low molecular weight marker (Biorad Laboratories, Richmond United States).

Figure 3. Life table showing survival (days of life) of Cpn-infected ($n=7$) and control mice ($n=7$) of group C.
* $p=0.02$

Figure 4. Different characteristics of myocardial infarction.

A: Multifocal areas (►) of ischemic necrosis. Magnification 100x

B: Fibrosis of septum of the right ventricle. Magnification 100x

C: Severe subendothelial fibrosis of the septum, note the atherosclerosis of the coronary artery (►).

►► indicates a non-atherosclerotic coronary artery. Magnification 400x

D: Martius Scarlet Blue staining showing plaque rupture in aortic root of Cpn-infected ApoE^{-/-}, LDLr^{-/-} mouse. Red staining indicates fibrin, yellow staining shows blood coagulates, while collagen is stained blue, magnification 100x

Cpn infection accelerates the incidence of plaque rupture

Since we observed that Cpn infection leads to a reduction of the fibrous cap area and an increase in lipid core content, we were interested whether plaque destabilization due to Cpn infection resulted eventually in the plaque rupture and premature death of the mice. To study this, 14 ApoE^{-/-}LDLr^{-/-} mice were either inoculated with Cpn or mock solution and observed until natural death had occurred. At the time of death, post-mortem histological examination was performed and multiple organs were screened for possible causes of death, such as plaque rupture, myocardial infarction or stroke.

The experiments with mice in group C showed that Cpn-infected mice had a decrease in life span with a mean survival of 86,4 weeks 95%CI(70.5-102.2) in the control mice and 56.8 weeks 95%CI(59.7-71.9) in the Cpn-infected mice (figure 3). Histological examination of heart sections showed characteristics of multifocal areas of ischemic necrosis(figure 4A), severe fibrosis of the right ventricle (figure 4B) and severe subendothelial fibrosis of the septum (figure 4C) all characteristics of previous myocardial infarcts. Plaque rupture of aortic root lesions (figure 4D) was also observed, these infarctions were not due to coronary embolism but to atherosclerotic narrowing of the coronary arteries.

Discussion

This study demonstrates that Cpn does not affect atherogenesis in ApoE^{-/-}LDLr^{-/-} mice with advanced atherosclerotic lesions with respect to total lesion size or type. Nonetheless, we found signs of plaque destabilization following Cpn infection as the fibrotic cap was significantly reduced while lipid cores were markedly increased. As signs of plaque rupture and myocardial infarction were observed in Cpn-infected and control mice at autopsy, we suggest that this accelerated formation of unstable plaques may account for the premature death of mice following multiple Cpn infections.

The large amount of data accumulated in recent years has reinforced the idea that infectious agents play a significant role in the pathogenesis of atherosclerosis and in the clinical manifestations of vascular disease. Sero-epidemiological^{1,2} and experimental data^{3,25,26} from a number of studies have linked Cpn to atherosclerosis. This pathogen may influence atherogenesis through a number of mechanisms, ranging from cell lysis²⁷⁻²⁹ to the stimulation of adhesion molecule expression³⁰ and cytokine production³¹ by infected cells. Moreover, we⁷ and others^{26,32} have demonstrated that Cpn promotes atherogenesis in atherosclerotic mice when infected at a young age when normally only initial lesions are present. Invigorating effects on both total lesion area as well as an acceleration in the formation of advanced lesions have been reported. In the present study, however, we did not find any effects of Cpn infection(-s) on either lesion area or lesion type. The reason for this discrepancy may be related to the fact that in 16 week-old ApoE^{-/-}LDLr^{-/-} mice, as used in the present study, advanced lesions are already the predominant phenotype while in younger mice (e.g. 8 week-

old ApoE3-Leiden used in our earlier study)⁷ initial plaques are the principal phenotype. Initial lesions mainly consist of macrophages infiltrating the vascular wall while advanced lesions contain a large necrotic core with extracellular lipids and cholesterol-ester crystals, extracellular matrix components (collagen) and smooth muscle cells. As macrophages are used as vehicle by Cpn for dissemination from the site of infection to different organs throughout the whole body (including the vessel wall)³³, it may be anticipated that it is more easy for Cpn to enter an initial atherosclerotic plaque and exert its pro-atherogenic effect than to enter an advanced lesion. This may explain why Cpn infections during the initial stages of atherosclerosis may accelerate the process while infections may have no effect on lesion progression when the plaques are already in a more advanced stage.

Whereas Cpn had no effect on total lesion size or lesion type, the percentage occupied by the fibrous cap was significantly reduced in Cpn-infected animals. At the same time, the fraction of the plaque occupied by the lipid core was significantly increased in Cpn infected mice when compared to control animals. As a thin fibrous cap and a large lipid core are the main characteristics of plaques vulnerable to rupture³⁴ these findings suggest that Cpn infections induce an unstable plaque phenotype. As plaque destabilisation may result in plaque rupture, thrombosis and acute clinical syndromes such as acute coronary syndromes and myocardial infarction, we infected or mock injected mice and waited until sudden death occurred. In line with our expectation, sudden death took place at much earlier time points in infected mice. Furthermore, at (post mortem) autopsy we indeed found signs of plaque rupture and (healed) myocardial infarcts in both groups. Taken together these findings suggest that Cpn infections induce an unstable plaque phenotype, thereby increasing the risk for future cardiovascular complications, such as plaque rupture and subsequent myocardial infarction or stroke.

MMPs are a family of enzymes that selectively digest individual components of the extracellular matrix, thereby playing an important role in maintaining the vessels integrity.³⁵ However, in certain environments these MMPs may contribute to pathologies such as restenosis,³⁶ to the de novo formation of atherosclerotic plaques³⁷ or to plaque rupture of by degrading the fibrous cap that surrounds them.³⁸ Interestingly, it has recently been demonstrated that Cpn is able to stimulate the production of MMP-2 and -9 by macrophages⁸. Furthermore, immunoreactivity for MMP-2 and MMP-9 were found to be increased in human atheromatous plaques, and predominantly in immunoreactive macrophages/mononuclear cells to Cpn.³⁹ Also, zymographic analysis revealed that activities of MMP-2 and MMP-9 were more increased in atherosclerotic tissues infected with Cpn compared to control tissues.^{10,11} Based on these findings we conjectured a role for these MMPs in the observed plaque destabilisation following Cpn infection. Various techniques were used to verify this hypothesis. Although a marked up-regulation of both MMP antigens in the lesions was observed, activity, measured by zymography or mRNA expression levels were similar in both groups. This suggests that a role for these specific MMPs is less likely which is in accordance with data from others. Rodel and co-workers demonstrated a lack of effect of Cpn

infection on MMP-2 and -9 mRNA levels in smooth muscle cells.⁹ Also, no association between the presence of Cpn DNA and increased activity of MMP-2 and -9 could be demonstrated in aneurysmatic abdominal aortas,⁴⁰ indicating a minor role for these specific MMPs in murine plaque destabilisation following Cpn infection. Nevertheless, in the mean time other corrosive enzymes, such as collagenases (MMP-1 and -8) and stromelysins (MMP-3), have been associated with Cpn infection and should be subject for further study. Furthermore, a limitation of the present study is that the activity of tissue inhibitors of the MMPs (TIMPs) was not investigated. Increased activity of these TIMPs may result in a failure to disclose an increased MMP activity in the vascular tissue. Another interesting topic for future study is MMP-2 and -9 expression at protein level by western blot. However, to study TIMPs activity and MMP-2 and -9 protein expression a new group of atherosclerotic aortic arches from ApoE^{-/-}LDLr^{-/-} mice are needed.

In conclusion, our results suggest that Cpn infection does not aggravate the atherosclerotic process in ApoE^{-/-}LDLr^{-/-} mice by increasing the size of the lesions but initiates a cascade of events, ultimately resulting in thinning of the fibrous cap and an increase of the necrotic core, both indicative of an unstable plaque phenotype. When mice were observed for a longer time period a reduction in survival occurred in the Cpn-infected mice due to cardiovascular complications. To our knowledge, this is the first study to demonstrate an *in vivo* mechanism that may account for the increased incidence of myocardial infarction among Cpn positive coronary heart disease other than the initially suggested aggravation of atherosclerotic plaque formation.

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CHAPTER 6

Anti-Chlamydia pneumoniae and anti-inflammatory properties of azithromycin in atherosclerotic mice

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Abstract

Objective: Since *Chlamydia pneumoniae* (Cpn) infections have been linked with atherosclerosis, we decided to study the effect of antibiotic therapy on lesion development in mice.

Methods: ApoE^{-/-}LDLr^{-/-} mice were infected twice (at 16 & 18 weeks) with Cpn or mock and treated with azithromycin (10mg/kg/day, sc) or sterile PBS. Mice were then sacrificed at 20 weeks of age and aortic arch sections were analyzed for Cpn DNA, lesion type, lesion area, fibrous cap area, necrotic core content, T-cells, and matrix metalloproteinases (MMP)-2 and -9.

Results: All mice developed atherosclerotic lesions (type 2 to 5b). Treatment of Cpn-infected mice with azithromycin resulted in formation of less complex atherosclerotic lesions, decreased lesion area, lipid core area, T-cells, and MMP-2. Fibrous cap area was increased after azithromycin treatment. Intriguingly, also non-infected control mice treated with azithromycin showed less complex lesions and a significant reduction in lesion area and necrotic core area. In Cpn-infected mice treated with azithromycin 57% (4/7) of vascular tissue samples remained Cpn DNA positive.

Conclusions: Here we demonstrate that azithromycin therapy not only prevents development of vulnerable plaques in Cpn-infected ApoE^{-/-}LDLr^{-/-} mice, but also in mock-infected mice. These data suggest that azithromycin has not only antibiotic properties but also anti-inflammatory.

Introduction

Although significant progress has been made in revealing important aspects in the etiology of atherosclerosis, the exact pathogenesis is not completely understood yet and the search for additional risk factors is ongoing.

One of the key events in lesion progression is inflammation of the vascular wall with subsequent activation of adhesion molecules,¹ recruitment of monocytes, T-cells, and release of cytokines (IL-2, IFN γ)² leading to recruitment of more inflammatory cells and ultimately the development of atherosclerotic lesions. Increase of the inflammatory process has been suggested to stimulate and sustain the inflammation leading to unstable plaque morphology. Lately, infections in particular with *Chlamydia pneumoniae* (Cpn) have been reported to act as moderators of atherosclerosis in several sero-epidemiological studies,³ animal studies,⁴ and in vitro experiments.⁵ We have demonstrated in an animal model that Cpn infection 1) augment the inflammatory process by increasing the number of T-lymphocytes in the plaque and 2) accelerating the formation of complex lesions.⁶ Also, 3) Cpn infection might alter the plaque phenotype from stable to vulnerable⁷ which may explain the correlation between myocardial infarction and Cpn.⁸

Since several lines of evidence have linked Cpn infection with progression of atherosclerotic disease, the question was raised whether antibiotic therapy would be an option to prevent or stabilize of the atherosclerotic process.⁹ To define the duration and quantity of antibiotic treatment several animal models, mice¹⁰ and rabbits¹¹, were used. Treatment with azithromycin¹² or clarithromycin¹¹ resulted in reduction of atherosclerotic lesion size. Nonetheless, the effect of treatment on plaque morphology has not been documented yet. Therefore, we infected ApolipoproteinE, low density lipoprotein receptor double knockout (ApoE^{-/-}LDLR^{-/-}) mice¹³ with Cpn or control and used azithromycin or sham therapy to study the effect of antibiotic treatment on atherosclerotic plaque morphology.

Methods

Mice

Thirty-two male specific pathogen free (SPF) ApoE^{-/-}LDLR^{-/-} mice on a C57Bl/6J background were used. Mice were housed under standard conditions at the Central Animal Facility of the Maastricht University and fed a normal chow diet (Hopefarms BV, Woerden, The Netherlands) and water ad libitum. All experiments were performed according to the institutional guidelines of the committee for the welfare of laboratory animals of the Maastricht University.

Chlamydia pneumoniae

Cpn strain TWAR 2043 (ATCC VR-1355) was cultured in Hep-2 cells as described previously.¹⁴ Bacterial titers were determined by titration in Hep-2 cells and stained with

fluorescein-isothiocyanate-conjugated (FITC) monoclonal antibody RR 402 (Dako, Glostrup, Denmark) directed against a protein component of the cell membrane of Cpn. Titers were expressed as number of inclusion forming units per milliliter (IFU/ml).

Study design

In humans, multiple Cpn infections occur throughout one's lifetime. To mimic this situation, mice were reinfected two weeks after the first infection and were treated with azithromycin (an antibiotic used as therapy for Gram negative bacterial infections, gift from Pfizer Research Laboratories, Groton, Conn., United States) or sham treatment, which consisted of phosphate-buffered saline (PBS).

As described previously,⁷ animals were infected 2 times with Cpn starting at the age of 16 weeks via intraperitoneal (ip) injection of Cpn (5.106 IFU) while control mice (mock) received sucrose-phosphate-glucose (SPG) solution only. Previously we demonstrated that the dissemination process following ip injections is similar to the process following intranasal inoculations, but there is better control over the amount of Cpn that is applied to the animal (unpublished data). All mice received a second inoculation (5.106 IFU Cpn or mock) at the age of 18 weeks and were afterwards treated with azithromycin (n=7 Cpn, n=8 mock) or sham therapy (PBS, n=8 Cpn, n=8 mock). Azithromycin therapy consisted of 7 consecutive subcutaneous (sc) injections of azithromycin (10 mg/kg), once a day, (figure 1). This dosage of azithromycin was chosen because it is known to give similar concentrations alike those achieved in humans after an oral dose of 500 mg a day.¹⁵ Sham therapy was started 1 day after infection and was administrated sc once a day for 7 consecutive days from week 16-17 and from week 18-19, (figure 1). All mice were sacrificed at the age of 20 weeks.

To determine the preferred way of administration of azithromycin in mice, a pilot study was performed before starting the present study. For this study, C57Black/6J mice were treated with azithromycin through sc administration once a day¹⁶ or orally via azithromycin in drinking water ad libitum. Plasma, lung and vascular tree were collected for determination of azithromycin concentrations by an Iso-sensitest agar (Oxoid Ltd Pasingstoke, Hampshire, UK) with *Micrococcus luteus* ATCC 9341 as the testorganism. Subcutaneous administration of azithromycin resulted in higher tissue levels than oral therapy (data not shown). Therefore, in the present study mice were treated with azithromycin or PBS through sc administration.

Statistical analysis

Difference in lesion size, fibrous cap area, T-cell number, lipid core area and MMP-2 and -9 immunoreactivity between the Cpn-infected and control mice treated with azithromycin or sham therapy were analyzed with the Mann-Whitney-U test. The same test was used to analyze differences in Cpn DNA of Cpn-infected mice that received sham therapy or azithromycin. Difference in lesion type between the Cpn-infected and control mice treated with azithromycin or sham therapy were analyzed with the Chi-square test. A p-value of <0.05 was considered statistically significant. Results are expressed as mean $\pm$ SEM.

Figure 1. Infection and treatment schedule for ApoE^{-/-}LDLr^{-/-} mice. Mice were infected with Cpn or mock at the age of 16 and 18 weeks. Treatment started 1 day after infection and consisted of 10 mg/kg azithromycin or sham (PBS) therapy sc, once a day, for 7 consecutive days from week 16-17 and week 18-19. All mice were sacrificed at the age of 20 weeks.

Results

Of the 32 mice used in the study, 1 mouse in group B (Cpn-infected and treated with azithromycin) died before reaching the time point of sacrifice. This mouse was excluded from the study, the cause of death remained unknown.

Presence of anti-Cpn antibodies and Cpn DNA

All Cpn-infected mice treated with either azithromycin or sham therapy developed antibodies at dilutions of 1:10 and 1:100, indicating that they had been infected successfully. Mock-infected mice treated with either azithromycin or sham therapy did not develop anti-Cpn antibodies.

Cpn DNA was detected in all aortic arches from Cpn-infected mice that received sham therapy, showing that intraperitoneal injection resulted in dissemination of Cpn to the vessel wall. In Cpn-infected mice on azithromycin therapy, 57% (4/7 vs. 8/8 in the sham treated mice, $p=0.03$) of all vascular tissue samples were positive for Cpn DNA, which demonstrates that antibiotic therapy does not completely eradicate Cpn from the vascular wall.

No positive results were observed in control-injected mice treated with azithromycin or sham.

Effect of antibiotic therapy on Cpn induced plaque progression and destabilisation

ApoE^{-/-}LDLr^{-/-} mice were infected and lesion characteristics were determined 2 weeks after the last inoculation. Aortic arches of all mice contained atherosclerotic lesions ranging from type 2 to 5b. No effect of Cpn on the frequency of initial or advanced lesion (figure 2A) nor on lesion size (figure 2B) was observed. Yet, azithromycin treatment led to a significant reduction in lesion size. The average lesion area for sham treated was: $0.24 \pm 0.03 \text{ mm}^2$ vs. $0.05 \pm 0.01 \text{ mm}^2$ for azithromycin treatment (figure 2B, $p<0.001$). Also, the frequency of advanced lesions was signifi-

cantly reduced following azithromycin treatment ($p=0.02$, figure 2A). Remarkably however, similar effects of azithromycin were observed in non-infected mice (sham therapy: $0.22 \pm 0.03 \text{ mm}^2$ vs. azithromycin therapy: $0.07 \pm 0.01 \text{ mm}^2$, $p<0.001$, figure 2B), suggesting a non-Cpn related effect of azithromycin on lesion development.

A significant increase was observed in the necrotic core of the lesions following Cpn infection, (mock: 0.12 ± 0.02 vs. Cpn: 0.24 ± 0.03 , $p<0.001$, figure 2C). Like for lesion size and frequency, azithromycin treatment also reduced the necrotic core content in both the Cpn infected group (sham: 0.24 ± 0.03 vs. azithromycin: 0.13 ± 0.03 , $p=0.03$, figure 2C) as well as in the mock group (sham: 0.12 ± 0.02 vs. azithromycin: 0.08 ± 0.01 , $p=0.01$, figure 2C).

Cpn infection significantly reduced the fibrous cap area, expressed as the fractional volume of the whole lesion (mock: 0.29 ± 0.03 vs. Cpn: 0.09 ± 0.01 , $p<0.001$, figure 2D), providing further evidence for Cpn induced plaque destabilisation. Azithromycin treatment, however, completely prevented the reduction of the fibrous cap area, (sham: 0.09 ± 0.01 vs. azithromycin: 0.33 ± 0.02 , $p<0.001$). In this case no effects of treatment were observed in the mock group (figure 2D).

Positive immunostaining for MMP-2 and -9 was mainly concentrated in the endothelial layer, the lipid core and the shoulder region of the lesions. Cpn infection resulted in a significant increase in MMP-2 positive staining (Cpn: 1.50 ± 0.24 vs mock: 0.83 ± 0.18 , $p<0.05$). Treatment with azithromycin resulted in a significant reduction in MMP-2 positive staining (sham: 1.50 ± 0.24 vs. azithromycin: 0.86 ± 0.15 , $p<0.05$). No significant effects of azithromycin on MMP-2 immunoreactivity were observed in the mock-infected group (sham: 0.83 ± 0.18 vs. azithromycin: 0.75 ± 0.27 , ns).

A significant increase in MMP-9 immunoreactivity was observed in lesions following Cpn infection (mock: 0.25 ± 0.17 vs. Cpn: 0.75 ± 0.17 , $p<0.05$). And although a slight reduction in MMP-9 immunoreactivity was observed in either the Cpn-infected group (sham: 0.75 ± 0.17 vs. azithromycin: 0.43 ± 0.22) or the mock-injected group (sham: 0.25 ± 0.17 vs. azithromycin: 0.33 ± 0.41) following azithromycin treatment, differences never reached the level of statistical significance.

In Cpn-infected mice, azithromycin therapy resulted in a significant decrease in T cell influx from $0.36 \cdot 10^{-4} \pm 0.59 \cdot 10^{-5}$ cells/lesion area following sham therapy to $0.79 \cdot 10^{-5} \pm 0.35 \cdot 10^{-5}$ cells/lesion area following azithromycin treatment, $p<0.001$, figure 2E. Also, in the mock-infected mice a slight reduction in T-cells after azithromycin therapy was observed from $0.31 \cdot 10^{-4} \pm 0.15 \cdot 10^{-4}$ cells/lesion area in the sham treated group to $0.23 \cdot 10^{-4} \pm 0.06 \cdot 10^{-4}$ cells/lesion area in the azithromycin group. This difference was not statistically significant, (figure 2E).

Figure 2. The effect of azithromycin therapy on atherosclerotic plaque phenotype of ApoE^{-/-}LDLr^{-/-} mice infected with Cpn or control. **A.** Lesion types presented as the mean frequency $\pm$ SEM at which that type of lesion was present. No effect of Cpn on the frequency of initial or advanced lesion was observed. The frequency of advanced lesions was significantly reduced following azithromycin treatment in the Cpn-infected and mock-infected mice. **B.** Lesion area was expressed as mean lesion area (mm²) of all plaques measured. No effect of Cpn on lesion area was observed. Lesion area was significantly reduced following azithromycin treatment in the Cpn-infected and mock-infected mice. **C.** Lipid core area was expressed as the fractional volume of the whole lesion occupied by each of them. **D.** Fibrous cap was expressed as the fractional volume of the whole lesion occupied by each of them. **E.** T cell content of the lesion was expressed as the number of T cells in the plaque divided by total lesion area.

Discussion

The association between Cpn infections and atherosclerotic lesion progression has been demonstrated in sero-epidemiological studies,³ animal models⁴ and in vitro studies showing presence of Cpn antigen, DNA¹⁷ and viable Cpn¹⁸ in atherosclerotic lesions. Potential mechanisms by which Cpn infection affects the atherosclerotic process, such as upregulation of adhesion molecules and continuous release of inflammatory mediators, have been reported.¹⁹

We⁶ and others^{20,21} have demonstrated that Cpn infection enhances the inflammatory process by increasing T-cell influx in the plaque and accelerating the formation of complex lesions. Moreover, plaque phenotype is shifted from stable to vulnerable plaque after multiple Cpn infections.⁷ However, in the present study, lesion area, lesion type and influx of T-cells of 20 week-old ApoE-/-LDLr-/- mice were not affected by infection with Cpn. In contrast, other studies,¹⁰ in which mice were infected at a younger age, demonstrated an increase in lesion area. Our mice were infected at 16 weeks, at which time point advanced lesion types are predominantly present. This suggests that Cpn infection in the presence of early atherosclerotic lesions results in modification of lesion type, lesion area and T-cell influx, whereas infection of mice with advanced lesions, as in the present study, did not result in these changes (figure 2 A, B, E). This explains the results of the present study where lesion area, size and T cell influx were, in contrast with other studies, not affected after Cpn infection.

For determination of atherosclerotic plaque morphology, not only lesion type, lesion area and T-cell influx are important, but more precise markers, as fibrous cap area and necrotic/lipid core area, must be included. The latter markers can be used to distinguish between stable and unstable lesions and define plaque phenotype. The present study shows that the necrotic core was increased and the fibrous cap area decreased following Cpn infection. Furthermore, MMP-2 and -9 immunoreactivity in the plaque were increased following infection suggesting a role for extracellular matrix degradation. Thus, Cpn infection modifies several markers of plaque stability, MMP-2 and -9, fibrous cap and necrotic core area, inducing the formation of a more unstable, and consequently more rupture prone plaque phenotype.

Since we are convinced that this is an important observation we posed the question as to whether Cpn eradication through antibiotic therapy will ameliorate the course of the atherosclerotic process. In the present study, we demonstrated that in Cpn-infected mice azithromycin therapy resulted in the formation of less complex atherosclerotic lesions, reduction in lesion and lipid core area and also reduction in T cell influx and presence of MMP-2 and prevented reduction of cap area. Although other animal studies reported a reduction in lesion area after azithromycin²² or clarithromycin¹¹ therapy, reports concerning the effect of treatment on atherosclerotic plaque phenotype are lacking. Thus, the results of the present study corroborate the role of Cpn in atherosclerotic lesion progression and the effect of azithromycin on this process.

The anti-Cpn effect of azithromycin has been confirmed in several other animal models. Muhlestein and coworkers infected New Zealand White rabbits on an athero-

genic diet with Cpn or saline at 3 weeks intervals and treated them with azithromycin or no therapy for 7 weeks.¹² Plaque area in the thoracic aorta was increased in the infected rabbits compared to both uninfected controls and infected rabbits receiving antibiotics. Using clarithromycin therapy after 3 Cpn infections (2 weeks apart) in rabbits, Fong and coworkers found a reduction in the number of lesions and in Cpn antigen content in the vascular tree.¹¹ Earlier studies performed by the same group,²³ reported similar results although azithromycin was used instead of clarithromycin with shorter treatment protocols.²²

These observations are in line with early small secondary prevention trials in patients,^{3,24} which showed a significant reduction in cardiovascular events (death, myocardial infarction or admission for unstable angina). However, results of clinical trials with larger patient numbers were less positive for patient mortality and morbidity.²⁵ For explanation of these contrasting findings, several implications and limitations of antibiotic trials should be taken into consideration. Not only incorrect hypothesis but also inadequate study size or design may explain negative outcomes of antibiotic trials.²⁶ Furthermore, effective eradication of Cpn by antibiotics is difficult or even impossible, since this pathogen enters a persistent state not refractory to antibiotic treatment. This has been demonstrated in Cpn-infected circulating human monocytes, which were treated with azithromycin *in vitro* and *in vivo* without achieving eradication of Cpn.²⁷ Kutlin and coworkers used continuously infected HEp-2 cells that were treated for 30 days with azithromycin, clarithromycin, or levofloxacin and found no elimination of Cpn.²⁸ Also, results from our study show that azithromycin therapy does not eradicate Cpn from the vascular wall since 4 out of 7 Cpn-infected mice remained Cpn DNA positive after azithromycin treatment.

Several animal studies^{10,29} demonstrated that antibiotic treatment beginning early after infection was more effective than delayed treatment. Our study confirms these observations, since we started azithromycin therapy one day after infection and found an effect on atherosclerotic lesion progression. This suggests that eradication of Cpn with antibiotics can be achieved in active Cpn infections while persistent infections are difficult to eradicate.

Interestingly, azithromycin therapy in the present study not only affected plaque phenotype in Cpn-infected mice, but showed similar effects in atherosclerotic lesions of mock-infected mice. This suggests that besides the anti-Cpn effect, azithromycin has anti-inflammatory properties that affect atherosclerotic plaque phenotype in ApoE^{-/-}LDLr^{-/-} mice. These observations are confirmed by Hall and coworkers³⁰ who demonstrated that stimulation of human monocytes and subsequent treatment with azithromycin *in vitro* resulted in reduction of TNF- α and IL-6. In contrast, a recent study in human hepatocytes did not display a direct anti-inflammatory effect of azithromycin.³¹ Besides *in vitro* evidence, azithromycin revealed anti-inflammatory capacities *in vivo*, as measured on the basis of reduced plasma concentrations of IL-6 and CRP.³² The ACADEMIC trial³³ reported a significant reduction in global rank sum score of 4 inflammatory markers (CRP, IL-1, IL-6, and TNF- α) after azithromycin therapy at 6, but not at 3 months follow up. Other studies^{34,35} with small patient num-

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CHAPTER 7

General Discussion

Introduction

Although the first reports suggesting a possible role for infections in cardiovascular disease were published already at the beginning of the 20th century^{1,2,3} it took until the late 1970's⁴ before the interest in infections as a potential risk factor for atherosclerosis was renewed. At that time, Julius and Catherine Fabricant⁵ demonstrated the prominent presence of atheroma in chickens infected with an avian herpes virus, suggesting a potential role for infections in the etiology of atherosclerosis. Yet, it was the seminal paper by Saikku and coworkers⁵ that stimulated many researchers to study the role of the respiratory pathogen *Chlamydia pneumoniae* (Cpn) in the progression of atherosclerotic cardiovascular disease. Since then, numerous sero-epidemiological studies have reported an association of Cpn antibodies with myocardial infarction, chronic coronary heart disease⁴ and an increased relative risk for cardiovascular complications.⁶ Furthermore, Cpn DNA⁷ as well as antigen^{7,8} and even viable Cpn⁷ has been detected in the atherosclerotic vascular tissue. Nevertheless, the majority of these studies were rather observational in nature and no clear molecular mechanisms by which Cpn might contribute to atherosclerosis were identified. Therefore, one of the main objectives of the present thesis was to elucidate potential mechanisms by which Cpn may aggravate atherosclerosis.

Atherosclerosis is a progressive disease that already starts at a rather young age and progresses with time. Initially, lesions are small and their organization is considerably different from advanced lesions, which can be found in the vascular tree at later age. As exposure to Cpn is extremely common, and respiratory infections occur repeatedly among most people it may be anticipated that Cpn can infect both early as well as advanced lesions. However, to our knowledge it has not been evaluated yet whether this affects lesions in a different way. Therefore we studied the effect of Cpn infection on lesion size and composition either in young mice with early lesion only, or in aged mice with severe atherosclerosis. Also, we evaluated the effect of frequent infection on lesion development, either in normal mice (**Chapter 4**) or in atherosclerotic mice (**Chapter 3,5**) Finally, we determined whether the effect of Cpn on atherosclerosis can be prevented by antibiotic treatment (**Chapter 6**).

Dissemination of Cpn in mouse model Although Cpn has recently been associated with extrapulmonary diseases like Alzheimer's disease,⁹ multiple sclerosis,¹⁰ reactive arthritis¹¹ and atherosclerosis,⁴ it is an obligatory intracellular pathogen, which predominantly causes respiratory infections such as pneumonia, bronchitis and pharyngitis.¹² As its natural route of infection is through the airways, it might be obvious to infect (atherosclerotic) mice intranasally by droplets administered onto the nostrils when you want to study the effect of infection on vascular pathology. Nevertheless, this route of infection has several disadvantages. E.g., intranasal inoculation stimulates sneezing which may result in less volume of Cpn-containing solution, and thus less infectious particles, in the lungs. Also, it has the risk of inoculation of the stomach instead of lungs. Overall, it is difficult to standardize the amount of inclusion forming units when mice become infected intranasally. In contrast, intratracheal inoculation does not seem to have the disadvantages of intranasal inoculation. However, dissemination profiles, in particular to the vascular wall, following intratracheal infection have

not been determined so far. Alternatively, intraperitoneal injections have been used to infect mice. Although this may not be the natural route of infection, it has been shown to result in the prominent presence of Cpn DNA in the vascular tree. To decide which route of infection is most appropriate to study the effects of Cpn on atherosclerosis, we determined systemic dissemination of Cpn to target organs, especially the vascular wall, after both intratracheal and intraperitoneal infection. In **Chapter 2**, we demonstrated that Cpn has the ability to disseminate systemically after intratracheal and intraperitoneal. Intraperitoneal Cpn infection induces systemic dissemination to the vascular tree that contains Cpn DNA up till 6 days after infection. Intratracheal Cpn infection results in systemic dissemination as well, although significantly lower Cpn DNA copy numbers were observed in the vascular tree at 4 and 6 days post infection. Thus, intraperitoneal inoculation of Cpn is the preferred inoculation route in mice to study the effect of Cpn infection on atherosclerotic lesion progression and was therefore used in future experiments.

Cpn and atherosclerosis

Animal models

To study the pathogenesis and mechanisms involved in atherosclerosis, different animal models, including mice, rats and rabbits, have been used in the past. Nevertheless, mouse models are generally favoured over rabbits or rats because of the possibility to easily create knockout and transgenic mouse strains. In this thesis, three different mouse strains have been used. First, the Apolipoprotein E (ApoE)3-Leiden strain was used in **Chapter 3** to study the effect of Cpn infections on lesion development. This strain develops advanced lesions only when fed a high cholesterol diet and lesion formation is quite protracted.^{13,14} This is in contrast to the Apolipoprotein E, low density lipoprotein receptor (ApoE-/LDLr-/-) double knockout strain, which develops advanced lesion already within 3 months even without additional diet (**Chapter 5**).¹⁵ Finally, we used the C57BL/6J strain, which develops early lesions only even when fed an additional high cholesterol diet (**Chapter 4**).¹⁶ The use of these three mouse strains allowed us to discriminate between the effects of Cpn on either initial or advanced lesion.

Effect of Cpn on early lesions

In **Chapter 3**, ApoE3-Leiden transgenic mice, fed a high fat/high cholesterol diet, were infected at a young age (9 weeks) when only initial lesions are present. When the effect of Cpn was evaluated shortly after infection (1 month), no effect was observed either on lesion size, number or type. This is in accordance with data from other groups who also could not demonstrate an effect on plaque development early after inoculation.¹⁷⁻¹⁹ In contrast, others have demonstrated that the effect of Cpn on atherosclerosis becomes more prominent later on and that repetitive inoculations might be required.¹⁷ Indeed, we were able to demonstrate a significant enlargement in lesion size in 14 months old C57BL/6J mice following multiple Cpn infections. Otherwise, no effect of Cpn on lesion size was observed in 8 and 11 months old ApoE3-Leiden transgenic mice. As these mice were infected only twice, this supports

the idea that multiple infections are needed before a prominent effect of Cpn on lesion size can be observed.

Apart from lesion size, we also evaluated the complexity of the lesion as this is generally considered as clinically more relevant than lesion size. Despite a prolonged atherogenic diet, no advanced lesions were found in 14 months old C57BL/6J mice following multiple Cpn infections suggesting that additional factors might be required for the development of advanced lesions. Interestingly, we only found slightly, and non-significantly, elevated cholesterol and triglyceride levels in these mice. This suggests that in the absence of high plasma cholesterol levels, repetitive Cpn infections are sufficient to increase lesion size, but that high cholesterol levels are required for the development of advanced lesions. This is supported by our findings in ApoE3-Leiden transgenic mice. In these mice, which develop significantly higher cholesterol and triglyceride levels than C57BL/6J mice, complex lesions were found in 8 and 11 months old mice. Furthermore, although two Cpn infections were obviously insufficient to increase lesion size, a significant acceleration in advanced lesion formation was observed at 6 and 9 months post infection. Summarizing, our results indicate that repetitive infections are mandatory before Cpn affects lesion size. Alternatively, once the circumstances are created for the formation of advanced lesions, less frequent Cpn infections seems to be needed to accelerate this process.

Effect of Cpn on advanced lesions

Clinical symptoms of cardiovascular disease usually results from plaque rupture, thrombus formation and ultimately occlusion of the vascular lumen. As Cpn has been frequently associated with stroke and myocardial infarction, it is hypothesised that Cpn plays a role in plaque destabilisation and increase the risk for future insults. To test whether Cpn affect the stability of an advanced lesion, we infected ApoE^{-/-}/LDLr^{-/-} mice with Cpn. In these mice, advanced lesion can already be found at a rather young age (~3-4 months) even when these mice are fed a normal chow diet. Thus, 16 week old mice were infected twice and sacrificed at an age of 20 weeks. Similarly, mice were infected once every 2 weeks and sacrificed at 40 weeks of age or observed until natural death. Surprisingly, neither 2 nor 6 consecutive Cpn infections resulted in significant alterations in lesion type or size. This suggests that, in contrast to our findings in infected mice with only initial lesions, Cpn does not alter size or complexity of already advanced lesions. The reason for this discrepancy remains to be established. If Cpn is more tropic to cells that contribute to fatty streaks, the early lesion in atherosclerosis, or if the microenvironment of the fatty streak favors the replication of Cpn more than that of a fibrous plaque, it is possible that Cpn cause more inflammation in early lesions than in advanced lesion. This is supported by our finding that the number of T-cells was significantly increased in lesions of mice infected already at a young age (**Chapter 3**). Also, early lesions from infected 14 months old C57BL/6J mice stained more positive for serum amyloid P than lesions from mock injected mice, suggesting higher levels of local inflammation (**Chapter 4**). Thus, Cpn effects on lesion size and/or type seem to be restricted to initial lesions.

Nevertheless, when advanced lesions in the infected ApoE^{-/-}/LDLr^{-/-} mice were studied in more detail, remarkable signs of plaque destabilization were found in lesions from infect-

ed mice. More precisely, in both groups (infected twice or 6 times) a significant increase in the size of the necrotic core was observed while the fibrous cap of the lesions was significantly diminished. Thus, Cpn infection modifies several markers of plaque stability, thereby inducing the formation of a more unstable, and consequently more rupture prone plaque phenotype. The clinical significance of this was illustrated in the survival experiment. When mice were infected 6 times and observed until natural death a significant reduction in life expectancy was observed in the infected group compared to the mock injected group. Furthermore, autopsy revealed signs of (healed) myocardial infarcts in some of the mice providing evidence that these mice might indeed have succumbed to complication of atherosclerosis.

It is well established that activated macrophages, which are essential components in atherosclerotic plaques, are able to produce and secrete matrix metalloproteinases (MMPs) like collagenases, elastases and stromelysins²⁰ that can degrade the fibrous cap, ultimately leading to a vulnerable plaque with a high risk of rupture. Recently, it now has been shown that Cpn is able to induce MMP activity directly in monocytes through an extracellular matrix metalloproteinase inducer (EMMPRIN)-dependent pathway and indirectly in smooth muscle cells via monocyte-derived cytokines.²¹ Furthermore, evidence suggests that exposure of human monocytes or smooth muscle cells to Cpn results in a significant enhancement in the production of various MMPs.²²⁻²⁴ In line with this we found a significant upregulation of MMP-2 and -9 immunoreactivity in plaques of ApoE-/-/LDLr-/- mice both after 2 and 6 inoculations which may provide a possible explanation for the observed destabilisation of the fibrous plaque. Although a marked up-regulation of both MMP antigens in the lesions was observed, activity, measured by zymography or mRNA expression levels were similar in both groups. This suggests that a role for these specific MMPs is less likely which is in accordance with data from others. Rodel and co-workers demonstrated a lack of effect of Cpn infection on MMP-2 and -9 mRNA levels in smooth muscle cells.²³ Also, no association between the presence of Cpn DNA and increased activity of MMP-2 and -9 could be demonstrated in aneurysmatic abdominal aortas,²⁵ indicating a minor role for these specific MMPs in murine plaque destabilisation following Cpn infection. Nevertheless, in the mean time other corrosive enzymes, such as collagenases (MMP-1 and -8) and stromelysins (MMP-3), have been associated with Cpn infection and should be subject for further study. Furthermore, a limitation of our study (**Chapter 5**) is that the activity of tissue inhibitors of the MMPs (TIMPs) was not investigated. Increased activity of these TIMPs may result in decreased MMP activity in the vascular tissue. Another topic for future study is MMP-2 and -9 expression at protein level by western blot. However, to study TIMPs activity and MMP-2 and -9 protein expression a new group of atherosclerotic aortic arches from ApoE-/-/LDLr-/- mice are needed.

Antibiotics as future therapy for atherosclerosis?

The macrolide class of antibiotics is well established and often recommended for use in the treatment of community-acquired respiratory tract infection caused for example by Cpn. The availability of these drugs and the suggested association between Cpn, inspired

researchers already in the late 1990' to test whether antimicrobial therapy may be helpful in the secondary prevention of CAD.⁶ Initial clinical studies reported that antibiotic therapy might lead to large reductions in secondary cardiovascular risk in patients with stable coronary heart disease or acute coronary syndromes (table 1).^{6,26-28} However, subsequent studies of intermediate size failed to confirm a large benefit,^{27,29-31} although these studies left open the possibility of limited-to-moderate clinical benefits. To test this possibility, large randomised trials involving thousands of patients were accordingly initiated (ACES, WIZARD, PROVE-IT, AZACS). Nevertheless, no significant benefits of antimicrobial therapy for the secondary prevention of cardiovascular events after acute coronary syndromes were observed, even when large patients groups were included or when therapy was given over the long term. Consequently, the results of these trials seriously questioned the validity of the infection hypothesis.

Nevertheless, although some of the initial comment on the first trials (small sample size, short treatment schedule of infection, short follow-up) were counteracted in the later trials, there are still some critical remarks concerning the trials that ought to be resolved before the hypothesis should be considered as false. The first issue that has to be clarified is time point at which the treatment is started. As it is not uncommon that a person becomes infected with Cpn already early in life, while the clinical signs and symptoms of atherosclerosis do not become manifest until the 6th decade, it is questionable whether treatment at this late time point is effective. Furthermore, Cpn infections are frequently sub-clinical, not treated or inefficiently treated with broad-spectrum antibiotics. Nevertheless, as our data in **Chapter 6** demonstrate, treatment of acute infection might be beneficial in preventing acute effects of Cpn infection.

Secondly, it can not be excluded that the antibiotic regimen is ineffective. Indeed, Gieffers et al. found that Cpn infection in vitro in monocytes from healthy volunteers was refractory to azithromycin as antibiotic treatment did not inhibit chlamydial growth within monocytes, and after withdrawal of antibiotic therapy, Cpn could be cultured from monocyte cell lines. Similar results were obtained with circulating monocytes from patients who had been treated for coronary heart disease.³² Accordingly when we treated our mice immediately after infection with azithromycin we were unable to eradicate Cpn completely in at least 4 out of 7 mice providing further evidence for the ineffectiveness of currently available antibiotics for the treatment of Cpn, although it remains to be determined whether quinolones show a similar ineffectiveness in Cpn clearance. Nevertheless, we were able to prevent the development of vulnerable plaques in infected mice demonstrating that azithromycin was effective at least to some extent. However, it remains to be established to what extent this effect is attributable, is it an antimicrobial effect or a more general anti-inflammatory effect, as has recently been suggested by others.³³⁻³⁶

Thirdly, if infections indeed contribute to atherosclerosis it is unlikely that only one micro-organisms is able to influence this process. In fact multiple micro-organisms have been associated with atherosclerosis over the past two decades. Also, several reports have suggested that multiple infections augment the risk conveyed by a single pathogen, the so called "pathogen burden" hypothesis.³⁷⁻³⁹ This hypothesis has been confirmed by a number of prospective studies demonstrating that an increased pathogen burden is inversely relat-

ed to the long-term prognosis in patients with documented coronary artery disease.^{40,41} Also, an increased pathogen burden may be an independent risk factor for endothelial dysfunction, one of the earliest manifestations of atherogenesis.⁴² This makes it doubtful that treatment with a single antibiotic might be sufficient to eliminate infections as a risk factor for atherosclerosis.

Finally, it has recently been suggested that the contribution of infection, and in particular Cpn, to atherosclerosis is independent of the direct effects of the microbe, but is due to cross-reactivity between microbial proteins and self-proteins. In particular, autoimmune reactions directed against heat shock protein 60 (HSP60) have been suggested as an important etiological factor in atherosclerosis.⁴³ HSP60 are phylogenetically highly conserved and there is an over 55% homology between bacterial hsp60 and the human homologue forming the basis for this cross-reactivity. Once infected, the organism will develop initiate and adaptive cellular and humoral immunity towards, among others, microbial HSP60 epitopes. As these epitopes share a homology with self HSP60, it may be anticipated that cross-reactivity develops. Now, in cases where endothelial cells are maltreated - e.g. by high blood pressure, high cholesterol or smoking - HSP60 is expressed on the endothelial surface and we have to 'pay' for our protective anti-HSP60 immunity by endothelial damage and the subsequent development of early atherosclerotic lesions.⁴⁴ In this alternative hypothesis explaining the effect of infections on atherosclerosis, it is doubtful that antimicrobial therapy for secondary prevention may be useful.

In summary, although the results of most large clinical trials evaluating the effects of antimicrobial therapy were rather disappointing, there is still a number of critical questions have to be addressed before the definite conclusion can be drawn that infection is not atherogenic. Furthermore, in contrast to the time point when treatment was started in most trials (usually following an initial cardiovascular event and not following an infection), our results suggests that antimicrobial therapy may be effective if treatment is started immediately after infection. However due to the difficulties in diagnosing Cpn infection in daily practice this might may be rather impractical to evaluate in clinical trials.

Concluding remarks and future perspectives

In this thesis, we tried to unravel some of the mechanisms involved in Cpn-induced development and/or progression of atherosclerosis by using several mouse models.

Before studying the effect of Cpn on atherosclerotic lesion progression, the "ideal" route of Cpn inoculation in mice was evaluated. We showed that intraperitoneal inoculation of Cpn and not intratracheal inoculation, induced higher Cpn DNA copy numbers in vascular tissue material which makes it the preferred inoculation route. When young ApoE3-Leiden transgenic mice were inoculated intraperitoneally with Cpn, no effect was seen on initial atherosclerotic lesions but an acceleration in advanced lesion type was observed. Moreover, multiplicity of infections was essential. In C57BL/6J mice following 10 Cpn infections, a significant enlargement in lesion size was observed, while in ApoE3-Leiden transgenic mice, inoculated only twice with Cpn, no effect on lesion size was observed. When old mice with advanced lesions were inoculated with Cpn, no effect on lesion type nor T-

cells was observed. Thus, Cpn infection in the presence of early atherosclerotic lesions results in modification of lesion type and T-cell influx, whereas infection of mice with advanced lesions modifies markers of plaque stability, inducing the formation of a more unstable plaque phenotype. These effects were more pronounced when mice were followed for a longer time period. A reduction in survival occurred in the Cpn-infected mice probably due to enhanced cardiovascular complications. The molecular mechanism that may explain these observations is the marked upregulation of MMP-2 and -9. Although we observed a marked up-regulation of both MMP antigens in the plaque of Cpn-infected mice, MMP activity and mRNA expression levels were similar in both groups. This suggests that a role for these specific MMPs is less likely. Nevertheless, other enzymes, such as MMP-1, -8 and MMP-3, have been associated with Cpn infection and should be subject for further study. Furthermore, in our study (*Chapter 5*) the activity of tissue inhibitors of the MMPs (TIMPs) was not investigated. Increased activity of these TIMPs may result in a failure to disclose an increased MMP activity in the vascular tissue. Another interesting topic for future study is MMP-2 and -9 expression at protein level by western blot. Finally, we determined whether the effect of Cpn on formation of unstable plaques can be prevented by antibiotic therapy. Indeed, we observed more stable lesions after treatment with azithromycin. However, these observations were also present in uninfected control mice treated with azithromycin, which suggests that azithromycin probably not only has anti-microbial but also anti-inflammatory capacities. Several other issues concerning secondary prevention through antibiotic therapy should be taken into consideration. Especially since the results of most large clinical trials evaluating the effects of antimicrobial therapy were rather disappointing. The first issue that has to be clarified is time point at which the treatment is started. We showed that treatment of acute infection might be beneficial in preventing acute effects of Cpn infection. However treating patients after every acute Cpn infection, which often is subclinical, is impracticable. Secondly, currently available antibiotics for the treatment of Cpn, might be ineffective. In our mice, which were treated with azithromycin immediately after infection we were unable to eradicate Cpn completely in at least 4 out of 7 mice. Moreover, if infections indeed contribute to atherosclerosis it is unlikely that only one micro-organisms is able to influence this process. It is more likely that "pathogen burden" (multiple different pathogens) is associated with progression of atherosclerotic cardiovascular disease.⁴⁰⁻⁴² This makes it doubtful that treatment with a single antibiotic might be sufficient to eliminate infections as a risk factor for atherosclerosis. Future intervention trials for further study of the role of pathogen burden in progression of atherosclerotic cardiovascular disease, may give us insight in this multifactorial systemic disease. Until then transgenic and knockout mouse strains can be used to evaluate new risk factors for progression of cardiovascular disease.

Name/Author (year)	N	Clinical condition	Therapy	Duration	Follow-up	End-points	Results
Peripheral vascular disease							
Masorin et al. ('01)	32	AAA	doxy. 150mg daily	3 months	18 months	aneurysm expansion	significant ↓ in expansion in treated group
Wiesli et al. ('02)	40	PAOD	roxi. 300mg daily	28 days	2.7 years	revascularisation	significant ↓ in revascularisation, ↑ walking distance, ↓ carotid plaque area
Parchure et al. ('02)	40	CAD	azithro. 500mg 3d, then 500mg weekly	5 weeks	5 weeks	FMD brachial artery	significant ↑ in FMD dilatation in treated group
Sander et al. ('02)	272	Ischemic stroke	roxi. 150mg 2t .daily	30 days	2 years	decrease IMT	significant ↓ IMT in Cpn+ treated group no difference in cardiovascular events, after 3,4 years no ↓ IMT
Vainas et al. ('05)	509	PAOD	azithro. 500mg daily	3 days	2 years	death, cardiovasc. events, ↓ ABPI	No effect on survival, ABPI
Coronary artery disease							
Gupta et al. ('97)	60	Post-MI	azithro. 500mg 3d., then 500 mg for 6 d.	9 days	18 months	death, MI, revasc.	significant ↓ in EP in treated group
Sinisalo et al. ('02)	148	UA, MI	clari. 500mg daily	3 months	18.5 months	death, MI, UA (1 EP) death, MI, UA, stroke, limb ischemia (2 EP)	↓ in 2nd endpoints at 18.5 months
Gurfinkel et al. ('97)	202	UA, MI	roxi.150mg twice daily	30 days	6 months	cardiac ischemic death MI, recurrent ischemia	At 30 days, significant ↓ in EP, no difference at 6 months
Anderson et al. ('99)	302	Stable CAD	azithro. 500mg 3d., then 500mg weekly	3 months	2 years	death, MI,stroke, UA ,	significant ↓ CRP, IL-6, no revasc. difference in EP
Stone et al. ('02)	325	ACS	amoxi+metro+ome azitro+metro+ome	1 week	1 year	MI, UA	↓ in endpoints with either therapy schedule
Zahn et al. ('03)	872	MI	roxi. 300mg	6 weeks	12 months	1 EP:total mortality 2EP:death, reinfarct. CVA, revasc., UA	no difference in 1 ep or 2nd ep
Neumann et al. ('01)	1010	Post-stenting	roxi. 300mg daily	28 days	6 months	restenosis, death, revasc., MI	significant ↓ restenosis in treated group
Cercek et al. ('03)	1400	UA	azitro. 500mg. 1 day, then 250mg 4 days	5 days	6 months	mortality, MI, revasc.	no difference in EP
Jackson et al. ('05)	4012	Stable CAD	azitro. 600mg weekly	1 year	3.9 years	CVD, MI, revasc., hospital for UA	no difference in EP
Cannon et al. ('05)	4162	Post-MI, UA	gatifloxacin 400mg daily for 2 weeks, then 10 day course monthly	2 years	2 years	death, MI, stroke, hospitalisation	no difference in EP
Coletta et al.('02)	7724	Post MI	azithro. 600mg 3d., then 600mg weekly	11 weeks	2 years	composite: mortality, recurrent MI, revasc., hospitalisation for MI	no difference in EP

Table 1: Summary of trials of the effect of antibiotic therapy on cardiovascular diseases. Update from Gelfand et al.⁴⁵

Name/Author (year)	N	Clinical condition	Therapy	Duration	Follow-up	End-points	Results
Ongoing trials							
MARBLE	~1240	Pre-CABG	azitro.500mg daily	Variable	3 months	cardiovascular events	
CLARICOR	~4600	Stable CAD	claritro. 500mg daily	2 weeks	2 years	CVD, MI, UA	
CROAATS	~270	Post-MI	azitro. 500mg 3 days cycles on day 1,10, 20	23 days	18 months	CVD, MI, revasc., hospital for UA	
roxi: roxithromycin			Masorin et al. ⁴⁶		Stone et al. ⁴⁷		
doxy: doxycyclin			Wiesli et al. ²⁸		Zahn et al. ²⁹		
azithro: azithromycin			Parchure et al. ⁴⁸		Neumann et al. ³⁰		
FMD: flow mediated dilatation			Sander et al. ^{49,50}		AZACS ^{51,52}		
IMT: intima media thickness			Gupta et al. ⁶		ACES ^{52,53}		
revasc.: revascularisation			Vainas et al. ³¹		PROVE-IT ^{54,55}		
ABPI: ankle brachial pressure index			Sinisalo et al. ²⁷		WIZARD ^{56,57}		
EP: endpoint			Gurfinkel et al. ²⁶		MARBLES ⁸		
PAOD: peripheral arterial occlusive disease			Anderson et al. ⁵⁹		CLARICOR ⁶⁰		
AAA: aneurysm of the abdominal aorta							
CAD: coronary artery disease							
MI: myocardial infarction							
ACS: acute coronary syndrome							
UA: unstable angina							
CABG: coronary artery bypass grafting							
amoxicillin (500mg 1dd1)+ metronidazole(400mg 2dd1)							
+omeprazole (20 mg 2dd1)							

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CHAPTER 8

Samenvatting

Samenvatting

Atherosclerose is een multi-orgaan aandoening met een chronisch progressief karakter. Er wordt algemeen aangenomen dat inflammatie van de vaatwand aan de basis van dit proces ligt. Naast bekende oorzaken van inflammatie, als roken en hypertensie zijn er aanwijzingen dat ook infecties met *Chlamydia pneumoniae* (Cpn) een rol spelen in de progressie van atherosclerose. In dit proefschrift worden verschillende mogelijke mechanismen beschreven waardoor Cpn infectie kan leiden tot progressie van atherosclerotisch vaatlijden. Daarbij is het effect van Cpn infectie op zowel vroege als complexe atherosclerotische lesies bestudeerd. Ook is het effect van multi-pele Cpn infecties op lesie ontwikkeling geanalyseerd in normale (**hoofdstuk 4**) en atherosclerotische muizen (**hoofdstuk 3 en 5**). Tot slot is het effect van antibiotische therapie op het remmen danwel voorkomen van progressie van atherosclerose na Cpn infectie bestudeerd (**hoofdstuk 6**).

Hoofdstuk 1 geeft een theoretische achtergrond van de etiologie en de pathofysiologie van atherosclerose voor zover relevant voor de beschreven studies. Hierbij wordt de bijdrage van inflammatie aan het atherosclerotisch proces in algemene zin beschreven en daarnaast wordt voornamelijk de rol van verschillende micro-organismen, met name Cpn, uiteengezet.

In **hoofdstuk 2** wordt de systemische verspreiding van Cpn na intratracheale (it) and intraperitoneale (ip) infectie beschreven. Het doel van deze studie was na te gaan welke route van inoculatie superieur is om disseminatie van het micro-organisme naar, met name, de vaatwand te bereiken. Daarbij zijn C57Bl6/J muizen, die als genetische achtergrond dienen bij het fokken van transgene en knockout muizen (ApoE3-Leiden, ApoE knockout, and LDL-receptor, ApoE dubbel knockout muizen), it of ip geïnjecteerd met Cpn waarna het aantal Cpn DNA kopieën in diverse organen werd bepaald. We vonden dat, hoewel Cpn een voornamelijk respiratoir pathogeen is, systemische verspreiding plaatsvond na zowel it als ip injectie. Daarbij werden na ip injectie significant meer Cpn DNA kopieën gemeten in de vaatwand dan na it inoculatie. Dit maakt ip inoculatie superieur aan it injectie. Daarom werden alle muizen uit de experimenten beschreven in dit proefschrift, ip geïnjecteerd met Cpn of controle.

In **hoofdstuk 3** wordt het effect van Cpn infectie op progressie van atherosclerose, aan de hand van type en grootte van de lesie, beschreven alsmede de rol van Cpn op het inflammatoire proces in de vaatwand. Daarvoor werden ApoE3-Leiden transgene muizen geïnjecteerd met Cpn en op verschillende tijdstippen na infectie opgeofferd. De resultaten laten zien dat Cpn infectie leidt tot (1) een versnelde vorming van complexe atherosclerotische laesies op 6 en 9 maanden na infectie en (2) een sterk verhoogde influx van T-cellen in de plaque op 1 maand na infectie. Aangezien geactiveerde T-cellen een belangrijke bron van pro-inflammatoire en pro-atherogene cytokines vormen, kan gehypothetiseerd worden dat de verhoogde influx van T-cellen

vroeg na infectie leidt tot een veranderde expressie van bepaalde cytokines. Op lange termijn zal dit resulteren in een versnelling van het atherosclerotisch proces zoals gemeten 6 en 9 maanden na infectie.

Om het effect van Cpn infectie op vroege atherosclerotische lesies te bestuderen werden C57Bl6J muizen gebruikt (**hoofdstuk 4**). Aangezien deze muizen vroege atherosclerotische lesies ontwikkelen zelfs na atherogeen dieet, was de vraag of additionele multiële (10 maandelijks) Cpn infecties zouden resulteren in de vorming van complexe lesies. We vonden dat Cpn niet resulteerde in progressie van lesie type maar resulteerde in een toename in lesie grootte (controle: $5022 \pm 1348 \mu\text{m}^2$ vs Cpn: $10\,821 \pm 2429 \mu\text{m}^2$; $p=0.04$). Daarmee gepaard gaande werd een toename van serum amyloid P (SAP) gemeten in atherosclerotische lesies (controle: $1.10^{-4} \pm 0.1.10^{-4}$ SAP positieve cellen/lesie oppervlakte vs Cpn: $10.10^{-4} \pm 1.10^{-4}$ SAP positieve cellen/lesie oppervlakte; $p=0.05$).

In **hoofdstuk 5** wordt de opbouw van complexe atherosclerotische lesies na Cpn infectie nader beschreven. We stelden de vraag of complexe lesies, ontstaan na Cpn infectie zoals beschreven in **hoofdstuk 3**, uiteindelijk zouden destabiliseren tot instabiele atherosclerotische lesies met op den duur een verhoogd risico op plaque ruptuur. Plaque ruptuur is het proces wat ten grondslag ligt aan ziektebeelden als hart- en herseninfarcten en acute perifere vaatafsluitingen. Er treedt afsluiting op van het desbetreffende bloedvat met ischemie van het aangedane weefsel en uiteindelijk weefselversterf.

In deze studie is gekozen voor ApoE,LDL-receptor dubbel knockout muizen die bekend staan om de snellere ontwikkeling van atherosclerotische lesies ten opzichte van ApoE3-Leiden muizen. Infectie van ApoE,LDL-receptor dubbel knockout muizen met Cpn resulteerde in een dunnere fibreuze cap, een toename in necrotische core en een toename van matrix metalloproteinase (MMP)-2 en -9 in de plaque, alle kenmerken van een instabiel plaque fenotype. Daarnaast vonden we een afname in overlevingsduur na Cpn infectie. De aanwezigheid van cardiovasculaire complicaties, geanalyseerd bij sectie van de proefdieren, was in beide groepen gelijk.

Om na te gaan of de Cpn-geïnduceerde destabilisatie van de atherosclerotische lesie geremd of voorkomen kon worden, zijn ApoE,LDL-receptor dubbel knockout muizen geïnfecteerd met Cpn of controle en behandeld met azitromycine of sham therapie (**hoofdstuk 6**). We vonden dat Cpn-geïnfecteerde muizen, behandeld met azitromycine, minder complexe lesies vormden dan sham behandelde muizen, met minder lesie oppervlakte, necrotische core, T cellen influx en MMP-2 expressie. Het aandeel van de fibreuze cap in het plaque volume was toegenomen na antibiotica behandeling. Opmerkelijk was dat deze bevindingen ook waargenomen werden bij niet geïnfecteerde controle muizen die behandeld waren met antibiotica. Dus azitromycine voorkomt niet alleen de ontwikkeling van instabiel plaques in Cpn-geïnfecteerde ApoE,LDL-receptor dubbel knockout muizen, maar ook in controle geïn-

jecteerde muizen. Dit suggereert dat azitromycine niet alleen antimicrobiële eigenschappen bezit maar ook anti-inflammatoire eigenschappen.

Tot slot worden in **hoofdstuk 7** de belangrijkste resultaten van dit proefschrift bediscuseerd in relatie tot de literatuur. Er zijn in dit proefschrift verschillende muismodellen gebruikt om enkele mechanismen te belichten die betrokken zijn bij Cpn-geïnduceerde progressie van atherosclerose. De resultaten tonen dat, in muisexperimenten, ip inoculatie van Cpn superieur is aan it inoculatie. Daarnaast suggereren de hier beschreven studies dat Cpn infectie in aanwezigheid van initiële lesies, zoals in de studie met ApoE3-Leiden muizen, resulteert in modificatie van lesie type en T cel influx, terwijl infectie van muizen met complexe lesies, zoals in de studie met ApoE,LDL-receptor dubbel knockout muizen, leidt tot veranderingen in plaque stabiliteit, naar een instabiel plaque fenotype. Dit proces kon voorkomen worden door vroege behandeling met azitromycine van Cpn-geïnfecteerde muizen, maar ook van niet geïnfecteerde controle muizen. Dit suggereert dat azitromycine zowel antimicrobiële als anti-inflammatoire eigenschappen bezit.

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Curriculum Vitae

Rajaa Ezzahiri is geboren op 8 februari 1976 te Breda. In 1994 deed ze eindexamen Gymnasium B aan het Newmancollege te Breda, waarna ze geneeskunde ging studeren aan de Universiteit Maastricht. Tijdens haar studie is ze actief bezig geweest als student-assistent (Dr Tordoir en Dr Lemson) en bij de oprichting van het eerste Maastricht Medical Student's Research Conference (congres voor studenten geneeskunde, commissie wetenschap). In 2001 behaalde ze het artsdiploma en werkte ze vervolgens als AGIKO aan dit proefschrift. In 2005 startte ze met de opleiding tot chirurg aan het Academisch Ziekenhuis Maastricht (opleider Prof Greve) en vanaf juli 2007 aan het Maxima Medisch Centrum Eindhoven (opleider Dr Prakken).